SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Mouse BTN1A1 expression in B16-F0-OVA cells and CRISPR-mediated knockout in MC38 cells. (A) Antigen-specific T cell responses were examined by measuring the percentage of surviving OT-I T cells (7.5 x 10^5) was analyzed by flow cytometry after co-culture with 3 x 10^5 irradiated of 293T-K^b-Ova (MHC-I-OVA) cells in a 6-well plate. **(B)** B16-F0-OVA cells were transduced using a lentivirus encoding mBTN1A1 gene and selected with puromycin (10 μg/ml). After single-cell sorting, clones expressing low, medium, and high levels of mBTN1A1, as determined based on the MFI and percent mBTN1A1 positivity, were selected by flow cytometry. **(C)** Cellular growth kinetics over time were measured with a real-time Incucyte imaging system. **(D)** Changes in body weight of B16-F0-OVA-engrafted C57BL/6 mice. These data correspond to animals in Figure 2A. **(E)** Changes in body weight of B16-F0-OVA-engrafted SCID mice. These data correspond to animals in Figure 2B. **(F, G)** A CRISPR approach was used to knock out murine BTN1A1 in MC38 cells. BTN1A1 knockout in cell lines derived from single clones (SC#3, SC#4, and SC#5) was confirmed by Western blotting with an anti-mBTN1A1 antibody (F) and by flow cytometry (G). **(H)** Growth kinetics of knock-out cells over time were determined using a real-time Inucyte imaging system. **P<0.01, Unpaired Student's t-test. EV: Empty vector; OVA: Ovalbumin; ICP: Immune checkpoint protein. Data are representative of at least two replicate experiments.

Figure S2. CRISPR-mediated generation of mBTN1A1 knockout mice. (A) Schematic of the Cas9/sgRNA-targeting sites in mouse BTN1A1. sgRNAs targeting mBTN1A1 exon 3 and exon 4 were microinjected into fertilized embryos of C57BL/6J mice. **(B)** After breeding and heterogenous intercrossing, genotyping was conducted by genomic PCR. WT and KO

mBTN1A1 isoforms produce ~3000 bp and ~200 bp PCR products, respectively, due to the deletion between exon 3 and exon 4 in the KO mice. **(C)** The PCR products were cloned into a TA cloning vector and sequenced to determine the deletion sites. Bases between exon 3 and exon 4 were deleted as shown in blue. **(D)** The expression of mBTN1A1 was assessed in the female mammary fat pads of btn1a1 WT, Herero, and KO mice. Anti-mBTN1A1 (STCM5) was used for Western blotting. **(E)** Immunohistochemical (IHC) analysis of snap-frozen lactating mammary fat pad sections. Frozen mammary fat pad sections from female knockout mice were fixed in acetone and stained with anti-mBTN1A1 (STC109). IHC images of wild type and BTN1A1-knockout mammary fat pad are shown. **(F, G)** mBTN1A1 and mBTN2A2 mRNA expression in wild type and mBTN1A1-knockout mice. Total RNA was extracted from the indicated organs and analyzed via qRT-PCR using TaqMan probes.

Figure S3. Phenotypic characterization of 12-month-old BTN1A1-KO mice. (A) Histological analyses of 12-month-old BTN1A1-KO mice was performed and compared with tissue samples from wild-type mice. Lymphoid proliferation and lymphocytic infiltration in BTN1A1-KO mice are indicated with arrows. (B) Representative tissue samples exhibiting lymphoid proliferation in the thyroid gland follicles (top left); the pancreas with an adjacent mesenteric lymph node exhibiting lymphadenopathy (top right); lung tissue exhibiting consolidation and the presence of macrophages, fibrin, and edema in the alveolar lumen, perivascular lymphocytic aggregates with hemorrhage, and histiocytic pneumonia (bottom left); and the pancreas with perivascular lymphocytic aggregates (bottom right).

