Bisecting GlcNAc expression by bone marrow stromal cells modulates TGF-β1-driven macrophage polarization in myeloid leukemias

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Bisecting GlcNAc expression by bone marrow stromal cells modulates TGF-β1-driven macrophage polarization in myeloid leukemias

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Methods

Cell lines and cell culture

Myeloid leukemia cell line KG1a, and BM-derived stromal cell lines HS5 and HS27a, were maintained as previously described.[1] Murine myeloid leukemia line (M-1), mouse M0 macrophages (RAW264.7) and human monocytic leukemia cells (THP-1) were obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). RAW264.7 were cultured in DMEM, while M-1 and THP-1 were cultured in RPMI 1640 (Biological Industries; Kibbutz Beit Haemek, Israel). All medium was supplemented with 10% fetal bovine serum (FBS) (Biological Industries), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). All cells were maintained at 37°C in a 5% CO_2 setting and routinely tested for Mycoplasma with the GMyc-PCR Mycoplasma Test Kit (Yeasen, cat. #40601ES20).

Isolation and culture of primary BM stroma cells

The isolation and culture of human BM stromal cells were described previously. [2] Briefly, the mononuclear cells were isolated from the bone marrow (BM) of MDS/AML patients and healthy donors (HD) using an equal volume of Ficoll solution (Solarbio, Beijing, China). These cells were subsequently cultured in MSC basal medium (MSCBM, Dakewe Biotech, Beijing, China) supplemented with 5% serum replacement (UltraGROTM-Advanced, Helios, USA) and 1% penicillin/streptomycin, and incubated at 37°C in a 5% CO_2 atmosphere. In the Passage 0 phase, stroma cell colony formation was monitored for 8-12 days until cells reached 70%-80% confluence, at which point they were subcultured to generate Passage 1. After an additional 12 days, a second

subculture was performed. During this period, flow cytometry was employed to assess the expression of stroma cell -specific markers ($CD44^+CD90^+CD105^+CD73^+CD45^-$). Furthermore, cell viability was evaluated using the trypan blue exclusion method, alongside appropriate controls to ensure experimental consistency. Stroma cells from Passage 2, Day 4 are routinely used for experiments.

For mouse BM stromal cells, we followed established methods for isolation and culture.[3, 4] In brief, bone marrow was collected from the femurs and tibias of C57BL male mice aged 6–8 weeks old. The cells were carefully suspended and passed through a 70-μm strainer, spun down, and resuspended in MEM-α (Biological Industries) supplied with 10% FBS with 1% penicillin/streptomycin. The fresh media were replaced the next day to remove the unattached cells (Passage 1). Stroma cells were characterized and identified by flow cytometry ($CD105^+CD90^+CD11b^-CD45^-$) at Passage 4 and then used in the following experiments.

Single-cell analysis

The scRNA-seq datasets of AML BM cells and healthy donor BM cells were acquired from the Gene Expression Omnibus (GEO) database (GSE116256). The scRNA-seq data was acquired from BM cells of 16 AML samples and 4 healthy donors. Information regarding the AML cells and cell preparation was retrieved from van Galen et al.[5] The data processing was performed using Seurat v4.4.0 following standard procedures.[6] First, the single-cell datasets from AML patients and healthy donors were integrated using the “merge” function in Seurat. Quality control (QC) measures were applied to filter out genes expressed in fewer than three cells, and cells were retained

if they had between 200 and 3000 unique feature counts and mitochondrial RNA content of less than 10%. Doublets were identified and removed using the scDblFinder package, with a predefined doublet percentage of 0.8% per 1000 cells.[7] After QC, a total of 18,924 high-quality cells were retained for downstream analysis.

The merged dataset was normalized using Seurat “NormalizedData” function with a global-scaling normalization method “LogNormalize”, and multiplied this by a scale factor (10,000 by default). And then scaled by performing Seurat “ScaleData” function with regression of the variation of “nCount_RNA” and “percent.mt”. Performing Seurat “JackStrawPlot” function and “ElbowPlot” function helped to select suitable dimensionality. Dimension reduction analysis was performed by Seurat “RunPCA” function, and non-linear dimensional reduction was performed by Seurat “RunUMAP” function. The Harmony algorithm was applied to correct for batch effects. Cell type annotation was performed based on classical marker genes combined with differentially expressed genes. A predefined gene set related to macrophage polarization[8] was collected and applied Gene Set Variation Analysis (GSVA)[9] to evaluate macrophage functional characteristics.

M0-M1/M2 polarization

Peripheral blood samples were collected from healthy donors. $CD14^+$ monocytes were sorted using microbeads according to the manufacturer’s instructions (Miltenyi Biotec, Somerville, MA, USA), cultured in RPMI 1640 with glutamine (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and exposed to 50 ng/mL CSF-1 for 6 days to generate their differentiation.

THP-1 cells were differentiated into M0 macrophages by incubating with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Beyotime, Nanjing, China) for 48 h. For M1 polarization, the THP-1 ("M0") were incubated with lipopolysaccharide (LPS, 100 ng/mL; PeproTech; Rocky Hill, NJ, USA) and IFN-γ (20 ng/mL, Beyotime) for an additional 24 h. For M2 polarization, the THP-1 ("M0") were incubated with IL-4 (20 ng/mL) and IL-13 (20 ng/mL; Aladdin, Shanghai, China) for 24 h.

In vitro co-culture model

Stromal cells were seeded in the upper chambers of 6-well plates and co-cultured with THP-1 ("M0") for 48 h. CD8+ T cells were isolated from PBMCs using an immunomagnetic bead separation kit (CD8 T cell isolation kit, cat. #480065, BioLegend, San Diego, CA, USA). The CD8+ T cells were then cultured with stromal cells educated TAMs for 48 h in the presence of human T-activator CD3/CD28 Dynabeads (cat. #11151D, Invitrogen, Life Technologies, Carlsbad, CA, USA).

