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BTN1A1, the Mammary Gland Butyrophilin, and BTN2A2 Are Both Inhibitors of T Cell Activation

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Butyrophilin (BTN) genes encode a set of related proteins. Studies in mice have shown that one of these, BTN1A1, is required for milk lipid secretion in lactation, whereas butyrophilin-like 2 is a coinhibitor of T cell activation. To understand these disparate roles of BTNs, we first compared the expression and functions of mouse Btn1a1 and Btn2a2. Btn1a1 transcripts were not restricted to lactating mammary tissue but were also found in virgin mammary tissue and, interestingly, spleen and thymus. In confirmation of this, BTN1A1 protein was detected in thymic epithelial cells. By contrast, Btn2a2 transcripts and protein were broadly expressed. Cell surface BTN2A2 protein, such as the B7 family molecule programmed death ligand 1, was upregulated upon activation of T cells. We next examined the potential of both BTN1A1 and BTN2A2 to interact with T cells. Recombinant Fc fusion proteins of murine BTN2A2 and, surprisingly BTN1A1, bound to activated T cells, suggesting the presence of one or more receptors on these cells. Immobilized BTN-Fc fusion proteins, but not MOG-Fc protein, inhibited the proliferation of CD4 and CD8 T cells activated by anti-CD3. BTN1A1 and BTN2A2 also inhibited T cell metabolism, IL-2, and IFN-γ secretion. Inhibition of proliferation was not abrogated by exogenous IL-2 but could be overcome following costimulation with high levels of anti-CD28 Ab. These data are consistent with a coinhibitory role for mouse BTNs, including BTN1A1, the BTN expressed in the lactating mammary gland and on milk lipid droplets. The Journal of Immunology, 2010, 184: 3514–3525.

The butyrophilin (BTN) genes, previously known as BT (1) and BTF (2) genes, are a group of partially characterized, MHC-associated genes with an unusual protein structure (2). They are type I membrane proteins with two extracellular Ig domains, and an intracellular B30.2 or PRYSRPR domain (3). There are three subfamilies of human BTN genes located in the extended MHC class I region: the single copy BTN1A1 gene and the BTN2 and BTN3 genes, which have undergone tandem duplication resulting in three copies of each type of gene, namely BTN2A1, BTN2A2, and BTN2A3; and BTN3A1, BTN3A2, and BTN3A3. Mouse Btn1a1 and Btn2a2 are single copy genes and orthologs of the human BTN1A1 and BTN2A2 genes, respectively. There is no ortholog of the human BTN3 genes in mice. BTN1A1 has been most extensively studied. It is required for the secretion of milk lipid droplets during lactation (4), and this function has been attributed to its cytoplasmic B30.2 domain (5, 6). Nothing is known of the function of the B30.2 domains of the other BTNs.

There is some sequence homology between the Ig domains of the BTN molecules and a number of Ig superfamily molecules known to function in T cell responses and T cell differentiation. The two extracellular Ig domains of the murine BTNs are most similar to mouse butyrophilin-like (BTNL)2 that coinhibits T cell activation (7). The level of identity between the proteins is ~40%. Murine BTNs also display ~30% identity with the B7S3/Skint gene family (8, 9), which mediate γδ T cell differentiation. The B7 family molecule that is most similar to murine BTN2A2 is murine B7-H3 (CD276), which is 26% identical to BTN2A2. The level of identity between the two murine BTN molecules themselves is 47%, suggesting that these proteins have conserved structures, as well as a common evolutionary origin. The similarity between the two proteins extends to both the extracellular and intracellular regions. The structural similarity between the BTNs and other T cell regulatory molecules suggests possible immunomodulatory functions for the BTN proteins.

The BTN2 gene, previously known as NG9 and BTL-II (7), is located adjacent to the MHC class II region in both humans and mice and encodes a negative regulator of T cell activation (10, 11). It has a different structure to the classical BTN genes, with four extracellular Ig domains and no B30.2 domain but a short cytoplasmic tail. The gene is preferentially expressed in the digestive tract (7). Although BTN2L2 is only present as a single copy gene in humans, in other mammalian species there is a cluster of BTN2L genes at this genomic locus (12, 13), but it is not known whether these additional genes are functional. This locus has been studied recently in relation to its genetic association with a variety of autoimmune diseases (13).

The B7S3/Skint genes, clustered on chromosome 1 in mice, are also homologous to the BTN genes (8, 9). In contrast to BTN genes, B7S3/Skint genes encode proteins with one or two extracellular Ig domains, three transmembrane domains, and a short cytoplasmic C terminus. Functional studies, using recombinant B7S3 (Skint2) protein, have demonstrated that the extracellular Ig domains inhibit

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Abbreviations used in this paper: BTN, butyrophilin; BTNL, butyrophilin-like; CHO, Chinese hamster ovary; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; Hu IgG, human IgG; MS, multiple sclerosis; NP-40, Nonidet P-40; PNGase F, peptide: N-glycosidase F; PD-L, programmed death ligand; TEC, thymic epithelial cell.
CD4 and CD8 T cell activation (9). The Skint1 gene was identified by analysis of a strain of FVB/N mice that have a deficiency in epidermal γδ T cells. The Skint1 transcript is expressed in the thymus and skin, and a truncation mutation of Skint1 disrupts the thymic selection of Vγ5Vδ1 T cells (8). The B7S3/Skint gene family may be rapidly evolving, because there are 10 intact paralogs of the locus in mice, but the homologous, single copy gene in humans is a pseudogene (8, 9).