Figure S4. Evaluation of *in vitro* and *in vivo* correlations between PD-L1 and mBTN1A1 expression in tumor-bearing mice. **(A-C)** qRT-PCR analyses were used to measure endogenous hBTN1A1 (**A**) and human PD-L1 (**B**) expression *in vitro* using gene-specific primers and the SYBR dye. **(C)** Negative correlation between BTN1A1 and PD-L1 in qRT-PCR analyses. Expression levels were measured via the $2^{\Delta\Delta Ct}$ method and are depicted as log2 transformed values for each transcript. Individual dots correspond to average values for the cell lines shown in (A-B). **(D-F)** The *in vivo* expression of PD-L1 and mBTN1A1 was assessed in mice bearing B16-OVA-EV or B16-OVA-mBTN1A1 tumors. Tumor (T) and non-tumor cells (NT) were isolated using a tumor dissociation kit and tumor cell isolation kit according to the manufacturer's instructions (Miltenyi Biotech) after implantation. **(D)** Cells were then lysed with RIPA buffer, and 20 μg of cell lysates were subjected to Western blotting under non-reducing conditions. Anti-mBTN1A1 (STCM5) and anti-PD-L1 (10F.9G2) were used for Western blotting. **(E, F)** Densitometric analyses were performed for immunoblots shown in (D) using the Image Lab software (Bio-Rad). Relative expression was calculated by normalizing band intensity with to that of β-actin, with lines demonstrating the expression of PD-L1 and mBTN1A1 in matched T or NT samples from individual mice. **(G, H)** Caco-2 cells were treated with IFN-γ (0, 10, 20, or 50 ng/ml) for 48 h in a 6-well plate. Cell lysates were subjected to Western blotting using anti-BTN1A1. MDA-MB-231 cells were used as a positive control for PD-L1 expression. Western blotting results (**G**) and densitometric quantitation of hBTN1A1 expression (**H**) are representative of three independent experiments. *P<0.05 (Student's t-test).

Figure S5. Co-culture of cancer cells with activated T cells induces BTN1A1 expression. **(A)** WT Caco-2 or BTN1A1-KO Caco-2 cells were incubated with primary donor T cells (1:10 ratio)

with or without anti-CD3 and IL-2 (activated and non-activated T cells, respectively) for 72 h or IFN-γ (100 ng/mL) for 48 h. The expression of hBTN1A1 was analyzed by qRT-PCR using from the total RNA isolated from these cells and gene-specific primers. **(B)** A549 and NCI-H226 cells were incubated with activated or non-activated T cells, and the expression of BTN1A1 was analyzed as described in panel A. **(C)** The expression of BTN1A1 proteins on the surface of the indicated cells was analyzed by flow cytometry using an AF488-conjugated anti-BTN1A1 antibody (STC810-AF488). *P < 0.05 (unpaired Student t-test). Data are representative of at least three repeat experiments

Figure S6. Expression of mBTN1A1 and mPD-L1 in tumors from LLC- and 4T1 cell-engrafted mice. (A) Representative histograms of mBTN1A1 (upper) and mPD-L1 (lower) expression on immune cells (CD45+) and tumor cells (CD45-CD31-) in LLC tumors harvested on the indicated days following the implantation of tumor cells. Red: isotype, blue: anti-BTN1A1 or anti-PD-L1. **(B)** Representative histograms of mBTN1A1 (upper) and mPD-L1 (lower) expression on immune cells (CD45+) and tumor cells (CD45-CD31-) in 4T1 tumors harvested on the indicated days following the implantation of tumor cells. Red: isotype, blue: anti-mBTN1A1 or anti-mPD-L1. **(C)** Mean fluorescence intensity (MFI) values for tumor cells (upper) and non-tumor cells (lower) from LLC tumors shown in (A). **(D)** MFI of tumor cells (upper) and immune cells (lower) from 4T1 tumors shown in (B). All data are representative of at least two independent experiments.

Figure S7. Screening of mouse surrogate anti-mBTN1A1 antibodies for *in vivo* efficacy. (A-D) To assess the activity of an anti-mBTN1A1 surrogate antibody (STC109) produced in rats, a

T cell killing assay **(A, B)** was performed as in (A-B). **P<0.01, rat IgG control vs. STC109 (50 mg/ml). T cell proliferation (**C**) and activation (**D**) were measured by flow cytometry after activation with beads conjugated with anti-CD3/anti-CD28/mBTN1A1-Fc or anti-CD3/anti-CD28/Fc (T cells: Beads = 2:1). CFSE-labeled T cells were used for T cell proliferation assays, and dye dilution in the presence of STC109 or rat control IgG was measured (C). Unpaired Student's t-test, ****P<0.0001, rat IgG vs. STC109. T cell activation was measured based on the population of $CD69^+$ T cells (D), revealing that STC109 significantly increased T cell proliferation and activation that was suppressed by bead-bound BTN1A. Unpaired Student t-test, *P<0.05 for rat IgG vs. STC109 50 mg/ml **P<0.01, no antibody vs. STC109. (**E**) WT and BTN1A1 knock-out Jurkat T-cells were activated by anti-CD3 (OKT3) and anti-CD28 for three days and PD-1 expression in cells was measured by western blot. Higher PD-1 expression was observed in the BTN1A1-KO activated T-cells when compared to the WT activated T-cells, suggesting that BTN1A1 negatively regulates PD-1 expression during T-cell activation. EV, Empty vector. All data are representative of at least two independent experiments.