In the co-culture experiment of T cells and stroma cell-educated TAMs, anti-human PD-L1 antibody (2.5 μg/mL; cat. #374502) or isotype control antibody (2.5 μg/mL; cat. #400165, BioLegend) were included in the culture medium. After co-culture, the cells were stained with the indicated antibodies and analyzed by flow cytometry.

For intracellular cytokine staining, CD8+ T cells were stimulated with Leukocyte Activation Cocktail (BD Biosciences, San Jose, CA, USA) for 5 h at 37°C, fixed and permeabilized, and stained with the indicated antibodies for flow cytometry analysis.

Secretome proteomics

Primary stroma cells isolated from BM of MDS/AML patients or HD were cultured in

medium with 10% FBS until 60−70% confluency. The cells (~ 2×10^6 cells) were washed with serum-free medium and incubated at 37°C for 16 h. post-incubation, the conditioned medium was collected and filtered using a 0.45 µm syringe filter (Millipore, Millipore Corp., Bedford, MA, USA) to remove any suspended cells. TFA (0.1%; Sigma-Aldrich, St. Louis, MO, USA) was added to the conditioned medium. Proteins from the conditioned medium were concentrated using tC2 reversed-phase Sorbent (Waters; Milford, MA, USA). The Sorbent, attached to a 10 mL syringe, was conditioned with 5 mL acetonitrile (ACN; Sigma-Aldrich) and equilibrated with 0.1% TFA. The protein sample in 0.1% TFA was loaded onto the cartridge, washed with 0.1% TFA to remove salts. Proteins were eluted with 3 mL of 60% ACN at 0.1 mL/min and concentrated to dryness using a speed vac concentrator.[10]

The concentrated proteins were denatured with 8 M urea, reduced with 5 mM DTT for 1 h at room temperature (RT), alkylated with 20 mM iodoacetamide (IAM) for 30 minutes in the dark at RT, and diluted with deionized water to reduce urea concentration below 2 M. The sample was digested with lysyl endopeptidase (Wako Pure Chemical, Osaka, Japan) for 4 h at 37°C, followed by an overnight digestion with trypsin (Promega Corporation, Madison, WI, USA) at 37°C. The mixture was acidified with 10% TFA to pH < 3 and purified using C18 cartridges (Waters Corp., Taunton, MA, USA). Two-dimensional liquid chromatography/mass spectrometry (LC-MS) was performed using LTQ Orbitrap MS (Thermo Fisher, San Jose, CA, USA) with data analysis by Proteome Discoverer[11] and quantification by MaxQuant (V. 1.5.2.8, maxquant.org).[12]

SDS-PAGE and western blotting

Cells were collected and lysed with RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM $MgCl_2$ and 5% glycerol) containing 1% PMSF. Protein concentration was determined using BCA Protein Assay Kit (cat. #P0011, Beyotime). Proteins (25 μg) from each lysate were separated by electrophoresis in a 10% polyacrylamide resolving gel and transferred onto a polyvinylidene difluoride (PVDF) membranes. After blocking with 3% BSA (cat. #ST023, Beyotime) in Tris-buffered saline containing 0.1% Tween-20 (TBST) at RT for 1 h, membranes were incubated at 4°C overnight in TBS-T containing following antibodies against PD-L1 (cat. #28076), CD80 (cat. #66406), CD163 (cat. #16646), TNF-α (cat. #60291, Proteintech Group, Chicago, USA), ERK (cat. #4695S), P-ERK (cat.cat. cat. #4370S), Smad3 (cat. #9523S,) and P-Smad3 (cat. #9520S, Cell signaling Technology, Danvers, MA, USA) followed by the addition of secondary antibody conjugated with HRP (cat. #A0208, cat. #A0216, Beyotime). Bands were visualized by enhanced chemiluminescence (ECL; Vazyme Biotech, Nanjing, China).

Lectin blotting

Proteins from each sample were separated by 8% SDS-PAGE and transferred onto PVDF membranes. Membranes were soaked in 3% (w/v) BSA in TBST for 2 h at 37 °C, probed with biotin-conjugated PHA-E (cat # B-1125, Vector Labs) overnight at 4 °C, and incubated with VECTASTAIN Elite ABC kit (cat # KT6100, Vector Laboratories) for 1h at RT. Bands were visualized and photographed as described above.

Co-immunoprecipitation (Co-IP)

The Co-IP assay was performed as described previously.[2] Cell lysates were incubated with antibodies against TGFβ1 (cat. #A21245, ABclonal Biotechnology), or Rab7 (cat. #A12308, ABclonal Biotechnology, Wuhan, China) at 4 °C overnight, and then incubated with Protein A/G agarose (cat. #sc-2003, Santa Cruz, CA, USA) at 4 °C for 12 h. Agarose was washed with PBS and collected by centrifugation at 2,000 g for 5 min. Samples were boiled in 1×SDS loading buffer for 10 min and subjected to western blotting analysis.

Enzyme-linked immunosorbent assay (ELISA)

One hundred μL conditioned medium in was placed in each well of a microtiter plate and incubated at 37 °C for 2 h. The plate was washed three times with 200 μL 0.05% Tween 20 in PBS and then blocked with 3% BSA (Beyotime) in PBS for 1 h at 37 °C. The antibody against TFGβ1 and the secondary antibody conjugated horseradish peroxidase (HRP) was added sequentially. After incubating at 37 °C for 1 h, 200 μL tetramethylbenzidine (TMB, Beyotime) was added and the intensity of chromogenic reaction was determined at 450 nm using a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoprecipitation MS (IP-MS)

Protein extraction, IP assay, and SDS-PAGE were performed as described previously[2]. Protein bands were stained with Coomassie Brilliant Blue R-250 (Thermo Fisher) and then decolorized. Target bands were diced into small pieces, destained with 25 mM NH_4HCO_3 in 50% ACN, reduced by 10 mM DTT/ 50 mM NH_4HCO_3 for 1 h

at RT, alkylated with 20 mM IAM/ 50 mM NH_4HCO_3 in the dark for 45 min, and digested with trypsin overnight at 37°C. Digested peptides were dissolved with 60% ACN/ 0.1% TFA and subjected to 2-D LC-MS analysis (Thermo Fisher).

