Supplemental Materials and Methods

Cell lines and reagents

The 4T1-luc2 cells were kindly provided by Prof. MO Lee (College of Pharmacy, Seoul National University, Seoul, Republic of Korea). LLC1 cells were obtained from Dr. HG Lee (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea). The 4T1, 4T1-luc2, and LLC1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Carlsbad, CA, USA) with 100 U/ml penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Gibco/Invitrogen). For depletion of lymphoid cells, hybridoma cell lines (J11d.2 and GK1.5) were obtained from the American Type Culture Collection (ATCC) and were grown in RPMI 1640 (Gibco/Invitrogen) containing 10% FBS and 100 U/ml penicillin-streptomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 in air. We purchased 4-[(1,4-dihydro-1,4-dioxo-2-naphthalenyl)amino]-benzenesulfonamide (ML-329) from Cayman Chemical (Ann Arbor, MI, USA), and 3-isobutyl-1-methylxanthine (IBMX) and 2-methoxyestradiol (2-ME2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-18 and IL-10 were purchased from PeproTech, Inc (Rocky Hill, NJ, USA).

Murine models of lung cancer

C57BL/6J mice at 6 to 8 weeks of age were purchased from Daehan Biolink and maintained on a 12-hour light-dark cycle at 23-25℃ temperature. The mice were housed in cages under specific pathogen-free conditions. LLC1-bearing mouse models were generated by subcutaneous injection of 5 x 10^5 LLC1 cells/mouse or tail vein injection of 2.5 x 10^5 LLC1 cells/mouse.

Tumor cell-conditioned medium (TCCM)

For collection of tumor cell supernatants, 5 x 10^5 4T1 or LLC1 cells were seeded in the complete medium in 10-cm dishes for 48 h. For enriched proteins, the cell supernatant was concentrated at 3,000 × g for 20 min at 4°C using an Amicon® Ultra centrifugal devices (nominal molecular weight limits of 3K) (Merck Millipore, Billerica, MA, USA).

Flow cytometry

Harvested cells were stained with fluorochrome-conjugated antibodies (online supplemental table 1) at 4℃ for 30 min. For intracellular staining, the cells were fixed and permeabilized with the Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. Then, the cells were stained with MITF (D-9, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or IFN-γ (XMG1.2, BD Biosciences). All samples were analyzed by a FACSCantoII™ flow cytometer (BD Biosciences). The flow cytometry data were quantified by FlowJo V.10 software (Tree Star, Ashland, OR, USA).

RNA isolation and real-time PCR

Total RNA was purified using TRI-reagent (Invitrogen) according to the manufacturer's instructions. For the reverse-transcription reaction, 2.5 μg of total RNA was converted into first-strand cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA), dNTPs, and oligo (dT) primers (Bioneer, Daejeon, Republic of Korea). For quantitative real-time PCR (qRT-PCR), amplification was performed using an ABI StepOnePlus™ real-time PCR thermal cycler with Power SYBR Green PCR Master Mix according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Cyclophilin was used as an internal control for each reaction. The specific primer sequences are shown in online supplemental table 2.

Western blot analysis

Cells were lysed in Pro-Prep™ reagent (iNtRON Biotechnology, Seongnam, Republic of Korea), for 30 min on ice. For immunoblotting, equivalent cell lysates were mixed with 5X sample buffer and separated by electrophoresis on an SDS-PAGE gradient gel. The separated proteins were transferred onto a PVDF membrane (Amersham Biosciences, Burkes, UK). The membranes were blocked with 5% skim milk solution for 1 h. The washed membrane was incubated with primary antibody overnight at 4°C. The antibody recognizing HIF-1α was purchased from Bethyl Laboratories Inc. (Montgomery, TX, USA), and the antibodies against MITF, pSTAT3, and STAT3 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Specific antibodies against arginase 1 and α-actinin were purchased from Santa Cruz Biotechnology. After this incubation, the membrane was washed with TBST and stained with the appropriate HRP-conjugated secondary antibodies from Santa Cruz Biotechnology or Sigma-Aldrich for 2 h at room temperature. Next, the membrane was enhanced with an Ez-Western Lumi Plus solution (ATTO Corporation, Tokyo, Japan) and visualized using an Ez-Capture MG (ATTO Corporation).

Measurement of ROS levels

Cells were pretreated with or without 10 mM NAC (Sigma-Aldrich) and stimulated with 100 ng/ml LPS (Sigma-Aldrich) or TCCM for 24 h. Then, 10 μM DCF-DA (Sigma-Aldrich) was added to the cells for 30 min under growth conditions, and DCF fluorescence was measured by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

The culture supernatants were collected and used to measure the mouse IL-10 levels using an IL-10 ELISA kit (Cat No: 900-TM53, PeproTech) according to the manufacturer's protocol. The absorbance was detected using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA).