Figure S8. Preparation of STC810, a monoclonal antibody specific for dimerized glycosylated human BTN1A1. (A) Cross-linking assay for native BTN1A1 in overexpressing-293T cells (left) performed using EDC and glutaraldehyde (Glu) followed by Western blotting with an anti-Flag antibody. Co-IP experiments were conducted with lysates from 293T cells co-transfected with pCDH-hBTN1A1-Flag and pCDH-hBTN1A1-Myc using anti-Flag M2 beads (right). **(B, C)** To assess the conformational specificity of STC810, Western blotting was performed using both the dimeric BTN1A1-Fc (Fc fusion protein with the extracellular domain of BTN1A1) and monomeric BTN1A1-His (His-tagged extracellular domain of BTN1A1).

Proteins (1 μg) were or were not treated with dithiothreitol (DTT, a reducing agent) and boiled. After separating the proteins via SDS-PAGE (left panel), Western blotting (right panel) with STC810 was performed. Under native conditions (without a reducing agent or boiling), STC810 preferentially recognized the dimerized Fc fusion protein, but not the monomeric His-tagged BTN1A1 ECD, suggesting that STC810 is a dimer-specific antibody. **(D)** BTN1A1 glycosylation site mutants and their stability in PC3 prostate cancer cells were assessed by stably transfecting PC3 cells with expression vectors harboring the N55Q and N215Q mutations disrupting hBTN1A1 site-specific glycosylation. Cells were treated with cycloheximide (CHX, 200 mM) 24 h post-plating. Cell lysates were prepared at several time points after CHX treatment and subjected to Western blotting analysis with an anti-Flag antibody. **(E)** Glycospecific and conformation-specific binding of STC810 to BTN1A1. Cell lysates from BTN1A1-expressing 293T cells were analyzed by Western blotting under native or denatured SDS-PAGE conditions. **(F)** To assess the specificity of STC810 for human BTN1A1, 293T cells transfected with various immune checkpoint genes were analyzed by flow cytometry using STC810. All proteins were Flag-tagged, and flow cytometry was performed with an anti-Flag antibody to assess protein expression. **(G)** Biacore analysis of STC810 binding to monomeric BTN1A1-His and dimeric BTN1A1-Fc. CM5 chip was immobilized with STC810, while various concentrations of BTN1A1 proteins (2 - 64 nM) served as analytes. STC810 exhibited K_D values of 7.8 nM and 0.33 nM for BTN1A1-His and BTN1A1-Fc, respectively. ECD, extracellular domain; WCL, whole cell lysate. All data are representative of at least three independent experiments.

SUPPLEMENTARY METHODS

CRISPR-Cas9 knockout cell line generation

For the CRISPR-mediated knockout of target genes in PC3, Caco-2, and Jurkat T cells, sgRNA1 (5'-TCACATCAGTATGCAAGTTC-3') and sgRNA3 (5'-GAGCAGATGCCCGAGTACCG-3') were co-transfected into cells using the NEON transfection system (ThermoFisher, Carlsbad, CA, USA) as per Integrated DNA Technology (IDT, Coralville, IA, USA) Alt-R CRISPR-Cas9 system-RNP electroporation Neon Transfection system protocols. A single cell was then sorted using BD FACSAria™ Fusion Flow Cytometer (BD, Franklin Lakes, NJ, USA) and confirmed by mismatch assay, sequencing after TA cloning, and Western blotting. For CRISPR knockout murine MC38 cells, a crRNA targeting murine *Btn1a1* (5'-AGCAGATGACGGAGTACCGC-3'; Mm.Cas9.BTN1A1. 1.AA, IDT) was reconstituted at 100 µM in Nuclease-Free Duplex Buffer (IDT). sgRNAs were then mixed at equimolar concentrations with Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550 (IDT) in a nuclease-free PCR tube. crRNA:tracrRNA duplexes were formed by heating samples at 95°C for 5 mins in a PCR thermocycler, then slowly cooling the samples to room temperature. Next, 9 µl of crRNA:tracrRNA duplexes were mixed with 6 µl (180 pmol) of TrueCut Cas9 Protein v2 (Invitrogen, Carlsbad, CA, USA), followed by incubation at room temperature for 10 min to form Cas9 RNPs. Then, 2 x 10^6 MC38 cells were resuspended in 100 µl of Buffer R (Neon Transfection System) and 15 µl of Cas9 RNPs were added to the resuspended cells followed by electroporation (1400 V, 20 ms, 2 cycles), and cells were then cultured in DMEM containing 10% FBS and 1x penicillin/streptomycin. At 3 days post-transfection, ATTO-positive MC38 cells were sorted by FACS. Loss of BTN1A1 was confirmed by mismatch assay, qPCR, Western blotting, and FACS.