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Tab. S1 Patients list

No.	Diagnosis	Age (years)	Gender	WBC (10^9/L)	Hemoglobin (g/L)	Platelets ($\times10^9$/L)	Neutrophil Count (ANC, 10^9/L)	BM Cellularity	BM Blasts (%)	Karyotype
1	AML	71	F	4.6	75	26	0.1	Hypercellular	41.0%	46,XX,der(7)(q36)
2	AML with MDS-related changes	65	M	5.8	151	98	3.7	Normal	25.0%	Complex karyotype
3	MDS-RS-MLD	57	M	2.8	88	61	1.5	Hypercellular	2.0%	Normal
4	AML	30	M	2.1	93	142	2.3	Hypercellular	29.5%	Normal
5	AML with MDS-related changes	90	F	1.5	96	176	5.2	Hypercellular	22.0%	46,XX,t(2;12)(p13;p15)[6]/46,XX[4]
6	AML	55	F	2.6	60	236	1.1	Hypercellular	65.0%	Normal

7	MDS-RS-MLD	65	M	0.9	104	50	0.5	Normal	4.0%	Normal
8	MDS-EB2	52	M	1.0	62	61	1.1	Normal	15.0%	Normal
9	MDS-EB2	68	M	2.2	75	29	1.8	Normal	19.0%	Normal
10	AML, Minimally Differentiated (M0)	51	F	0.3	59	17	0.1	Hypercellular	58.0%	Normal
11	MDS-EB1	70	M	1.0	52	18	0.7	Hypercellular	5.5%	Complex karyotype
12	AML, Acute Myelomonocytic Leukemia (M4)	79	M	1.0	54	13	1.3	Normal	44.5%	Normal
13	MDS-EB1	81	F	2.2	78	70	1.5	Hypocellular	7.5%	Normal
14	AML	62	F	7.4	83	33	0.8	Hypercellular	21.0%	Normal
15	AML, Acute Myelomonocytic Leukemia (M4)	31	F	4.8	72	23	0.9	Hypercellular	63.0%	Normal
16	MDS-EB2	43	M	3.3	50	15	1.0	Normal	15.0%	Normal
17	MDS-EB1	44	F	3.0	93	74	0.8	Normal	5.0%	Normal
18	MDS-EB2	75	F	3.5	84	9	0.9	Hypercellular	14.0%	Complex karyotype
19	AML	53	F	21.1	71	82	0.1	Normal	23.5%	Normal
20	MDS-EB1	61	F	3.2	53	52	1.9	Hypercellular	8.5%	Normal
21	MDS-EB2	58	F	3.2	78	37	2.1	Normal	18.0%	Normal
22	AML	68	M	1.5	75	9	0.5	Hypercellular	21.5%	Normal
1	HD	36	F							
2	HD	45	F							
3	HD	28	F							
4	HD	61	F							
5	HD	28	F							
6	HD	26	F							
7	HD	23	F							
8	HD	57	M							
9	HD	60	M							
10	HD	28	M							

Fig. S1 (**A**) Dot plot showing the expression of representative marker genes across different bone marrow (BM) cell types. Dot size indicates the proportion of cells expressing each gene, and color intensity reflects the scaled expression level. (**B**) Uniform Manifold Approximation and Projection (UMAP) plots displaying the expression of CD36, CD163, and MRC1 in macrophages. (**C**) Macrophage (M1) functional scores based on single-cell RNA sequencing (scRNA-seq) data from the BM of healthy donors (HDs) and patients with acute myeloid leukemia (AML), analyzed using gene set variation analysis (GSVA). Statistical significance was determined using the Wilcoxon rank-sum test.(**D**) Stacked bar chart showing the proportion of different immune cell types in each sample.(**E**) Confocal microscopy analysis of CD80 and programmed death-ligand 1 (PD-L1) in BM sections from HDs and patients with myelodysplastic syndromes or AML (MDS/AML).(**F**) Flow cytometry (fluorescence-activated cell sorting, FACS) analysis of $CD8^+$ T cells ($CD45^+CD3^+CD8^+$) in BM samples from HDs (n = 6) and MDS (n = 6)/AML (n = 6) patients.(**G**) Confocal microscopy of CD8 expression in BM from HDs and MDS/AML patients.

