Supplementary information, Data S1. Detailed methods

Tumor growth and treatments

In the tumor growth experiments, the cells were suspended in 10 μl of a 1:1 DMEM (Hyclone) and growth factor-reduced Matrigel (BD bioscience) mixture and injected into the mammary fat pads of 6-week-old female nude mice or Rag1-knockout mice. In co-injection experiments, the amount of growth factor-reduced Matrigel was reduced to minimize any effects of growth factors from the growth factor-reduced Matrigel, as described in the previous report [1]. Thus, the cells were suspended in 10 μl of a 4:1 DMEM and growth factor-reduced Matrigel mixture and injected into the mice. MDA-MB-231 cells were co-injected with primary TAMs at a ratio 1:10. For prophylactic treatment, 1×10^4 tumor cells were injected into the breast fat pad of mice. These mice were then allocated to different experimental groups randomly and treated by 10 μg control-IgG, BTN3A3-Fc, BTN3A1-Fc or 50 μg isotype control mAb, anti-BTN3A3 (5E08) mAb i.p. every three days. For anti-BTN3A3 treatment, 1×10^5 tumor cells were injected into the breast fat pad of mice. The tumor volume was calculated. The mice were divided into groups with equal tumor volume and treated by introtumoral injection with 50 μg isotype control IgG or anti-BTN3A3 (5E08) mAb every three days.
For measurement, single tumors were measured weekly using a caliper. The tumor volume was calculated using the equation $(L \times W^2) \times 0.52$ where “L”=length and “W”=width (measured by two observers blinded to the group allocation).

Flow cytometry analysis and cell sorting

For sorting or analysis of tumor cells and tumor infiltrating myeloid cells, stained cells were analyzed on LSR Fortessa (BD Biosciences) and were sorted on FACS Aria Ⅲ (BD Biosciences) with routinely 95% cell sorting purity. For mouse tumor infiltrating myeloid cells analysis, single-cell suspensions from breast tumors were stained with anti-CD45 FITC, anti-CD11b PE-Cy7, anti-MHCⅡ PE, anti-Ly6C APC, anti-Ly6G APC-Cy7, F4/80 APC-R700, anti-CD11c BV421 in PBS containing 0.2% BSA after

pre-incubated with anti-mouse CD16/CD32 antibody. For sorting BTN3A3^{high} or BTN3A3^{low} tumor cells from human xenografts, single-cell suspensions from breast tumors were stained with anti-mouse IgG PE, in PBS containing 0.2% BSA, following incubation with anti-BTN3A3 (5E08) or isotype control for 30 min at 4℃ temperature after pre-incubated with anti-mouse CD16/CD32 antibody and human Fc inhibitor (eBioscience). The GFP^{+} cells were divide to BTN3A3^{high} or BTN3A3^{low} tumor cells. For human tumor infiltrating myeloid cells and tumor cells, single-cell suspensions from surgical specimens were stained with isotype control, anti-BTN3A3 (5E08) or anti-LSECtin (CCB059) in PBS containing 0.2% BSA after pre-incubated with human Fc inhibitor, then were incubated with anti-mouse IgG APC. Single-cell suspensions were then stained with anti-CD45 PE, anti-CD31 PE, anti-CD3 FITC, anti-CD19 FITC, anti-CD56 FITC, anti-CD11b PE-Cy7, anti-CD14 V500, anti-CD15 PE-CY594, anti-CD11c BV421, anti-BDCA-1 PerCP-Cyanine5.5. Before sorting, single-cell suspensions were stained with Fixable Viability Dye (eBioscience) to distinguish live cells. The Median Fluorescence Intensity (MFI) less the MFI of isotype control equals the DeltaMFI.