Several other homologous genes are related to the BTN family. These include the Scuona blood group Ags encoded by the ERMAP gene (14) and the MHC-associated, multiple sclerosis (MS) autoantigen MOG (15, 16). The IgV domains of murine MOG and murine BTN1A1 share 44% amino acid identity, and treatment of rodents with a preparation of the IgV domain of bovine BTN1A1 has been shown to modulate the symptoms of experimental autoimmune encephalomyelitis (EAE), a disease model of MS (17, 18). BTN-related proteins in avian species include the chicken BG molecules, which are encoded in the extended BF/BL region of the MHC-containing B complex (19) and the Tvc gene, which is a receptor for subgroup C avian sarcoma and leukemia viruses (20).

The Ig domains of the BTN protein family display weak sequence similarity with coinhibitors of the B7 family, which negatively regulate the activity of T cells (21). These inhibitory ligands include programmed death ligand (PD-L)1 (also known as B7-H1 and CD274); PD-L2 (also known as B7DC and CD273); murine B7-H3 (CD276); B7-H4 (also known as B7× and B7S1); and VSIG4 (22).

The intracellular B30.2 or PRYSPRY domain, encoded by some BTN genes, is also present at the carboxy terminus of many of the numerous tripartite motif (TRIM) genes. This 200 aa sequence, comprising a β-sandwich fold, may have diverse functions (23, 24).

**FIGURE 1.** Expression of BTN1A1 and BTN2A2 transcripts and proteins. **A**, Expression of Btn1a1 and Btn2a2 mRNA in tissues from adult female C57BL/6 mice. Real-time PCR analysis of Btn1a1 was conducted using primers specific for exons 5 and 8 of Btn1a1 (left and center panels) and primers specific for exons 7 and 8 of Btn2a2 (right panel) using cDNA synthesized from total RNA. The PCR products were analyzed by agarose gel electrophoresis as depicted below the quantitative PCR data. Data for Btn1a1 expression in virgin mammary gland (left panel) was replotted with data for lactating mammary gland (center panel). For all three panels, the expression levels of both sets of transcript were normalized to the tissue with the lowest level of expression; heart for Btn1a1 and liver for Btn2a2, and the respective levels in these tissues were set to 1. Data are the means and SDs of triplicate determinations. **B**, Specificity of anti-BTN serum determined by Western blotting. Far left panel: Detection of full-length, HA-tagged BTN proteins from transfected CHO cells with an anti–HA-specific mAb. Gels were loaded as follows: untransfected CHO cells (lane 1), HA-BTN1A1 (lane 2), HA-BTN2A2 (lane 3), and HA-BTNL2 (lane 4). Center panels: Duplicate blots were probed with anti-BTN1A1 antiserum (center, left) and anti-BTN2A2 antiserum (center, right), and only the relevant protein Ags were detected. Far right panel: BTN2A2 protein is glycosylated. Cell lysates of untransfected (lane 1) and BTN2A2-transfected HEK 293T cells were either not treated (lane 2), mock treated (lane 3), or treated with PNGase F (lane 4), and BTN2A2 protein was detected with anti-BTN2A2. **C**, BTN2A2 is expressed in a panel of C57BL/6 adult mouse tissues. Western blots of mouse tissues (25 μg protein/lane) were probed with anti-BTN2A2. The testis sample was harvested from an adult C57BL/6 male mouse, and the lactating mammary tissue was obtained on day 2 of lactation from a female CD1 mouse. **D** and **E**, BTN1A1 and BTN2A2 are expressed in the thymic stroma and on TECs. Thymic sections were stained with anti-keratin (left panels) and anti-BTN1A1 (D, center panel) and anti-BTN2A2 (E, center panel) antisera. Overlaid images revealed coexpression of the proteins on TECs (right panels). Original magnification ×200.
FIGURE 2. Detection of BTN2A2 by flow cytometric analysis. A, Anti-BTN2A2 antiserum was specific for BTN2A2. CHO cells were transfected with the following expression constructs: pIRES2-EGFP vector, BTN1A1/pEYFP-N1, HA-BTN2A2/pIRES2-EGFP, and HA-BTNL2/pIRES2-EGFP. Cells were stained with purified preimmune serum (top row) or anti-BTN2A2 (bottom row) and detected with Alexa 647-conjugated goat anti-rabbit IgG. B, BTN2A2 was detected on murine naive CD19+ B cells; CD3+ NK1.1+ NK cells; CD11b+, Ly-6Glow/GR1low peritoneal macrophages; and CD11c+ splenic DCs, but there was negligible expression on naive CD3+ T cells. Cells were stained with anti-BTN2A2 (black line) and preimmune serum (dotted line, filled histogram). Bound Abs were detected with a goat anti-rabbit IgG Alexa Fluor 647 conjugate. C, BTN2A2 is upregulated on CD3+CD25+ T cells after in vitro activation of splenocytes with anti-CD3 and anti-CD28 (left panel) or treatment of splenocytes with Con A (right panel) as described in Materials and Methods. Cells were stained with anti-BTN2A2 (black line) and preimmune serum (gray line), and the data shown were the result of gating on CD3+, CD25+ live cells. D, Anti-BTN2A2 serum immunoprecipitates BTN2A2 but not BTN1A1 protein. Immunoprecipitations using control rabbit IgG (lanes 1, 4, and 7), anti-HA (lanes 2, 5, 8), or anti-BTN2A2 (lanes 3, 6, 9) were performed using lysates of untransfected CHO cells (lanes 1–3), HA-BTN1A1 expressing CHO cells (lanes 4–6), and HA-BTN2A2 expressing CHO cells (lanes 7–9). Immunoprecipitated proteins were detected by Western blot with
In the case of BTN1A1 the B30.2 domain of the protein interacts with xanthine oxidase (5, 6). The pairing of these two molecules may facilitate the formation of milk fat globules (4, 25). Nothing is known of the role of this domain in other BTNs, and it is possible that BTN1A1 has evolved a specific nutritional function in lactation (26). The B30.2 (PRYSPRY) domain of TRIM genes may mediate protein–protein interactions. For example the TRIM5αe protein targets retroviral capsid proteins through its PRYSPRY domain (27) and the PRYSPRY domain of TRIM21 binds to the Fc portion of IgG (28).