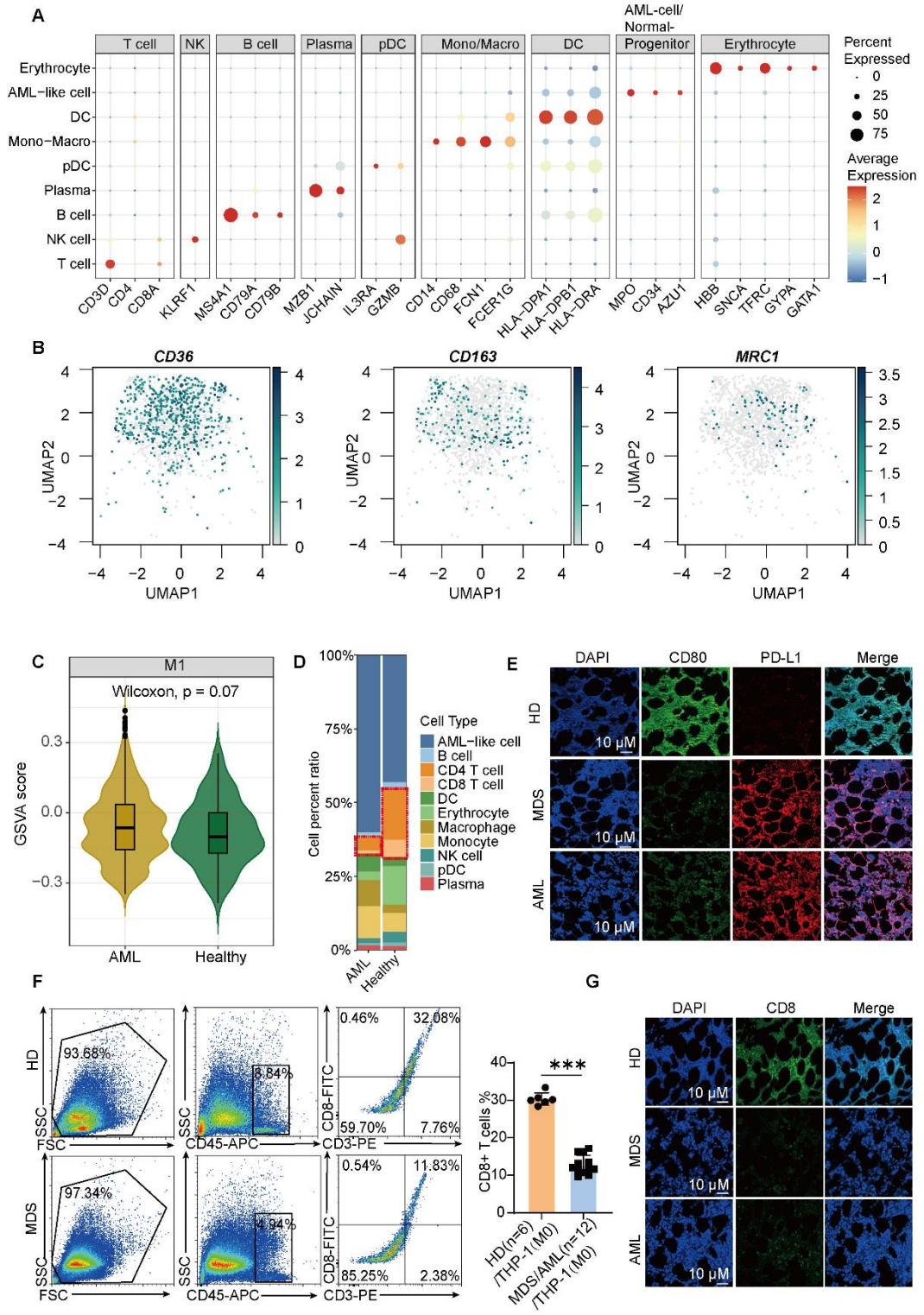


Fig. S2 (**A**) Schematic diagram of in vitro co-culture model. (**B**) Flow cytometry (fluorescence-activated cell sorting, FACS) characterization of bone marrow (BM) stromal cells derived from patients with myelodysplastic syndromes (MDS). (**C**)

Volcano plot showing differentially secreted proteins from MDS BM stromal cells (fold change > 2 or < 0.5; $p < 0.05$).(**D**) Western blot analysis of latency-associated peptide transforming growth factor β1 (L-TGFβ1) and mature transforming growth factor β1 (m-TGFβ1) in BM stromal cells.(**E–H**) Expression of programmed death-ligand 1 (PD-L1), CD163, p-Smad3, Smad3, p-ERK and ERK in THP-1-derived undifferentiated macrophages (M0) treated with recombinant TGFβ1 (rTGFβ1) at indicated concentrations (E), for various time points (F, G), or after co-culture with MDS-derived BM stromal cells (H).(**I**) THP-1 (M0) macrophages were treated with either dimethyl sulfoxide (DMSO), rTGFβ1, or rTGFβ1 combined with anti-human programmed death-ligand 1 antibody (a-hPD-L1), and then co-cultured with activated $CD8^+$ T cells. Production of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in $CD8^+$ T cells was measured by fluorescence-activated cell sorting (FACS) after 48 hours.