Immunofluorescence staining

Immunofluorescence staining analysis of LSECtin, BTN3A3, CD68, CD45, CD31 and CD90 expression in tumor biopsies obtained from breast cancer patients or human xenografts were done as described previously [1, 2]. Briefly, 5 μm thick tumor biopsy sections were deparaffinized in xylene and subsequently rehydrated with 100%, 95%, and 75% ethanol, and deionized H_2O. Sections were then placed in antigen-retrieval solution (10 mM sodium citrate, pH 6.0) and boiled 3 min, and then cooled down to room temperature. Subsequently, sections were stained with 1:200 dilution of secondary antibody at room temperature for 30 minutes, following incubation with a 1:100 dilution of goat IgG for 1 hour at room temperature and stained with dilution of anti-LSECtin, anti-CD68, anti-BTN3A3, anti-CD45, anti-CD31, or anti-CD90 antibodies overnight at 4℃. Before analysis the sections were stained with the

fluorescent dye 4'-6-diamidino-2-phenylindole (DAPI) at room temperature for 5 minutes. LSECtin, BTN3A3, CD68, CD45, CD31 and CD90 expression were detected and analyzed using the Zeiss LSM800. The overlap coefficient (>0.65) showed co-localization of LSECtin and CD68 in the clinical specimens.

Co-culture of breast cancer cells with macrophages

In co-culture system with cell-cell contact fashion, 231-NC or 231-sh4 cells were mixed with primary TAMs or THP-1 cell lines at a 1:10 ratio under mammosphere conditions (low-adhesion plate, mammosphere medium described above). In the transwell co-culture system, primary TAMs or THP-1 cell lines were seeded in the transwell insert membrane (with pores of 0.4 μm diameter, Corning). After 1 day culture, tumor cells with GFP were sorted by FACS. The collected cells were extracted RNA for analysis of stemness-related TFs expression.

Adhesion assay

At 30 hours post-transfection, the culture medium was aspirated and the transfected monolayers were washed with DMEM. The cells were then digested by the trypsin (37 ℃, 2 minutes). Digestion was terminated by DMEM with 10% fetal bovine serum before wash twice by the DMEM. The cells was first incubated by the LSECtin-Fc protein at different concentration using DMEM with 3% fetal bovine serum, wash at least three times. For the anti- LSECtin mAb blockade, LSECtin-Fc recombinant protein at 2 μg/ml was pre-incubated with anti-Human LSECtin mAb (Abcam) or isotype control IgG (Abcam) on ice, 60 minutes. For the BTN3As-Fc block, LSECtin-Fc (2 μg/ml) was pre-incubated with BTN3A-Fc (20 μg/ml) on ice, 60 minutes. For the anti-BTN3A3 (5E08) mAb blockade, breast cancer cells were incubated with anti-BTN3A3 (5E08) mAb or isotype contrl IgG (purified from the normal mouse serum with the same method of anti-BTN3A3 mAb purification) at 10 μg/ml. After the cells were incubated by the anti-Human LSECtin mAb (CCB059) (on ice, 90 minutes), washed three times in precooling DMEM. Goat anti-mouse IgG (Biolegend) was then

to incubate the cells (on ice, 30 minutes), wash three times. The cells finally were filtered for flow cytometry.

Expression cloning

Human spleen cDNA library (Invitrogen) screening were performed with transfected HEK293 (human embryonic kidney cell line) cell line obtained from the American Type Culture Collection (Manassas, VA), as described for other ligands [3-6]. Before transfection, the library was transformed into E.coli DH5α Electro-Cells (Takara, Japan) to amplify and get the plasmid concentration more than 1μg/ml to enhance the transfection efficiency (Qiagen). Mammalian Transfection System-Calcium (Promega) was used to transfect the library into HEK293 cells. After adhesion assay, the cells contained positive cDNA library were sorted by the flow cytometry and the positive cDNA were extracted, as described previously [4]. After five successive rounds of sorting, the predominant plasmids from the binding cells were extracted for sequencing.

Western blot analysis

Western blot analysis was performed as described previously [7]. The cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 150mM NaCl, 5mM EDTA, and 0.5% Nonidet P-40) and centrifuged at 16,000×g for 30 minutes to remove debris.