Generation of BTN1A1 knockout mice

Btn1a1 KO mice were generated in the Genetically Engineered Murine Model (GEMM) Core of the University of Virginia using the CRISPR/Cas9 system. Briefly, Cas9 mRNA and sgRNAs (sgRNA1 targeting Exon 3, CCGTATACCTCAAAGTGGC, and sgRNA2 targeting Exon 4, TGCATCCAGAATATCCTCCT) were microinjected into fertilized embryos of C57BL/6NJ mice (Figure 1A). Homozygous KO mice were born from a heterozygous intercross and used for phenotypic analyses in parallel with age- and sex-matched wild-type (WT) littermates as a control group. All mice were genotyped 2 weeks after birth via PCR with specific primers (forward CF1, 5'-CCACCCCGAGTTCCTTTCTT-3' and reverse CR1, 5'-AACCCAGCACTAGTTCCACT-3'), and animals were housed according to their sex after weaning. Mice were fed and housed as above.

Human and murine T cell isolation

Frozen peripheral blood mononuclear cells (PBMCs; StemCell Technologies) or PBMCs isolated from Buffy coats (New York Blood Center) were thawed in a 37°C water bath and counted. Cell pellets were then resuspended in 40 µL of buffer per 10^7 total cells. For negative selection, 10 µL of Pan T Cell Biotin-Antibody Cocktail per 10^7 total cells was added, mixed well, and incubated for 5 min in a refrigerator (2-8°C). Then, 30 µL of buffer per 10^7 total cells was added, followed by 20 µL of Pan T Cell MicroBead Cocktail per 10^7 total cells, after which samples were mixed well and incubated for 10 min in the refrigerator (2-8°C). Subsequently, the mixtures were subjected to magnetic cell separation by applying cell suspensions onto pre-equilibrated LS Columns. The flow-through containing unlabeled cells was collected as the T cell-enriched fraction. To collect residual T cells, columns were washed with 3 mL of buffer and combined

with the initial flow-through. For analyses of murine T cells, mice were euthanized, and spleens were isolated and passed through a 70 μm cell strainer (BD Falcon). After erythrocyte lysis, cell suspensions were washed and subjected to pan-T cell isolation according to provided protocols (Pan T Cell Isolation Kit II, 130-095-130, Miltenyi).

Bead-based assays

Dynabeads® were washed with 1 ml of Buffer 1 (0.1 M sodium phosphate buffer, pH 7.4–8.0 or 0.1 M sodium borate buffer, pH 7.6–9.5). To achieve approximately 200 μg of anti-CD3 anti-CD28 antibodies and hBTN1A1-Fc, hPD-L1-Fc or hIgG1-Fc, 1 mL (4×10^8) of beads were used. Beads were resuspended in Buffer 1 (1 mL minus the corresponding antibody volume) and 200 μg of appropriate antibodies and proteins were added to a total volume of 1 mL, followed by incubation for 16–24 h at room temperature with gentle tilting and rotation. Beads were then washed with 1 ml of Buffer 2 (Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% bovine serum albumin [BSA] and 2 mM EDTA, pH 7.4), mixed, and incubated for 5 min at 2-8°C with gentle tilting and rotation followed by the magnet-facilitated removal of the supernatant fraction. This process was repeated twice. To deactivate the remaining free tosyl groups, beads were incubated for 24 h at room temperature in 1 mL of Buffer 3 (0.2 M Tris supplemented with 0.1% BSA, pH 8.5). The beads were washed with 1 mL of Buffer 2 twice and resuspended in 1 mL of Buffer 2 (4×10^8 beads/mL). T cells were counted, and 2-3 x 10^7 cells were added to a 50 mL tube, rinsed with PBS, and resuspended in RPMI-1640 containing 10% FBS and 1x penicillin/streptomycin at 1x10^7 cells/mL. Cells were stained with CFSE at a final concentration of 2.5 μM and gently resuspended. Following a 15 min incubation in a 37°C water bath, 45 mL of RPMI-1640 containing 10% FBS and 1x penicillin/streptomycin was added to these cells to neutralize any