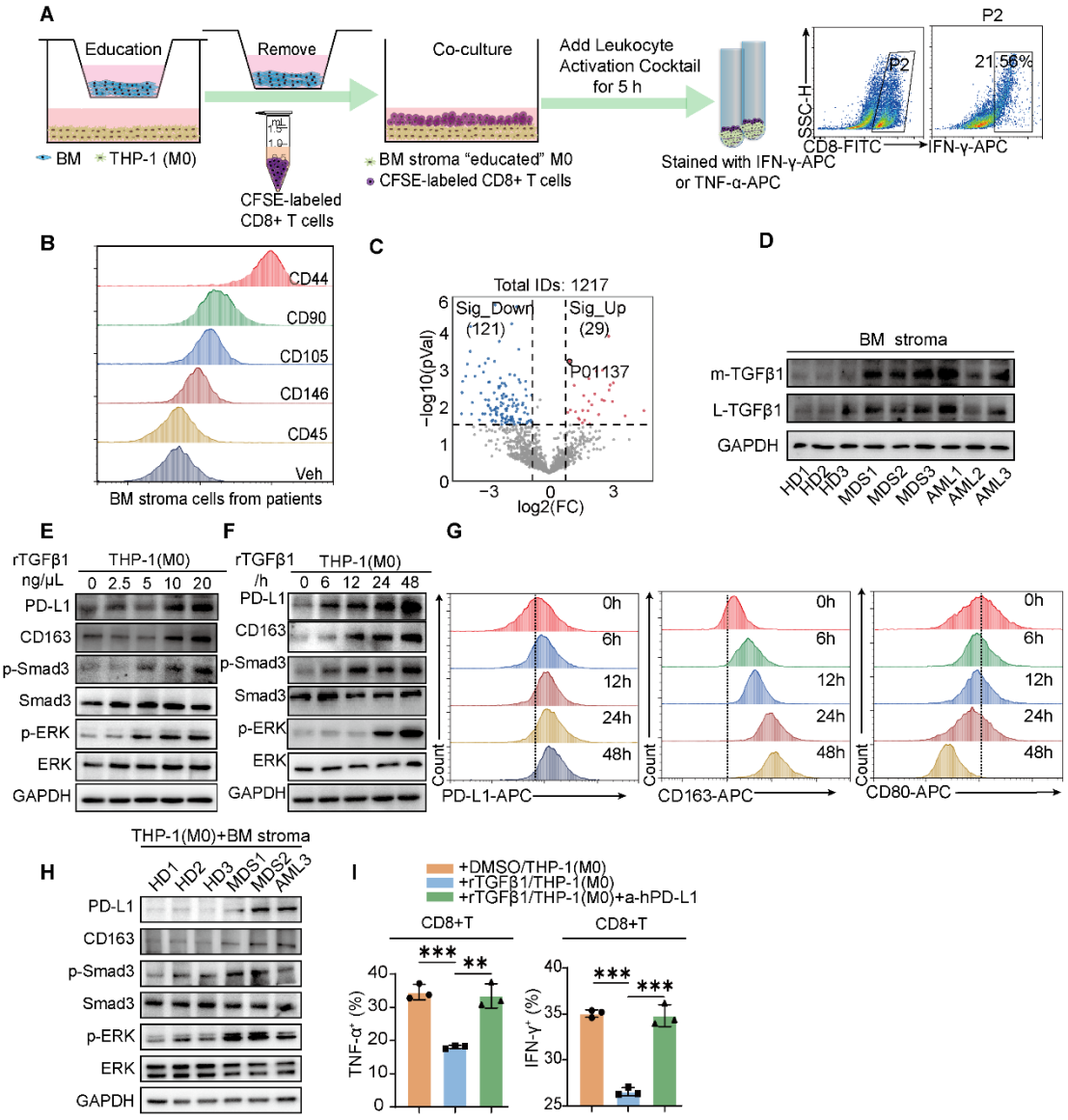


Fig. S3 (**A**) RAW264.7 murine macrophages were treated with either dimethyl sulfoxide (DMSO), recombinant transforming growth factor β1 (rTGFβ1), or rTGFβ1 combined with anti-mouse programmed death-ligand 1 antibody (a-mPD-L1), and co-cultured with $CD8^+$ T cells isolated from healthy donors (HDs) for 48 hours. Production of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) by $CD8^+$ T cells was measured by flow cytometry (fluorescence-activated cell sorting, FACS). (**B**) Schematic of in vivo mouse model. (**C**) Relative expression of $F4/80^+CD11b^+$ macrophages in bone marrow (BM) following clodronate liposomes treatment. (**D**)

Immunohistochemical staining of $CD8^+$ T cells in murine BM.

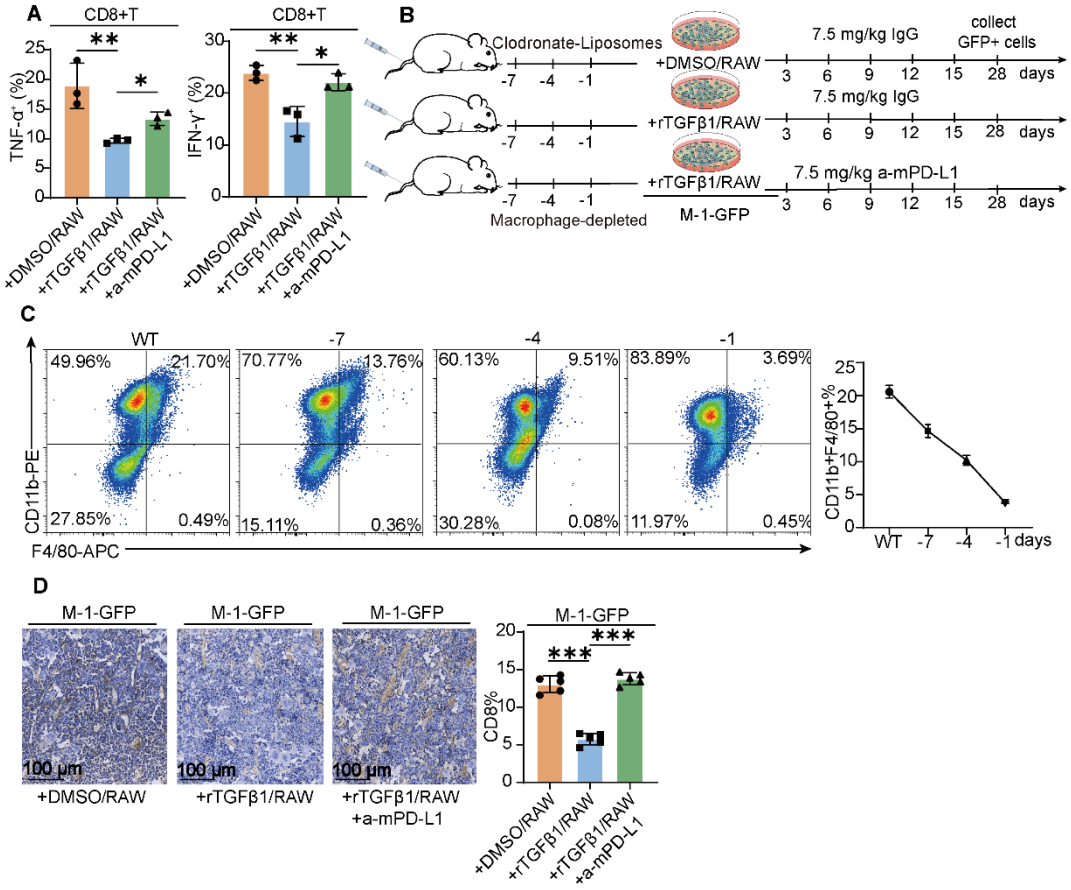


Fig. S4 (**A**) Lectin blot showing bisecting N-acetylglucosamine (GlcNAc) levels in forskolin (FSK)-treated and untreated normal bone marrow (NBM) stromal cells. (**B**) Western blot analysis of polarization-associated genes in THP-1-derived undifferentiated macrophages (M0) co-cultured with FSK-treated or untreated NBM stromal cells. (**C–E**) Flow cytometry (fluorescence-activated cell sorting, FACS) analysis of CD163, CD80, and programmed death-ligand 1 (PD-L1) in macrophages from the same co-culture. (**F**) Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production by $CD8^+$ T cells after co-culture with macrophages educated by NBM or FSK-treated NBM stroma. (**G**) Lectin blot showing bisecting GlcNAc levels in HS27a cells and HS27a cells overexpressing N-acetylglucosaminyltransferase III (MGAT3),