Several studies have attempted to identify cells with counter receptors for the BTNs. Recombinant human BTN3A1 protein binds to B and T cell lines (29), and similar experiments with human BTN2A1 revealed that a particular glycoform of BTN2A1 binds to a lectin molecule, DC-SIGN, found on dendritic cells (DCs) (30). Binding of BTN2A1 to DC-SIGN is dependent on high-mannose glycosylation of the protein when expressed by tumor cells. In two recent studies, recombinant murine BTN2L2 protein also bound an unidentified receptor on B and T cells (11), distinct from the known receptors for B7 molecules (10). Both groups demonstrated that activation of mouse T cells through engagement of CD3 is inhibited by BTN2L2 binding to its putative receptor on T cells.

In view of the evidence for BTN2L2 as a co-inhibitory receptor, we set out to investigate the functions of murine BTN1A1 and BTN2A2. We first examined the expression patterns of murine BTN1A1 and BTN2A2 and then probed their ability to co-inhibit activation of T cells.

Materials and Methods

Clones and sequence analysis

FANTOM clones 9130020N19 and D030063K05, encoding full-length cDNA sequences for murine (C57BL/6) Btn2 and Btn2a2, respectively, were purchased from K.K. DNAFORM (Yokohama, Japan).

Quantitative PCR

Total RNA was extracted from tissue from 6- to 11-wk-old C57BL/6 female mice with TRZol according to the manufacturer’s instructions (Invitrogen, Paisley, U.K.). For each tissue, cDNA was synthesized from 3 μg total RNA with Superscript II (Invitrogen) using an anchored oligo dT primer. Quantitative PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Poole, U.K.), according to the manufacturer’s instructions in an ABI 7500 Fast Real-Time PCR System. Primers spanning the junction of Btn1a1 exon 5 (Ts) and exon 8 (B30.2) were chosen from PrimerBank (31) ID 7304935a2. Primer sequences were exon 5, forward, 5’TACTGCGCTTAGTTCCTCAC-3’ (764–786 nt), and exon 8, reverse, 5’GACGCTGAATCTTCCAATCGAACT-3’ (997–975 nt). Primers spanning the junction of Btn2a2 exon 5 (7heptad repeat) and exon 8 (B30.2) were designed by Sigma-Aldrich. Primer sequences were exon 5, forward, 5’TGGGACGCAACCTTCTCACATG-3’ (913–934 nt) and exon 8, reverse, 5’ACACATGACGGCCTAATCGACAT-3’ (1049–1070 nt). The tissue distribution of both transcripts was confirmed by RT-PCR using second pairs of primers, annealing elsewhere in the transcript; namely, exons 4 and 5 of Btn1a1 (PrimerBank ID 7304935a1), and exons 2 and 3 of Btn2a2 (PrimerBank ID 28565275a1). To confirm the specificity of PCR primers, the DNA from several PCR reactions was separated by agarose gel electrophoresis to ensure that single PCR products, of the correct size, were generated. Amplicons for all primer pairs were also sequenced. To compare the level of Btn1a1 transcripts in different tissue samples, standard curves were constructed from 2-fold dilutions of cDNA synthesized from RNA extracted from the mammary gland of a C57BL/6 female mouse on day 5 of lactation (a gift from Dr. C. Watson, Department of Pathology, Cambridge University, Cambridge, U.K.). In the case of Btn2a2, cDNA was prepared from RNA extracted from a stable line of LTR fibroblast cells that had been transfected with an expression construct for BTN2A2 (Btn2a2/pcDNA 3.1/myc-His).

Production of Fc fusion proteins

DNA sequences encoding the extracellular portions of murine BTN1A1 (aa 28–245), BTN2A2 (aa 30–234), BTN2L2 (aa 26–457), murine MOG (aa 30–155) and a control protein, human KIR3DL3 (aa 22–323) were amplified and cloned into the mammalian expression vector Sigplg Plus consisting of the CD33 signal sequence and a human IgG1 Fc tag. The plasmids were transfected into HEK 293 cells. Stably transfected cell lines were maintained in RPMI 1640 with 2% low IgG FCS (Invitrogen). Secreted Fc fusion proteins were purified on protein A-Sepharose columns. Proteins were secreted predominantly as dimers. Reduced monomers migrated at their predicted sizes.

Ab production

A polyclonal rabbit antiserum against the exoplasmic domains of mouse BTN2A2 (anti-BTN2A2) was raised commercially using BTN2A2-Fc protein as immunogen (Covalab, Villeurbanne, France). An IgG fraction was purified from 1ml of the final bleed using protein A-Sepharose, and the Fc reactive species removed using human Ig G agarose (Sigma-Aldrich). A sample of preimmune serum (1 ml) was treated in the same way.

A polyclonal rat antiserum against the exoplasmic domains of mouse BTN1A1 (anti-BTN1A1) was raised commercially (Protein Expression and mAb Facility, Babraham Institute, Cambridge, U.K.). The Fc tail was cleaved from BTN1A1-Fc protein at an endogenous thrombin site (P1 residue is R243) and only the Ig domains were used as immunogen.

Animal husbandry

Female C57BL/6 mice were bought at age 6 wk from Harlan Laboratories (Loughborough, U.K.) and maintained under barrier conditions in the Biological Services facility of the Department of Pathology, University of Cambridge (Cambridge, U.K.). They received standard laboratory food and water ad libitum. All animal experiments were approved by the Ethical Review Committee of the University of Cambridge.