RNA extraction and real-time quantitative PCR

Total RNA was isolated from established human cell lines, patient specimen and human xenografts using the RNeasy Plus Mini Kit (Qiagen). Standard cDNA synthesis reactions were carried out using Reverse Transcription System (Promega) and reverse transcribed products were amplified with the SYBR Green real-time PCR (Toyobo). Expression of *Lsectin* by TAMs or *Btn3a3* by breast cancer cells from human TNBC xenografts or clinical specimens assayed by Realtime-PCR. Realtime-PCR was performed in a real-time PCR machine (iQ5, Biorad) using the primers listed below. The primers of Homo *Btn3a1*, *Btn3a2*, *Btn3a3* and *Lsectin* were purchased from Qiagen.

Results were first normalized to internal control β-actin mRNA. Primers sequences (5' to 3'):

Human *Sox2*	Forward	GCTCGCAGACCTACATGAAC
	Reverse	GGGAGGAAGAGGTAACCACA
Human *Oct4*	Forward	GCTCGAGAAGGATGTGGTCC
	Reverse	CGTTGTGCATAGTCGCTGCT
Human *Nanog*	Forward	TCTGGACACTGGCTGAATCCT
	Reverse	CGCTGATTAGGCTCCAACCAT
Human *β-actin*	Forward	GCATCCCCCAAAGTTCACAA
	Reverse	AGGACTGGGCCATTCTCCTT
Mouse *Lsectin*	Forward	GGTGCCCATCTGGTGATTGT
	Reverse	CAGTGGCTGAAGTTGAGTGAGG

Antibodies and chemicals

The antibodies used in the current study are listed below. Recombinant Human LSECtin-his Protein was purchased from R&D Systems. Insulin, EGF, heparin and hydrocortisone were purchased from Sigma-Aldrich. Basement Membrane Matrix, Growth Factor Reduced was purchased from BD Bioscience. Antibodies used in flow cytometry, Western blot analysis, immunofluorescence and immunoprecipitation:

Monoclonal Antibody	Company	Clone
Anti-β-Actin antibody	Sigma-Aldrich	AC-74
Anti-Mouse CD16/CD32 Purified	eBioscience	93
Anti-Mouse CD45 FITC	eBioscience	30-F11
Anti-Mouse CD45 PE	eBioscience	30-F11
Anti-Mouse CD45 PerCP-Cyanine5.5	eBioscience	30-F11
Anti-Mouse MHC Class II (I-A) PE	eBioscience	NIMR-4
Anti-Mouse CD11b PE-Cyanine7	eBioscience	M1/70
Anti-Mouse Ly6C APC	eBioscience	HK1.4
Anti-Mouse NK1.1 PE	eBioscience	PK136

Anti-Mouse gamma delta TCR APC	eBioscience	eBioGL3
Anti-Human CD45 PE	eBioscience	2D1
Anti-Human CD31 PE	eBioscience	WM59
Anti-Human CD3 FITC	eBioscience	OKT3
Anti-Human CD19 FITC	eBioscience	HIB19
Anti-Human CD56 FITC	eBioscience	TULY56
Anti-Human CD11b PE-Cyanine7	eBioscience	ICRF44
Anti-Human CD277	eBioscience	eBioBT3.1
Mouse IgG1 κ Isotype Control Purified	eBioscience	P3.6.2.8.1
Mouse IgG2a Isotype Control Purified	eBioscience	eBM2a
Anti-Mouse Ly6G APC-Cyanine7	BD Bioscience	1A8
Anti-Human CD14 V500	BD Bioscience	M5E2
Anti-Human CD15 PE-CF594	BD Bioscience	W6D3
Anti-Mouse CD3 PE-Cyanine5	Biolegend	145-2C11
Anti-Mouse MHCⅡ PerCP-Cyanine5.5	Biolegend	M5/114.15.2
Anti-Human LSECtin	Abcam	EPR13724
Anti-Human CD31	Abcam	P2B1
Anti-Human CD45	Abcam	MEM-28
Anti-Mouse F4/80	Abcam	BM8
Anti-Human CD68	Dako	EBM11
Anti-Human BTN3A3	In house	5E08
Anti-Human BTN3A3	In house	4G09
Anti-Human LSECtin APC	In house	CCB059
Anti-Human LSECtin	In house	CCB059
Anti-Human LSECtin	In house	CFD051