termed HS27a-M3. (**H–J**) Western blot and FACS analysis of macrophage markers in THP-1 (M0) macrophages co-cultured with HS27a or HS27a-M3 stromal cells.(**K**) FACS analysis of PD-L1 expression in the same cells.(**L**) TNF-α and IFN-γ production by $CD8^+$ T cells after co-culture with macrophages educated by HS27a or HS27a-M3 stromal cells.(**M**) Bisecting GlcNAc levels in HS5 cells and HS5 cells with MGAT3 knockdown (HS5-shM3).(**N–P**) Western blot and FACS analysis of macrophage markers in THP-1 (M0) macrophages co-cultured with HS5 or HS5-shM3 stromal cells.(**Q**) FACS analysis of PD-L1 expression in macrophages co-cultured with HS5 or HS5-shM3.(**R**) Cytokine production by $CD8^+$ T cells after education by bone marrow stroma derived from patients with MDS/AML.

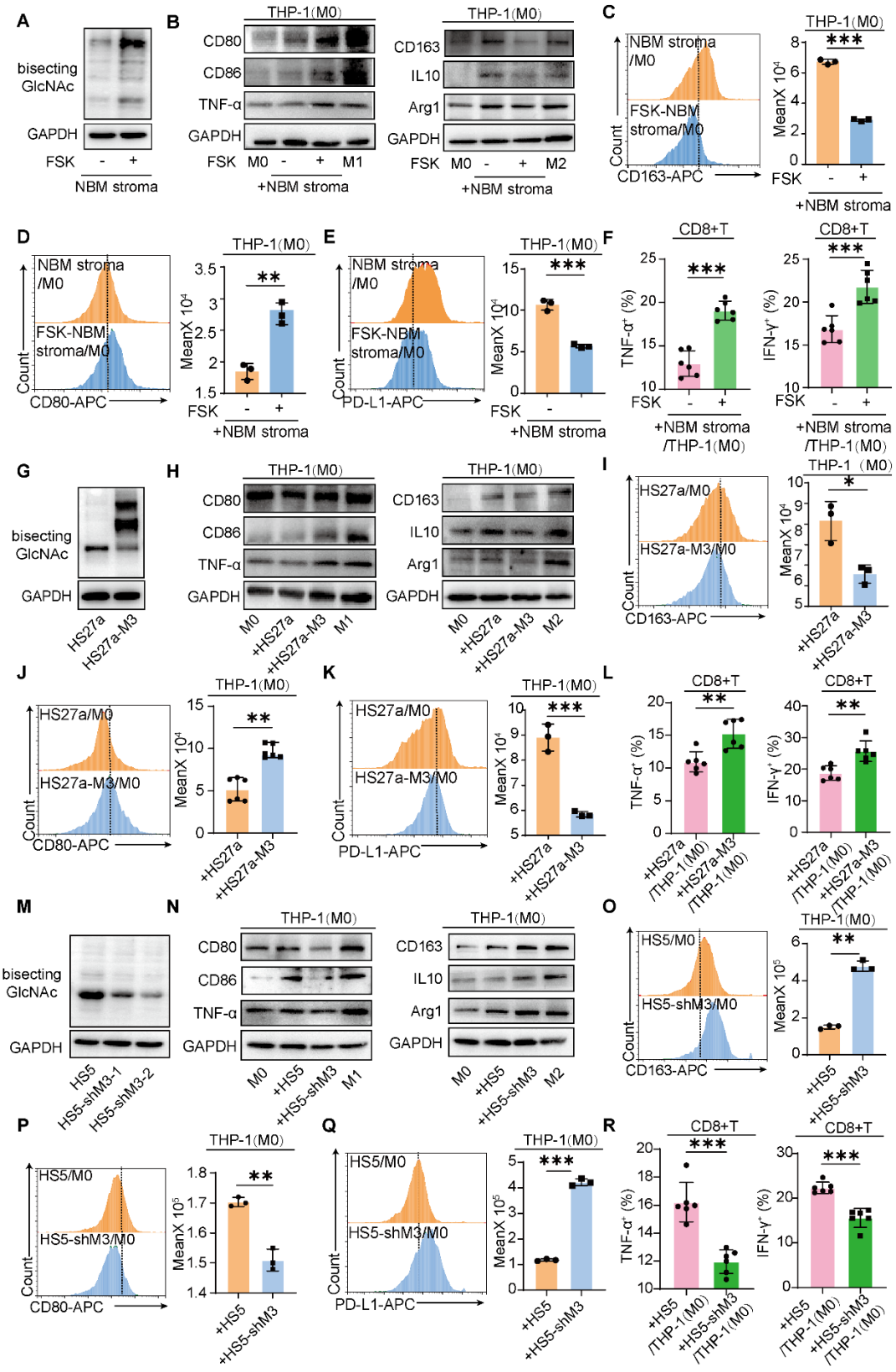


Fig. S5 (**A**) Relative expression of interleukin-10 (IL-10) in macrophages co-cultured with HS5, HS5 with MGAT3 knockdown (HS5-shM3), HS27a, HS27a overexpressing

MGAT3 (HS27a-M3), normal bone marrow (NBM) stroma, and forskolin-treated NBM stroma (FSK-NBM). (**B**) Flow cytometry (fluorescence-activated cell sorting, FACS) characterization of murine bone marrow (BM) stromal cells. (**C, D**) Expression of bisecting N-acetylglucosamine (GlcNAc), latency-associated transforming growth factor β1 (L-TGFβ1), and mature transforming growth factor β1 (m-TGFβ1) in HS27a, HS27a-M3, HS5, HS5-shM3, NBM, FSK-NBM, and murine NBM (mNBM) cells. (**E, F**) Enzyme-linked immunosorbent assay (ELISA) measurement of m-TGFβ1 protein levels in the above groups. (**G**) Half-life of L-TGFβ1 after cycloheximide (CHX) treatment. (**H, I**) FACS and western blot analysis of macrophage markers and signaling molecules in cells treated with transforming growth factor β1 receptor inhibitor (TRi).