Transfection of 293T and Chinese hamster ovary cells, preparation of cell lysates, and peptide: N-glycosidase F digests

Cells were transfected with jetPEI transfection reagent according to the manufacturer’s instructions (Polyplus Transfection, Illkirch, France). For the preparation of total cell lysates, cells were incubated with Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 0.15M NaCl, and 0.5M EDTA) containing protease inhibitors (Roche, Burgess Hill, U.K.) for 20 min at 4°C, and then centrifuged at 13,000 × g for 20 min. Supernatants were stored at −20°C. N-linked carbohydrates were removed by incubation with peptide: N-glycosidase F (PNGase F) (New England Biolabs, Hitchin, U.K.) for 5 h, according to the manufacturer’s instructions.

Preparation of mouse tissue lysates

Tissues were harvested from adult female C57BL/6 mice. Lactating mammary tissue was harvested on day 2 of lactation from an adult female CD1 mouse. Tissues were homogenized in 2 ml TBS solution (0.01 M Tris-HCl [pH 7.5] and 0.14 M NaCl) containing protease inhibitors (Roche). Samples were clarified through cheesecloth, centrifuged at 1000 × g for 10 min and the postnuclear supernatants stored at −20°C. The protein concentration of the lysates was measured with the Bio-Rad protein assay using BSA as a standard.

Cell surface biotinylation and immunoprecipitation

Biotinylation with EZ-Link Sulfo-NHS-Biotin was performed at 4°C according to the manufacturer’s instructions (Fierce, Rockford, IL). Splenic B cells were purified with an EasySep PE Selection Kit (StemCell Technologies) using anti-CD19-PE. Bone marrow derived DCs were the gift of Prof. A. Cooke (Department of Pathology, Cambridge University, Cambridge, U.K.). Naive and activated T cells were purified from splenocytes by cell sorting 10^6 CD3^+ live cells on a DakoCytoMation MoFlo MLS high-

anti-HA mAb. E, Semiquantitative verification of surface expression of BTN2A2 protein on purified primary B cells, bone marrow-derived DCs, and T cells activated for 72 h with either anti-CD3 and anti-CD28 or Con A. Cell surface proteins were biotinylated, and BTN2A2 was immunoprecipitated from cell lysates of 0.5 × 10^6–2 × 10^6 cells with anti-BTN2A2, but not the preimmune serum, as detected by streptavidin-HRP. Naive and activated T cells were purified from splenocytes by cell sorting, and BTN2A2 protein was immunoprecipitated from equivalent volumes of T cell lysate.
speed cell sorter (DakoCytomation, Carpinteria, CA). T cells were activated for 72 h by incubating splenocytes in flasks coated with 1 μg/ml anti-CD3 and 0.5 μg/ml anti-CD28, or by activating splenocytes with 2 μg/ml Con A and 20 U/ml IL-2. Cells were lysed with NP40 lysis buffer. Each immunoprecipitation was performed with 0.5 × 10^9–1 × 10^9 primary cells, or 2 × 10^6 cultured cells, using low stringency buffer (0.01 M HEPES [pH 7.5], 0.25 M NaCl, 0.1% NP-40, and 0.005 M EDTA) with 3.5 μg purified anti-BTN2A2 antisera or preimmune serum and protease inhibitors (Roche). Proteins were immunoprecipitated with protein A-Sepharose (Sigma-Aldrich), and washed twice with low stringency buffer and twice with RIPA buffer (0.05% Tris-HCl [pH 7.5], 0.15 M NaCl, 1% NP-40, 0.1% SDS, 0.5% NaDOC, and 0.001 M EDTA).

**SDS PAGE and Western blotting**

Proteins were denatured in lithium dodecyl sulfate sample buffer (Invitrogen) and separated on NuPAGE 4–12% Bis-Tris gels with MOPS running buffer (Invitrogen), and transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, U.K.). Western detection was performed in PBS with 0.1% Tween 20 (PBS-T). Blocking steps and Ab incubations were performed in PBS-T with the addition of 5% milk. Detection was performed with a swine anti-rabbit HRP Ab (DakoCytomation), or an anti–HA-tag-HRP monoclonal (3F10) (Roche). Blots of biotinylated proteins were blocked with 3% BSA in PBS-T, and detected with streptavidin-HRP (Sigma-Aldrich). All blots were developed with ECL Western blotting reagent (GE Healthcare).

**Immunohistological studies**

Immunofluorescence on tissue sections of thymi from 4 mo old C57BL/6 mice was carried out as described previously (32). Sections were incubated sequentially with anti-BTN sera, or appropriate negative control serum, followed by a biotinylated rabbit anti-rat secondary Ab (Jackson ImmunoResearch Laboratories, Newmarket, U.K.) to detect anti-BTN1A1 Abs, or biotinylated swine anti-rabbit secondary Ab (DakoCytomation) to detect anti-BTN2A2 Abs. Both were visualized with streptavidin-conjugated (Roche) or biotin-streptavidin (Sigma-Aldrich). All sections were developed with ECL Western blotting reagent (GE Healthcare).