Survival analysis

The Cancer Genome Atlas (TCGA) TARGET genotype-tissue expression (GTEx, https://commonfund.nih.gov/gtex) database was used to analyze the expression of MITF in tumors and verify its association with overall survival and disease-specific survival in breast cancer patients. The association between MITF expression and the overall survival of lung squamous cell carcinoma patients was analyzed via the Q-omics software program (v1.02, https://qomics.sookmyung.ac.kr). Statistical survival analysis was performed by using the log-rank test with a *p* value.

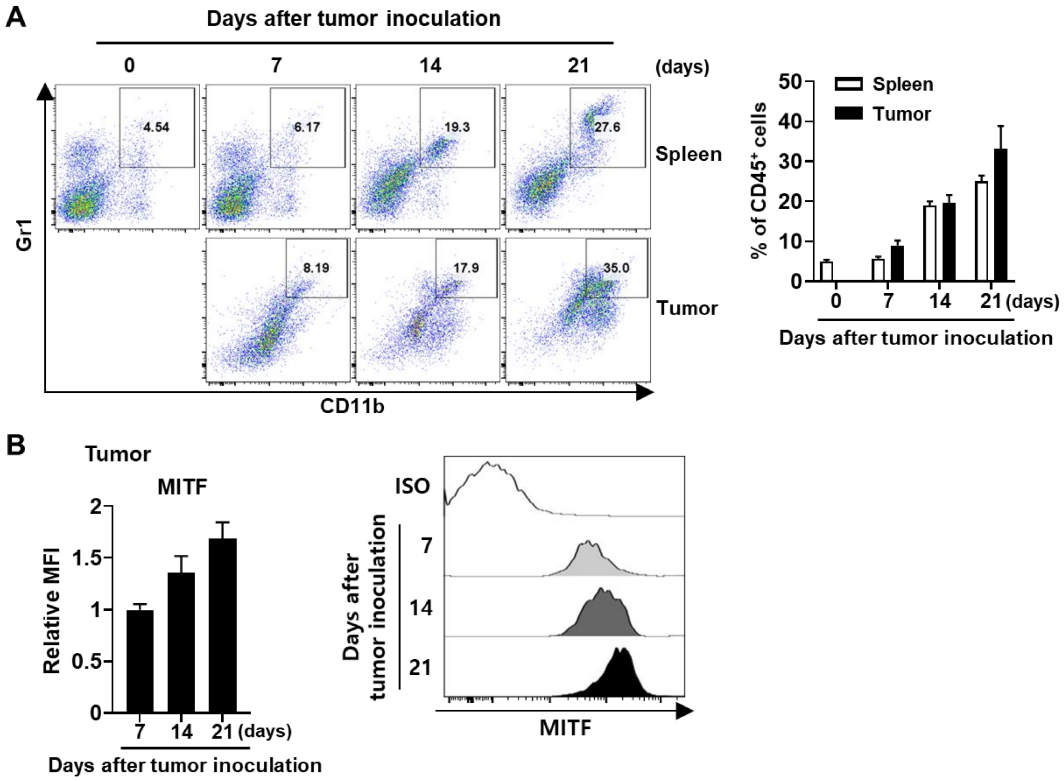
Supplemental table 1.

Antibodies	Source
CD45 (clone 30-F11)	Tonbo Biosciences, 60-0451-U100
CD11b (clone M1/70)	eBioscience, 11-0112-85
Ly6G/Ly6C (Gr1; clone RB6/8C5)	eBioscience, 17-5931-82
Ly6G (clone 1A8)	eBioscience, 12-9668-82
Ly6C (clone HK1.4)	eBioscience, 17-5932-82
PD-L1 (clone MIH5)	eBioscience, 12-5982-82
CD8a (clone 53-6.7)	eBioscience, 17-0081-82
F4/80 (clone BM8.1)	eBioscience, 45-4801-82
CD4 (clone GK1.5)	eBioscience, 17-0041-81
CD11c (clone N418)	BD Biosciences, 17-0114-81