Polyclonal Antibody	**Company**	**Catalog number**
Anti-Human BTN3A3 antibody	Sigma-Aldrich	HPA007904
Anti-Mouse IgG APC	eBioscience	17-4010
Anti-Mouse IgG PE	Biolegend	405307
Anti-Rabbit IgG PE	Biolegend	406421
TRITC Conjugated Goat anti-Rabbit IgG(H+L)	ZSGB-BIO	ZF-0316
FITC Conjugated Goat anti-Mouse IgG(H+L)	ZSGB-BIO	ZF-0312

Rabbit Anti-Mouse LSECtin	In house	——

Generation of stable cells using lentiviral infection

The lentivirus were used to knock down or over expression of human BTN3A3. To knock down BTN3A3 expression, the lentivirus was purchased from Genepharmagps (Suzhou, JS, China). The negative control sequence of shRNA-mediated knockdown in MDA-MB-231 and MDA-MB-468 was TTCTCCGAACGTGTCACGTTTC (NC). The mature antisense sequences were GCCACAGATGGATCTCATATC (sh3) and CCCTTCTGCAACAACCAATCA (sh4). To overexpress BTN3A, the lentiviral-based pHAGE-EF-ZsG-DEST plasmid was used to construct vectors for BTN3A1, BTN3A2, BTN3A3, BTN3A3 (E153D), BTN3A3 (E158K), BTN3A3 (I221T), BTN3A3 (N224S), BTN3A3 (△IgC), BTN3A3 (△IgV), BTN3A3(ECD)BTN3A3(ICD). To generate lentivirus, we transfected HEK293T cells with pHAGE-ZsG (for vector control virus), pHage-ZsG-BTN3A1, pHage-ZsG-BTN3A2, pHage-ZsG-BTN3A3, pHage-ZsG-BTN3A3 (E153D), pHage-ZsG-BTN3A3 (E158K), pHage-ZsG-BTN3A3 (I221T), pHage-ZsG-BTN3A3 (N224S), pHage-ZsG-BTN3A3 (△IgC), pHage-ZsG-BTN3A3 (△IgV) or pHage-ZsG-BTN3A3(ECD)BTN3A3(ICD) combined with psPAX2 and pMD2.G. Twenty-four hours after transfection, the medium were replaced with fresh medium and then collected at 24 hour intervals. The collected medium containing lentivirus was centrifuged to eliminate cell debris and was filtered through 0.45μm filters. Cells were seeded at 60 % confluence 12 hours before infection, and the medium was replaced with fresh medium containing concentrated lentivirus. After infection for 24 hours, the medium was replaced with fresh medium.

Recombinant protein purification

The recombinant soluble BTN3A-Fc, human LSECtin-Fc, mouse LSECtin-Fc produced by HEK293T grown in chemically defined medium (Invitrogen) was purified with Protein G agarose columns (GE Healthcare), as described previously [8].

Bioinformatics analysis

The TCGA data set was obtained from the TCGA Data Portal. Patients with incomplete records of mRNA expression or pathology data were excluded. Kaplan-Meier survival curve generation and tests, gene expression comparison across different molecular subtypes and correlation analysis were performed using Prism 5.0 software (GraphPad).

Statistical analysis

No statistical method was used to predetermine sample size. Mice were assigned at random to treatment groups for all mouse studies and, where possible, mixed among cages. For mouse studies, investigators were blinded to the group allocation. Investigators were also blinded when assessing the outcome by immunofluorescence staining, gene expression and sphere-formation. For each data set, the data meet the assumptions of the tests, including normal distribution and homogeneity of variances. Data were analyzed using Prism 5.0 software (Graphpad) and presented as mean values ±SEM.

For image quantification and adhesion rate comparison, statistical significance was assayed by unpaired Student's t test. Statistical differences in the sphere-forming assay was determined by unpaired Student's t test. Statistics analysis of tumor growth were by two-way ANOVA and unpaired Student's t test to compare the means. P values were considered significant as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Supplementary information references

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