**Flow cytometric analysis**

Transfected Chinese hamster ovary (CHO) cells were harvested, two days after transfection, with PBS containing 0.5 mM EDTA. Lymphocytes were harvested from the spleen and lymph nodes of naive mice by treatment of tissues through a 70 μm cell filter. Activated T cells were obtained by treating splenocytes with 2 μg/ml Con A and 20 U/ml IL-2, or by incubating splenocytes in flasks which had been coated with anti-CD3 (145-2C11) at 1 μg/ml and anti-CD28 (37.51) (BD Pharmingen, San Diego, CA) at 0.5 μg/ml. Splenic NK cells were purified with an EasySep PE Selection Kit (StemCell Technologies, Sheffield, U.K.) using anti–NK-1.1-PE (PK136). Splenic B cells were purified by following the protocol of the ECL Western blotting reagent (Roche) digestion of the spleen, using a CD11c-Positive Selection Kit (StemCell Technologies). Peritoneal macrophages were harvested by washing the peritoneal cavity with HBSS (Sigma-Aldrich) with 2% FCS. FeRs on DCs and macrophages were preblocked with 100 μg/ml mouse IgG (Sigma-Aldrich) and Fc blocking Abs (StemCell Technologies). Cells were stained with 10 μg/ml preimmune or anti-BTN2A2 purified serum in PBS with 1% goat serum (Sigma-Aldrich). A goat anti-rabbit IgG Alexa Fluor 647 secondary Ab (Molecular Probes, Eugene, OR) was used for detection. Where indicated the last 16 h of the assay prior to harvesting onto glass fiber filters. Radioactivity was measured using a 1450 MicroBeta TriLux liquid scintillation and luminescence Counter (Wallac, Gaithersburg, MD). In some assays cell proliferation was measured using the redox indicator Alamar blue (AbD Serotec, Oxford, U.K.), which was added (20 μl/well) at the start of the proliferation assay. Fluorescence (excitation 360 nm; emission 390 nm) was measured using a Synergy HT plate reader. The IL-2 and IFN-γ concentration of tissue culture supernatants was measured after 72 h by ELISA (eBioscience, San Diego, CA).

**Statistical analysis**

T cell proliferation assays were performed in quadruplicate. Data were analyzed by single-factor ANOVA and pairwise t tests between the control-FC fusion protein and BTN-Fc proteins using the Data Analysis tool pack in Microsoft Excel 95.

**Results**

Expression of BTN1A1 and BTN2A2

Before embarking on functional studies, we examined expression of mouse BTNs in detail. Murine Btn1a1 and Btn2a2 mRNAs were measured by quantitative RT-PCR using gene-specific primers and a panel of RNA samples purified from tissues of virgin and lactating female mice. The specificity of all primer pairs was verified (see Materials and Methods). We found >100-fold higher levels of Btn1a1 transcript in lactating mammary tissue, over virgin mammary tissue, demonstrating strong induction of the transcript during lactation. The transcript was also present in spleen and thymus albeit ~10–20 times lower than the level in virgin mammary tissue. Very low levels of the transcript were present in several other tissues (Fig. 1A). National Center for Biotechnology Information expressed sequence tag databases include entries for human BTN1A1 mRNA in tonsillar lymph nodes (e.g., GenBank IMAGE clone BX106561), corroborating our PCR data.

Using Btn2a2-specific primers to perform quantitative RT-PCR on the same panel of cDNAs as used for the Btn1a1 studies, we found the highest level of expression in brain, lymph node, and thymus (Fig. 2A), with very low levels of the transcript evident in other tissues as observed for Btn1a1. These results are similar to our previous studies on the orthologous human BTN2A1 gene where the transcript was detected in all tissues (30).

We developed polyclonal antisera (Materials and Methods) to study the expression of BTN1A1 and BTN2A2 proteins. Both antisera were specific, as assessed by Western blotting of cell lysates from transfected cells (Fig 1B). The major protein species migrated with an apparent molecular mass of ~65 kDa, which is more slowly than the predicted molecular mass of the proteins (58.5 kDa). We also observed additional higher molecular mass bands, suggesting the presence of variably glycosylated isoforms of the proteins. It has already been shown that BTN1A1 protein from several proteins.
mammalian species is glycosylated (33, 34). We examined whether BTN2A2 is glycosylated by treating cell lysates from transfected cells with PNGase F. Following digestion, BTN2A2 protein migrated with an apparent molecular mass of 59 kDa. These data show that the antisera recognized different glycoforms of their specific protein Ags.

A large panel of mouse tissue lysates was screened for BTN1A1 and BTN2A2 expression by Western blotting. BTN1A1 protein was detected in lactating mammary tissue, reflecting the strong induction of this gene in lactation, but was not detectable in other tissues using this technique (data not shown). By contrast, BTN2A2 was readily observed, migrating as a major band of ~65 kDa in all tissues (Fig. 1C). The high level of BTN2A2 transcript detected in brain by quantitative PCR was not reflected at the protein level in the Western result for the whole brain homogenate. This anomaly could be due to differential regulation of BTN2A2 mRNA translation or posttranslational turnover of the protein in the brain.

Members of the related B7S3/Skint gene family are expressed in the thymus, where they regulate γδ T cell development (8). We performed immunohistochemistry on sections of murine thymi to look for BTN protein expression. Both BTN1A1 and BTN2A2 proteins were detected in the thymic stroma (Fig. 1D, 1E in red). The staining pattern was reticular in nature, reminiscent of thymic epithelial cells (TECs). Double-staining experiments using keratin as a panepithelial marker (in green) demonstrated a high degree of colocalization between BTN1A1 and keratin (Fig. 1D in yellow).

**FIGURE 3.** BTN2A2 protein is expressed in a range of rodent cell lines. A, Cells were stained with anti-BTN2A2 (black line) and preimmune serum (dotted gray line, filled histogram) as described in Materials and Methods. Cell lines were from the following lineages: NSO and WEHI-231, B cells; EL4 and BW5147, T cells; RAW 264.7, monocyte macrophage; LTK and 3T6, fibroblast; and RBL, rat basophilic leukemia. B, BTN2A2 protein was immunoprecipitated from a panel of murine cell lines. Cell surface proteins were biotinylated and BTN2A2 was immunoprecipitated from cell lysates with anti-BTN2A2 antiserum but not the preimmune serum.
Expression of BTN1A1 appeared most prominent in medullary areas of the thymus and at the corticomedullary junction. Cells expressing BTN2A2 were frequently clustered together, but there was also some reticular staining. Double staining with anti-keratin Ab also revealed colocalization of BTN2A2 with TECs situated at the corticomedullary junction (Fig. 1E in yellow). The majority of the expression of BTN2A2 in the thymus appeared to be restricted to this region but was not confined solely to TECs.