free dye. Following a 5 min incubation, cells were collected and resuspended in pre-warmed RPMI-1640 containing 10% FBS and 1x penicillin/streptomycin. Human/murine T cells (0.2 x 10^6 cells/well in 96-well plates) were co-incubated with the conjugated beads at a ratio of 1:1 (T cells: Beads) for 4 d, with T cell aggregation being imaged on days 2, 3, and 4. On Day 4, cells were harvested by centrifugation at 300×g for 10 min and were washed twice with FACS staining buffer for downstream analysis.

Live-cell imaging analyses of growth kinetics, T cell clustering, and tumor cell apoptosis

To monitor the growth kinetics of engineered cell lines, a live cell imaging system (Incucyte ZOOM) was used in phase-contrast mode. B16-F0-Ova expressing mouse/human BTN1A1, and MC38-BTN1A1 ^{WT} and MC38-BTN1A1^{KO} cell lines were seeded at 2,000 cells/well in 100 µL in a 96-well flat-bottom plate (Corning® 96 Well Clear Flat Bottom Microplate, #3596). The next day, the plate was positioned in the Incucyte ZOOM instrument and allowed to equilibrate for 30 min before scanning. The imaging schedule was as follows: repeat scanning (10x) every 4 h for up to 60 h; Objective 10x; Vessel Type: Corning 356407; Scan Mode: Standard; Scan Pattern: 3 images per well; and Channel: Phase. After scanning, the growth kinetics were analyzed with the Incucyte ZOOM image analysis software. For T cell clustering experiments, 0.2 x 10^6 T cells/well were plated in a 96-well plate in the presence of anti-CD3 (10 mg/mL), IL-2 (10 ng/mL), and hBTN1A1-Fc (5, 10, 50 mg/mL). Human IgG1 was used as a negative control in place of BTN1A1-Fc. Images of activated T cells were taken with the IncuCyte ZOOM imager every 3 h. The Incucyte ZOOM software was used to automatically score and quantify cluster formation. Clusters were defined as cell aggregates occupying an area of at least 2000 mm^2 with an eccentricity (non-circularity) of 0.8 and were expressed as the number of clusters

per square millimeter. Four different locations were imaged in each well over the entire experimental time course.

BTN1A1 induction in MC38 cells

Cytokines present in human milk may influence BTN1A1 expression in mammary epithelial cells during lactation. Certain cytokines and growth factors (IL-7, IL-8, MIP-2, M-CSF, and IGF-I) are known to be expressed in lactating breast tissue. The MC38, CT-26, EMT-6, 4T1, B16-F0, and LLC murine cell lines were therefore treated with IL-7 (100 ng/mL), IL-8 (50 ng/mL), MIP-2 (200 ng/mL), M-CSF (10 ng/mL), and IGF-1 (20 ng/mL) for 24 hours. Cells were then harvested in a cell lysis buffer, and 30 μg of protein from these cells was used for Western blotting performed with the anti-mBTN1A1 monoclonal antibody RS200A (STCube).

Anti-BTN1A1 antibody production

Hybridomas producing monoclonal antibodies reactive against glycosylated human BTN1A1 were obtained via the fusion of SP2/0 murine myeloma cells with spleen cells isolated from human BTN1A1-immunized BALB/c mice (n=6) (Antibody Solutions, Inc., Santa Clara, CA, USA) according to a standardized protocol. Before fusion, sera from the immunized mice were validated for binding to the BTN1A1 immunogen through a FACS analysis. Monoclonal antibody (mAb)-producing hybridomas were then generated. Those hybridomas that produced antibodies were again tested for specificity. To this end, over 100 candidate mAb-producing hybridomas were selected, grown in ADCF media, and their monoclonal antibody-containing supernatants were concentrated and purified. The purified mAbs were tested for their ability to bind BTN1A1 using flow cytometry and in a live-cell imaging assay in which HEK293T cells

expressing BTN1A1 were incubated with anti-human BTN1A1 and with a fluorescently-labeled anti-human Fc secondary antibody. Antibody binding was quantified using the Incuyte ZOOM instrument (Essen Bioscience) every hour, as per the manufacturer's instructions.