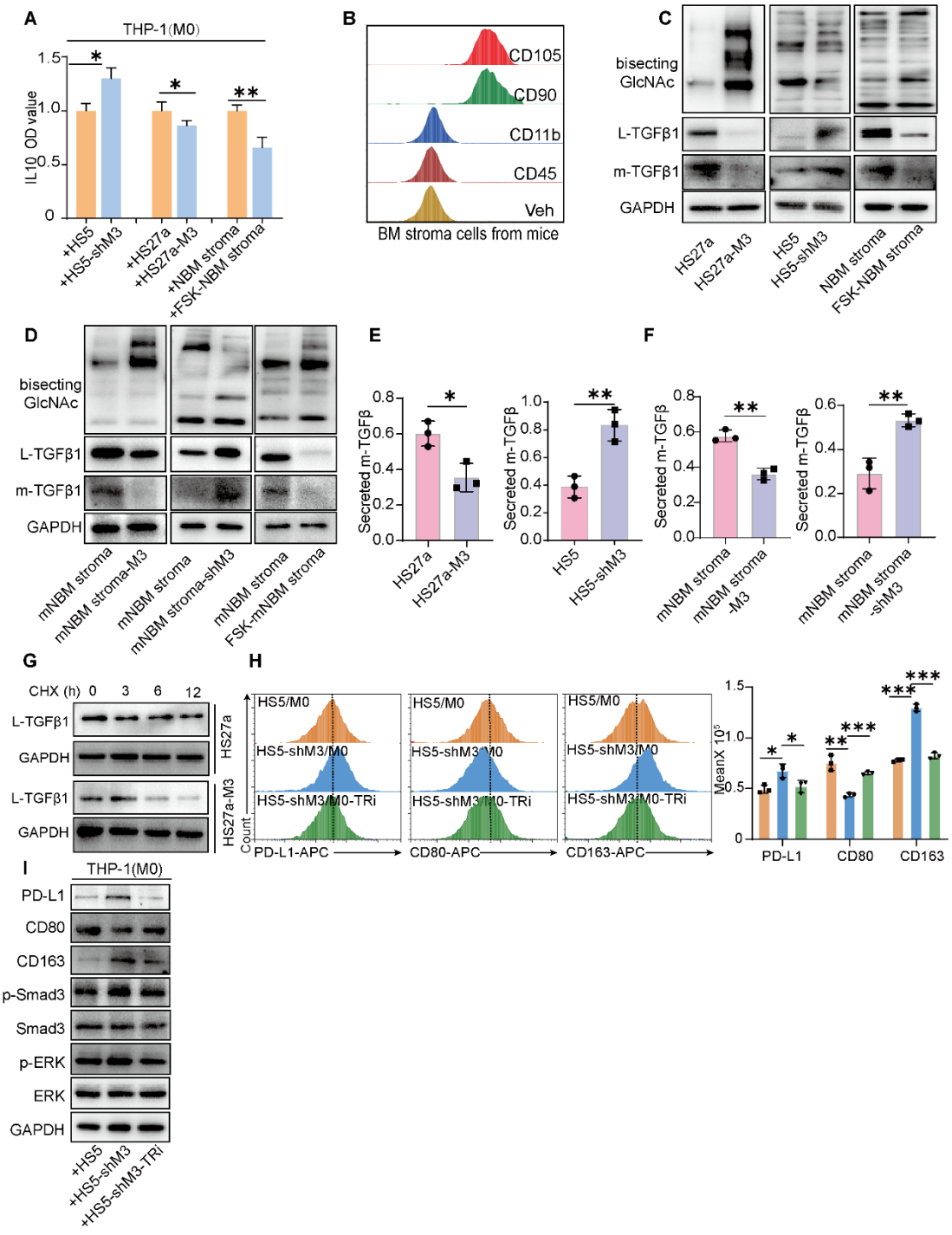
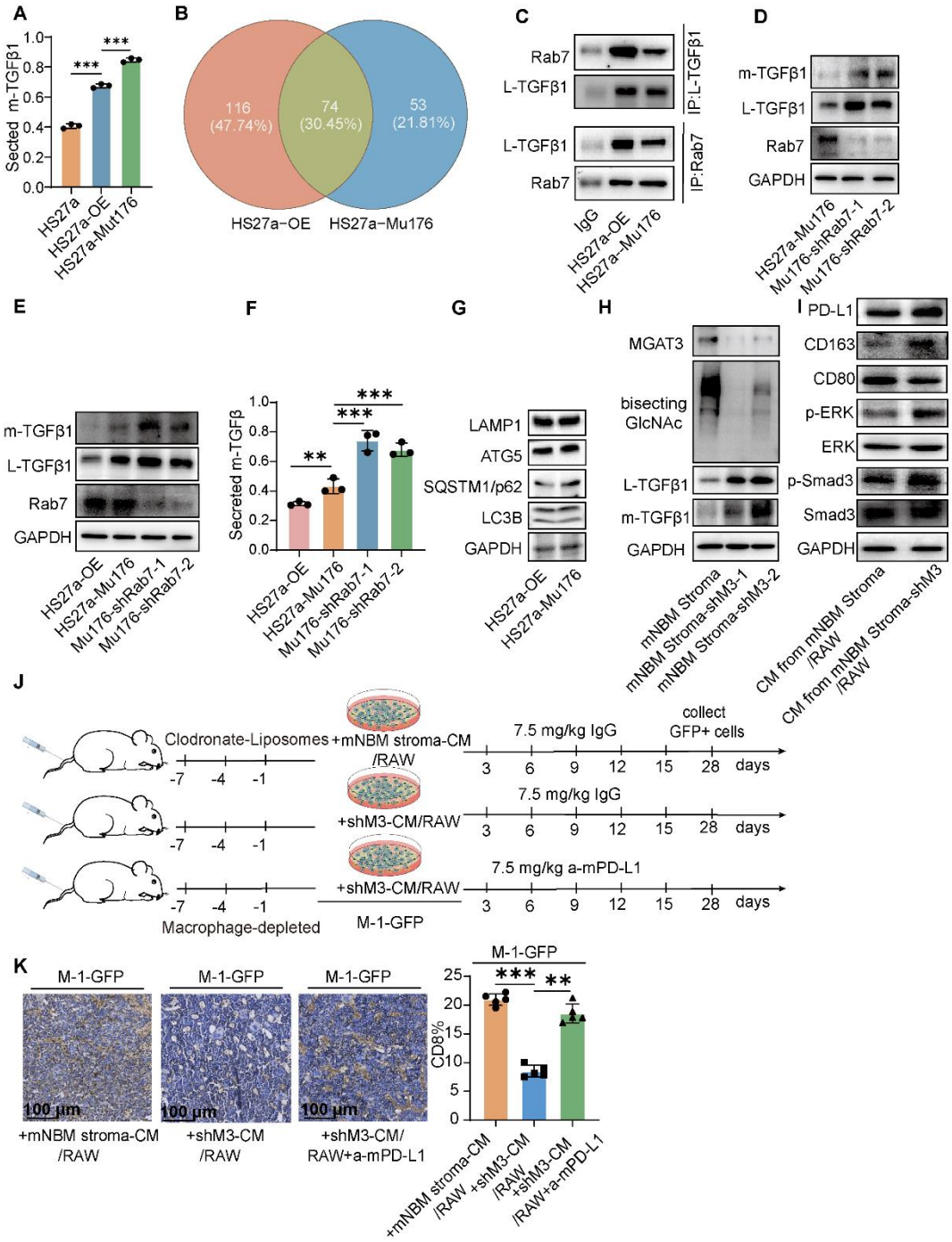