The widespread occurrence of BTN2A2 protein (Fig. 1C) was also consistent with expression of the protein in circulating cells of the vascular system, as observed for human BTN3A1 (29). Therefore, we explored whether BTN2A2 protein was expressed on naive murine lymphocytes and myeloid cells. In flow cytometric analysis, the anti-BTN2A2 antiserum recognized transfected murine BTN2A2, with negligible cross-reaction with murine BTN1A1 and BTN2F proteins (Fig. 2A). Flow cytometric analysis of stained lymphoid and myeloid cells from spleen, lymph node, and the peritoneal cavity revealed significant BTN2A2 expression on naive B cells, splenic NK cells and DCs, and peritoneal macrophages (Fig. 2B). There was negligible expression of the protein on naive murine CD3+ T cells. Several inhibitory members of the B7 family are expressed on activated T cells, including PD-L1 (21). We observed a similar upregulation of surface expression of BTN2A2 on in vitro-activated CD3+CD25+ T cells after 24 h of stimulation. Furthermore, expression continued to increase over a period of 3 d (Fig. 2C).

To confirm our flow cytometric staining data, we performed a series of immunoprecipitation experiments. Using anti-BTN2A2 antiserum, we immunoprecipitated HA-tagged BTN2A2 protein, but not HA-BTN1A1 protein, from lysates of transfected CHO cells (Fig. 2D). BTN2A2 protein could also be immunoprecipitated from lysates of surface biotinylated B cells, DCs, and activated T cells using the anti-BTN2A2 antiserum but not the preimmune serum (Fig. 2E). Multiple bands were observed in DCs, indicating possible modification or differential splicing. Our findings reflect those for human BTN2A1 (30) and BTN3A1 (29), for which expression of BTN protein was detected on lymphoid and myeloid cells from peripheral blood.

We also tested by flow cytometric analysis whether murine BTN2A2 protein was expressed on mouse and rat cell lines (the sequences of murine and rat BTN2A2 are 91% identical). We detected the protein on a panel of hematopoietic and fibroblast cell lines (Fig. 3A), and biotinylated BTN2A2 protein could also be immunoprecipitated from cell lysates (Fig. 3B). Consistent with this, Compte et al. (29) showed that BTN2A1 is expressed on a large panel of hematopoietic and cancer cell lines, and we have shown expression of BTN2A1 on embryonic fibroblast and kidney cell lines (30).

In light of these data, we conclude that murine BTN1A1 protein is not restricted to mammary tissues, as it is also found, albeit at lower levels, in the thymic stroma. BTN2A2 protein is expressed on TECs as well as lymphoid and myeloid cells and is increased on the surface of T cells by activation. With this in mind, we set out to characterize expression of receptors for these molecules.

Expression of a putative receptor for BTN1A1 and BTN2A2 on activated T cells

Previous studies have shown that a putative receptor for BTN2L2 is expressed on activated T cells (11). We investigated whether similar receptors for BTN1A1 and BTN2A2 are expressed on stimulated T cells. We purified soluble mouse BTN1A1-Fc, BTN2A2-Fc, and BTN2L2-Fc fusion proteins, which make up the extracellular Ig domains of each BTN protein fused to the Fc domain of human IgG1. The proteins were biotinylated and were used in binding studies with CD3+ T cells that had been stimulated for 72 h with Con A and IL-2. Biotinylated human IgG did not bind to either naive or activated T cells (Fig 4, filled histograms), and neither did the biotinylated Fc domain (data not shown). By contrast, all three biotinylated BTN-Fc fusion proteins bound to activated, but not naive, T cells from lymph nodes (Fig. 4, open histograms) or spleen (data not shown).

BTN1A1 and BTN2A2 inhibit CD4+ and CD8+ T cell activation

BTN2L2 was reported to inhibit T cell proliferation (10, 11). To investigate a possible immunomodulatory role of the other mouse BTN molecules, we performed in vitro T cell proliferation assays with primary mouse T cells in the presence of BTN1A1-Fc, BTN2A2-Fc, and murine MOG-Fc fusion proteins. When CD4+ T cells, purified from mouse spleen and lymph nodes, were activated with immobilized anti-CD3 Ab 145-2C11, inhibition of T cell proliferation was observed in the presence of both BTN1A1-Fc and BTN2A2-Fc fusion proteins. The degree of inhibition, between 40 and 65%, was similar to that observed with BTN2L2-Fc (Fig. 5A). No inhibition was observed when anti-CD3 was presented in the presence of human IgG, nor with a control Fc fusion protein containing the three Ig domains of human KIR3DL3 (control-Fc), nor with the purified Fc tail alone, demonstrating that the inhibitory effects of the BTN-Fc fusion proteins were specific to the extracellular domains of these proteins. Furthermore, no inhibition of proliferation was observed with a murine MOG-Fc fusion protein, showing that the inhibitory effect was a property of the BTN protein fold. The three BTN-Fc fusion proteins inhibited proliferation across a range of
FIGURE 5. Inhibition of CD4+ T cell proliferation by BTN1A1 and BTN2A2.