Surface plasmon resonance (SPR)

SPR-based analyses of K_D values were performed using a Biacore X100 instrument (GE Healthcare). Mouse IgG1 was immobilized on a research-grade CM5 chip using standard procedures, and antibody was flowed over the chip at 2 mg/mL in HBS-EP buffer. Next, 6 concentrations of BTN1A1, each a 2-fold dilution, were passed over the chip. Sensorgram data were analyzed with the Biacore X100 evaluation software (v 2.0.1) with 1:1 binding kinetics.

Promoter-luciferase reporter assay

DNA corresponding to the human BTN1A1 promoter (997 bp upstream of ORF) and the human PD-L1 promoter (952 bp upstream of ORF) was amplified from the PC3 cell genome by PCR. The promoter region was identified using the LightSwitch™ Promoter Reporter GoClone® Collection (SwitchGear Genomics, Carlsbad, CA). The PCR products were cloned into the pGL4 luciferase reporter plasmid (Stratagene, Santa Clara, CA). Luciferase reporter constructs (200 ng) were cotransfected with 12 ng of pRL-TK Renilla into PC3 cells in which BTN1A1 was knocked out or overexpressed using FuGene HD (Promega, Madison, WI). After a 24 h incubation, luciferase activity was assayed in a Dual-Luciferase Assay (Promega, Madison, WI)

OPAL Multispectral Imaging

For multiplexed IHC analyses using the OPAL protocol (OPAL 4-color fIHC kit, Perkin Elmer NEL794001KT), slides were deparaffinized in xylene and rehydrated using a graded ethanol series. Antigen retrieval was performed in citrate buffer (pH 6.0, low pH buffer) using microwave treatment (MWT; boil for 2 min in 100% in a 1200 W inverter MW and stop when boiling, followed by 15 min at 20% power). After blocking with the OPAL antibody blocking solution, slides were incubated with a primary rabbit polyclonal antibody (Atlas Antibodies HPA011126) specific for BTN1A1 (1:100) for 1 h in a humidified chamber at room temperature, followed by detection using the SuperPicTure™ Polymer Detection Kit (Invitrogen 87-8963). Visualization was accomplished using fluorescein TSA Plus (1:50, 520 OPAL), after which the slides were placed in citrate buffer (pH 6.0) and heated using MWT. The slides were then incubated with a primary rabbit antibody specific for PD-L1 (1:200) for 1 h in a humidified chamber at room temperature, followed by detection using the rabbit SuperPicTure™ Polymer Detection Kit. Her2 was visualized using Cy5 TSA Plus (1:50, 570 OPAL). Slides were again placed in citrate buffer (pH 6.0) and heated using MWT. Nuclei were subsequently stained with DAPI (1:2000), and slides were mounted using Vectashield® HardSet™ Antifade Mounting Medium. Image acquisition was performed with a Vectra Quantitative Pathology Imaging System (Akoya Biosciences).

Flow Cytometry

Cells overexpressing BTN1A1 or empty vector control constructs were isolated via trypsinization and resuspended in Cell Staining Buffer (CSB) (BioLegend, San Diego, CA, US) at a concentration of $2x10^6$ cells/mL, after which 50 µL of cells were added per well of a 96-well round-bottom plate to which 50 µL of 20 µg/mL primary antibody was added, followed by

gentle mixing and incubation for 1 h at 4°C in the dark. Cells were washed with CSB, incubated with anti-mouse IgG-PE conjugate (10 µg/mL) and DAPI (1:100) for 30 min at 21°C in the dark. Cells were then washed, and data were acquired with a Guava EasyCyte HT (Millipore Darmstadt, DE) or FACS Celesta (Becton Dickinson, Franklin Lakes, NJ, US) flow cytometer.