Fig. S6 (**A**) Levels of mature transforming growth factor β1 (m-TGFβ1), latency-associated TGFβ1 (L-TGFβ1), and bisecting N-acetylglucosamine (GlcNAc) in HS27a overexpressing either wild-type or N176D-mutated L-TGFβ1 (termed HS27a-OE and HS27a-Mu176, respectively), measured by enzyme-linked immunosorbent assay (ELISA). (**B**) Proteomic identification of L-TGFβ1-interacting proteins in HS27a-OE and

HS27a-Mu176 cells. (**C**) Immunoprecipitation (IP) analysis of L-TGFβ1 and the endosomal regulator Ras-related protein Rab7. (**D–F**) Western blot analysis of Rab7, L-TGFβ1, and m-TGFβ1 expression in HS27a-OE, HS27a-Mu176, and HS27a-Mu176 cells with Rab7 knockdown (HS27a-Mu176-shRab7). (**G**) Expression of lysosomal markers LAMP1, autophagy-related protein 5 (ATG5), p62, and microtubule-associated protein 1A/1B-light chain 3B (LC3B) in HS27a-OE and HS27a-Mu176 cells. (**H**) Expression of N-acetylglucosaminyltransferase III (MGAT3), bisecting N-acetylglucosamine (GlcNAc), L-TGFβ1, and m-TGFβ1 in murine normal bone marrow (mNBM) stromal cells and mNBM cells with MGAT3 knockdown (shM3).(**I**) Western blot analysis of programmed death-ligand 1 (PD-L1), CD163, CD80, p-Smad3, Smad3, p-ERK, and ERK in macrophages co-cultured with mNBM or shM3-transduced mNBM stromal cells.(**J**) Schematic diagram of the in vivo mouse model. (**K**) Immunohistochemical staining of $CD8^+$ T cells in murine bone marrow (BM).


A
Sected m-TGFβ1
HS27a
HS27a-OE
HS27a-Mut176
B
116 (47.74%)
74 (30.45%)
53 (21.81%)
HS27a-OE
HS27a-Mu176
C
Rab7
L-TGFβ1
IP:L-TGFβ1
IP:Rab7
IgG
HS27a-OE
HS27a-Mu176
D
m-TGFβ1
L-TGFβ1
Rab7
GAPDH
HS27a-Mu176
Mu176-shRab7-1
Mu176-shRab7-2
E
m-TGFβ1
L-TGFβ1
Rab7
GAPDH
HS27a-OE
HS27a-Mu176
Mu176-shRab7-1
Mu176-shRab7-2
F
Secreted m-TGFβ
HS27a-OE
HS27a-Mu176
Mu176-shRab7-1
Mu176-shRab7-2
G
LAMP1
ATG5
SQSTM1/p62
LC3B
GAPDH
HS27a-OE
HS27a-Mu176
H
MGAT3
bisecting GlcNAc
L-TGFβ1
m-TGFβ1
GAPDH
mNBM Stroma
mNBM Stroma-shM3-1
mNBM Stroma-shM3-2
I
PD-L1
CD163
CD80
p-ERK
ERK
p-Smad3
Smad3
GAPDH
CM from mNBM Stroma /RAW
CM from mNBM Stroma-shM3 /RAW
J
Clodronate-Liposomes
+mNBM stroma-CM /RAW
+shM3-CM/RAW
+shM3-CM/RAW
7.5 mg/kg IgG
7.5 mg/kg IgG
7.5 mg/kg a-mPD-L1
collect GFP+ cells
days
Macrophage-depleted
M-1-GFP
K
M-1-GFP
100 μm
+mNBM stroma-CM /RAW
+shM3-CM /RAW
+shM3-CM/ RAW+a-mPD-L1
CD8%
+mNBM stroma-CM /RAW
+shM3-CM /RAW
+shM3-CM /RAW+a-mPD-L1