A. CD4+ T cells were activated with plate-bound anti-CD3, coated at 1 µg/ml, in the presence of BTN1A1-Fc, BTN2A2-Fc, or BTN1L2-Fc fusion proteins, or control proteins, all coated at 10 µg/ml. The control proteins were human IgG (Hu IgG), the Fc domain alone (Fc tail), and KIR3DL3-Fc (control-Fc). MOG-Fc did not inhibit proliferation. B. CD4+ T cells were activated with increasing doses of anti-CD3 in the presence of BTN-Fc fusion proteins or control Fc protein, and inhibition by the BTN proteins was observed at all anti-CD3 concentrations. C, CD4+ T cells were activated with plate-bound anti-CD3 with increasing amounts of BTN protein as described in Materials and Methods. D, BTN-Fc fusion proteins inhibit CD4+ T cell proliferation when the proteins are bound to the plate but not when present in solution at 10 µg/ml. Statistical analysis by single-factor ANOVA indicated a significant difference between the data groups when the proteins were presented plate bound (p < 0.0001) but not when added in solution (p = 0.27). E, Potent costimulation of CD4+ T cells with anti-CD28 abrogates inhibition by BTN1A1-Fc and BTN2A2-Fc. Plates were coated with anti-CD3 (1 µg/ml) and anti-CD28 (0.5 or 2 µg/ml) together with goat anti-human Fc (10 µg/ml). BTN-Fc and control proteins were coated at 10 µg/ml. Data are the means of quadruplicate samples, and error bars indicate the SD of the mean.

anti-CD3 concentrations (Fig. 5B), and the negative effect on cell division was dose dependent (Fig. 5C). Previous data on BTN2L demonstrated that the inhibitory effect of the Fc fusion protein was only evident when the protein was presented bound to the plate, rather than in solution (11). We also found that BTN1A1-Fc and BTN2A2-Fc inhibited proliferation when plate bound but not when added to the media at a concentration of 10 µg/ml (Fig. 5D).

Ligation of CD28 molecules on T cells provides a potent costimulatory signal for T cell activation. Therefore, we included a stimulatory anti-CD28 Ab, together with anti-CD3 and the BTN-Fc fusion proteins in the CD4+ T cell proliferation assays. Coating the 96-well plates with modest concentrations of anti-CD28 (0.5 µg/ml) stimulated proliferation of the CD4+ T cells but did not block the inhibition effected by the BTN-Fc fusion proteins. At higher concentrations of anti-CD28 (2 µg/ml), there was a further increase in T cell proliferation in all the samples, and this stimulatory effect was sufficient to overcome the inhibition of the BTN-Fc fusion proteins seen previously (Fig. 5E). This result is consistent with modulation of cellular activation by integration of signals from multiple receptors, as is the case in other situations, such as NK cell activation (35).

To examine the effect of the BTN-Fc fusion proteins on cytokine production by CD4+ T cells, we measured the concentration of IL-2 in tissue culture supernatants after 72 h of stimulation with anti-CD3. We frequently observed that samples incubated in those wells of the 96-well plate, which had not had received a second coating of fusion proteins (i.e., the anti-CD3 samples), had higher levels of IL-2 than the negative control samples. This effect was also observed by Arnett et al. (10) in their studies using the same two-step coating method. By contrast, the lowest levels of IL-2 secretion were always recorded in the presence of BTN1A1-Fc and BTN2A2-Fc, and this was significantly lower than the level recorded with the control-Fc fusion protein or MOG-Fc (Fig. 6A). Strikingly, at this time point (72 h), BTN1A1-Fc and BTN2A2-Fc routinely inhibited IL-2 production to a greater extent than BTN1L2-Fc. We also measured IL-2 production by CD4+ T cells at earlier time points. After 24 h, the amount of IL-2 in tissue culture supernatants was below the level of detection of our ELISA system (data not shown). At 48 h, we observed lower levels of IL-2 than we recorded at 72 h, and that treatment with any of the three BTN proteins significantly reduced the IL-2 concentration in the media (Fig 6B). IL-2 production by the CD4+ T cells was strongly dependent on the concentration of anti-CD3 used for coating the plates; at low levels of anti-CD3 (0.5 µg/ml), very little IL-2 was secreted even after 72 h of incubation (Fig. 6C). In fact, the level of IL-2 in the BTN-Fc–treated samples was below the level of detection of the ELISA. Notably, the inhibition of IL-2 production by BTN1A1-Fc and BTN2A2-Fc was observed at all anti-CD3 concentrations (Fig. 6C).
We also measured whether the BTN-Fc proteins inhibited IFN-γ production by CD4+ T cells after 72 h of incubation. Although we saw some variation in IFN-γ production in our negative control and MOG-Fc samples, all three BTN fusion proteins always inhibited IFN-γ secretion (Fig. 6D).

Nguyen et al. (11) demonstrated that the addition of exogenous IL-2 was not sufficient to overcome the inhibitory effect of BTNL2 on T cell proliferation. We also observed that inhibition by all three BTN-Fc fusion proteins was not overcome by culturing CD4+ T cells at all concentrations of anti-CD3. CD4+ T cells were activated with plate-bound anti-CD3 (1 μg/ml) in the presence of BTN-Fc, MOG-Fc, or control-Fc fusion proteins (10 μg/ml). After 72 h, BTN1A1-Fc and BTN2A2-Fc inhibited IL-2 secretion at all concentrations of anti-CD3. CD4+ T cells were stimulated with anti-CD3 (1 μg/ml) in the presence of BTN-Fc or control proteins (10 μg/ml) with IL-2 (4 ng/ml; 20 U/ml). Incorporation of [3H]thymidine was measured after 72 h. The data are representative of more than three experiments, with error bars indicating the SD of the mean of quadruplicate samples.

Discussion
All three BTN proteins, BTN1A1, BTN2A2, and BTNL2, appear to engage receptors on T cells and inhibit T cell activation (Fig. 5) (10, 11), consistent with a conserved function, namely to raise the
threshold for T cell activation. At present, little is known about the identity of the putative inhibitory receptor for the BTN proteins on T cells. Binding studies with biotinylated BTN-Fc fusion proteins demonstrated that the cognate receptor is not expressed at appreciable levels on naive T cells (Fig. 4). However, inhibition of cellular metabolism by BTN proteins was evident within 24 h of T cell activation (Fig. 8A), suggesting that the receptor may be present at a low level on naive T cells and/or is rapidly upregulated in response to ligation of the TCR.