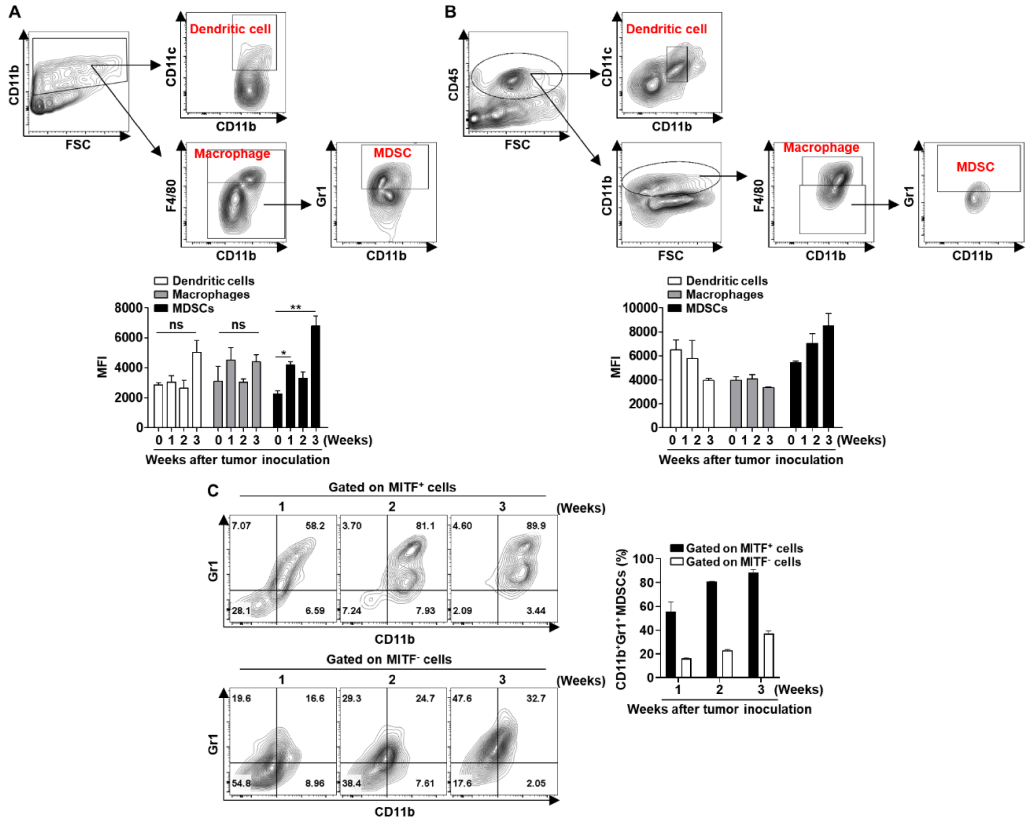
Supplemental table 2.

Name	Sequence
iNOS sense	5'- GGGCAGCCTGTGAGACCTT -3'
iNOS anti-sense	5'- TGAAGCGTTTCGGGATCTG -3'
IL-10 sense	5'- GATGCCCCAGGCAGAGAA -3'
IL-10 anti-sense	5'- CACCCAGGGAATTCAAATGC -3'
TGF-β sense	5'- GGACTCTCCACCTGCAAGAC -3'
TGF-β anti-sense	5'- GACTGGCGAGCCTTAGTTTG -3'
MITF sense	5'- CGCCTGATCTGGTGAATCG -3'
MITF anti-sense	5'- CCTGGCTGCAGTTCTCAAGAA -3'
HIF1A sense	5'- GAAATGGCCCAGTGAGAAAA -3'
HIF1A anti-sense	5'- CTTCCACGTTGCTGACTTGA -3'
Cyclophilin sense	5'- ATGGTCAACCCCACCGTGT -3'
Cyclophilin anti-sense	5'- TGCTGTCTTTGGAACTTTGTC -3'