Cross-linking assay

293T cells were transfected with the pCDH-BTN1A1-FLAG vector for 48 h, and RIPA buffer (ThermoFisher) was then used to prepare cell lysates. The lysates were crosslinked with different concentrations of EDC (1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) or glutaraldehyde. Western blot analyses (20 µg per well) were performed under reducing (DTT) and denaturing (boiling) conditions. Proteins were detected using an anti-FLAG antibody and an appropriate HRP-conjugated secondary antibody.

BTN1A1-overexpressing B16-F0-Ova and MC38-BTN1A1$^{-/-}$ tumor growth analyses and CD8^{+} T cell depletion

B16-F0-Ova and B16-F0-BTN1A1 (low, medium, and high expression) or MC38-BTN1A1^{WT}and MC38-BTN1A1^{KO} tumor cells were subcutaneously (s.c.) injected into the flanks of SCID or C57BL/6 mice. Tumor volumes were monitored over time as above. To determine the extent to which the effects of BTN1A1 depend on specific host T cells, CD8^{+} T cells were depleted via the injection of an anti-CD8 antibody (clone 2.43; BioXcell, cat no. BE0061) at -1-, 3-, and 7-days post-MC38-BTN1A1^{WT} or MC38-BTN1A1^{KO} engraftment. The depletion of CD8^{+} T cells was confirmed via flow cytometry analyses by staining splenocytes with antibodies specific for CD4 and CD8 at the end of the study.

Tumor-infiltrating leukocyte collection

Tumors were harvested from syngeneic model mice and minced into 2-4 mm fragments that were dissociated in a gentleMACS C Tube (Miltenyi 130-096-334) containing an enzyme mix using the gentleMACS dissociator (Miltenyi 130-095-937), which was operated with a gentleMACS Program (m_impTumor_02). Samples were then incubated for 40 minutes at 37°C with continuous rotation using the MACSmix tube rotator. After attaching the C Tube upside down onto the sleeve of the gentleMACS Dissociator, the gentleMACS Program (m_impTumor_03) was run to achieve further dissociation. The dissociated samples were subjected to a short spin at 300 ×g to collect the sample at the bottom of the tube, after which samples were resuspended and applied to a MACS SmartStrainer (70 µm) on a 15 mL tube. This strainer was washed with 10 mL of RPMI-1640 or DMEM, and the cell suspension was centrifuged at 300 ×g for 7 minutes.

Sample preparation and NanoString / RNA-seq analysis

PC3 and BTN1A1-OE PC3 cells were treated with IFN-γ for 48 hours. Subsequently, total RNA was extracted, and gene expression analysis was performed using the nCounter® PanCancer IO 360™ Panel, following the manufacturer's guidelines (NanoString Technologies, Seattle, WA, US). Similarly, Caco-2 and BTN1A1-KO Caco-2 cells were also treated with IFN-γ for 48 hours, then total RNA was extracted.

RNA Sequencing

Total RNA was processed for library preparation employing polyA selection. Sequencing was performed on the Illumina HiSeq platform, producing 2x150bp paired-end reads. This sequencing service was outsourced to GeneWiz Inc (https://www.genewiz.com/). The obtained raw sequence reads underwent quality assessments and subsequent processing using Trimmomatic v.0.36 to eliminate potential adapter sequences and low-quality nucleotides. Following this quality control step, the cleaned reads were aligned to the Homo sapiens GRCh38 reference genome available on ENSEMBL using the STAR aligner (v.2.5.2b). The results of this alignment were stored as BAM files. Gene-specific hit counts were determined using featureCounts from the Subread package v.1.5.2. Emphasizing accuracy, only unique reads localized within exon regions were incorporated into the counts. Annotations were sourced from the gene_id feature in the relevant annotation file. For experiments that utilized strand-specific library preparation, the counting process took this specificity into account.

Gene Expression Analysis

The DESeq2 package was employed for the in-depth quantitative analysis of gene expression variances between predetermined sample groups. The Wald's test was pivotal in deriving p-values and log2 fold changes. To designate a gene as differentially expressed, it needed to fulfill criteria of an adjusted p-value less than 0.05 and an absolute log2 fold change greater than 1. A gene ontology (GO) analysis was undertaken using the software GeneSCF v.1.1-p2 to deduce the functional implications of the differentially expressed genes. The goa_human GO dataset served as the reference for classifying genes based on their integral biological processes. This comprehensive analysis yielded a catalog of gene clusters defined by shared gene ontology terms, providing insights into the predominant biological pathways and processes influenced.