A single receptor may engage all three BTN molecules, or they could have separate receptors, a consideration that may also be applied to the additional human gene, BTN3A1 (29). The receptor is unlikely to be any of the known inhibitory receptors on T cells, namely CTLA4, PD-1, or BTLA (10, 29). Nor would it be DC-SIGN, the C-type lectin molecule recently shown to bind human BTN2A1 in cancer cell lines, as binding was mediated by high mannose modifications in the glycans of human BTN2A1 and was specific to tumor cell lines (30).

The expression of BTN1A1 and BTN2A2 throughout the stroma of the thymus (Fig. 1D,1E) suggests that BTN proteins may be involved in regulating stages of thymopoiesis, as observed for Skint 1 (8). TECs are unique in that they are highly heterogeneous and, unlike other epithelial cells, are arranged in a three-dimensional network. Several studies have shown that mammary and thymic epithelia express various markers in common (37, 38). It is interesting to note that BTN1A1 and BTN2A2 were expressed on TECs at the junction of the cortex and medulla, which is the site where T cell precursors enter the thymus via blood vessels (39).

The inhibition of T cells by BTN1A1 was unexpected (Fig. 5), because this protein has previously only been linked to the secretion of milk-lipid droplets during lactation (4). However, this function is applied...
probably contingent upon the intracellular portion of the protein; an interaction between the cytoplasmic B30.2 domain of BTN1A1 and xanthine oxidase (5, 6) is postulated to facilitate the budding of milk fat globules from the cell apex of mammary epithelial cells. Negative regulation of T cell activation by BTN1A1 would be mediated via IgV and IgC domains and could indicate an additional unsuspected role for the protein. This exoplasmic surface of BTN1A1 is displayed on the mammary epithelium and secreted milk-lipid droplets (33), and BTN1A1 is also a constituent of exosomes, 50-nm membrane-bound vesicles, which are present in breast milk and have been shown to inhibit T cell activation (40). What could be the value of the neonate of a molecule that modulates T cell activation in milk? In humans, BTN1A1 protein is rapidly degraded in the stomach of breast-fed infants (41), suggesting that its immunological functions in the neonate are limited. However, until the half-life of BTN1A1 in the gastrointestinal tract is measured and the BTN1A1 breakdown products are characterized, we cannot rule out a role for BTN1A1 in neonatal immunity.

Another possibility is that BTN1A1 expression in lactation may regulate maternal T cell function in the mammary gland and milk. T cells are present in lactating mammary tissue and enter the milk in several mammalian species, including cow, rat, and human (42). Human milk T cells can be activated by Con A to produce IL-2, and T cells are present in lactating mammary tissue and enter the milk in late stages of gestation; thus, BTN1A1 may be expressed in milk at that time. We cannot rule out a role for BTN1A1 as an inhibitor of T cell activation in milk, because BTN1A1 is also present in human milk (43). BTN1A1 may be expressed in milk by maternal T cells, which can be activated by Con A to produce IL-2, and T cells are present in lactating mammary tissue and enter the milk in late stages of gestation. However, until the half-life of BTN1A1 in the gastrointestinal tract is measured and the BTN1A1 breakdown products are characterized, we cannot rule out a role for BTN1A1 in neonatal immunity.

Maternal T cells are transferred to the neonate during breastfeeding, by trans-epithelial migration, and BTN1A1 protein may prevent activation of the cells prior to and during transfer. Such maternally inherited T cells, acquired during breastfeeding, protect mice against rejection of semiallogeneic skin grafts (47) and affect delayed-type hypersensitivity (48). Studies on EAE, a model for the disease MS, have demonstrated a protective effect of BTN1A1 protein on the development and severity of the disease (17, 18). Immunization with BTN1A1 protein has been shown to cause T cell anergy in response to MOG, as well as the differentiation of a protective source of regulatory cells (18). These effects were ascribed to molecular mimicry between BTN1A1 and the myelin protein MOG (17). BTN1A1 and MOG presumably have different functions in vivo, and in the EAE experiments BTN1A1 protein may have mediated a more direct effect on the immune system, by regulating T cell activation. It also remains possible that dietary exposure to BTN1A1 may modulate the pathogenic autoimmune response in MS.

There are similarities between the expression patterns of BTN proteins and those of other members of the B7 family. B7 molecules are typically expressed on APCs, and some are upregulated on activated T cells (21). Expression of PD-L1 on activated T cells has functional consequences, as blockade of the PD-L1:PD-1 pathway enhances T cell proliferation and cytokine production (49). We detected BTN2A2 protein on B cells, macrophages, and DCs as well as activated T cells. Recent studies have demonstrated that the inhibitors PD-L1, PD-L2, B7-H3, and B7-H4 are found on a variety of tumors, in which their expression may be linked to tumor immune evasion (50). We (30) and others (29) have shown expression of BTN proteins on cancer cell lines. This raises the possibility that expression of BTN proteins may also occur during cellular transformation and may promote immune tolerance of tumors.

Considering all the data so far, it seems likely that all BTNs influence immune homeostasis and T cell development in the thymus. Regarding BTN2A2, expression in the periphery may help to prevent autoimmune tissue destruction, possibly by damping down nonspecific T cell responses. It is of interest in this regard that in the Mdr 1a mouse model of inflammatory bowel disease, there is increased expression of BTN2L2 transcript and protein in inflamed colonic tissue (10). Genetic studies have also implicated BTN2L2 in a variety of human autoimmune diseases (13). Increased expression of BTN2A2 on activated T cells is of particular interest as these cells also appear to express the putative receptor. It is possible that BTN2A2 interaction with its receptor regulates T cell activity as clones expand, either in trans (receptor on one T cell interacting with ligand on another T cell) or cis (receptor and ligand on the same T cell). The potential of BTN1A1, expressed in milk, to influence T cells is intriguing. Clearly, a critical next step is to identify the receptors for BTN molecules.

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Disclosures

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References