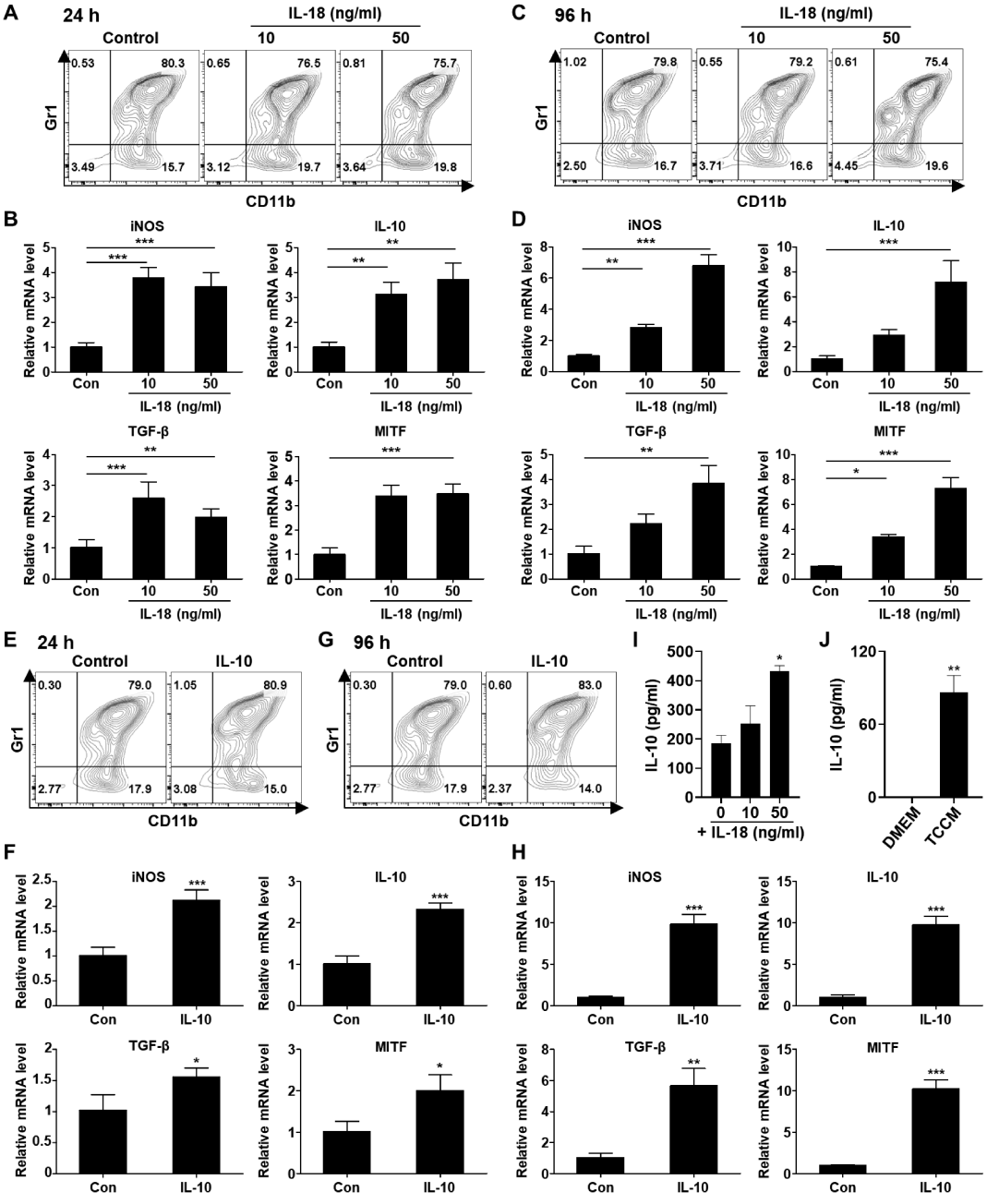
Supplementary figures



Supplementary figure 1. MDSC population and MITF expression in splenocytes and tumor tissues obtained from 4T1-bearing mice. (A) Percentage of MDSCs in spleens and tumor tissues derived from the normal mice or tumor-bearing mice on Days 0, 7, 14, and 21 after 4T1 tumor inoculation (n = 3-4). The graph represents the MDSC proportion, gated on the basis of $CD45^+CD11b^+Gr1^+$ cells. (B) The graph represents MITF expression after intracellular staining of cells gated by $CD45^+CD11b^+Gr1^+$ expression from tumor tissues of tumor-bearing mice (n = 3). MFI, mean fluorescence intensity.

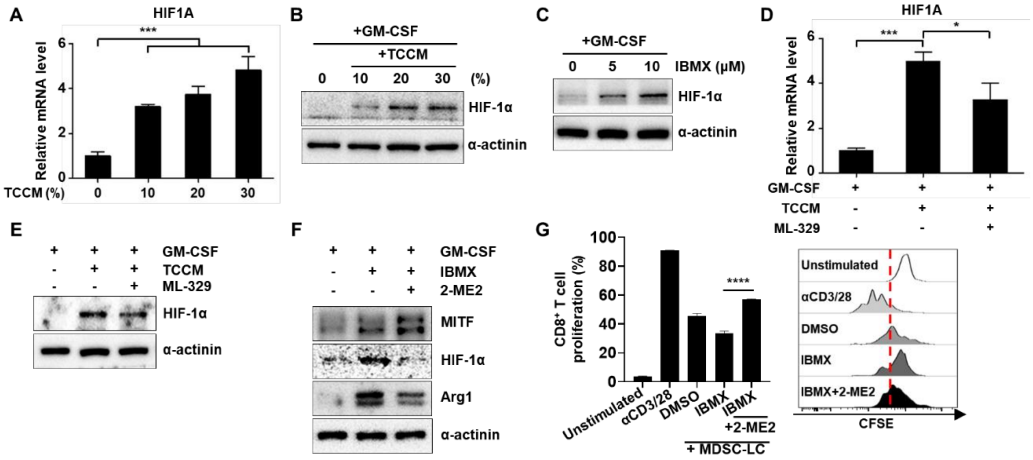


Supplementary figure 2. MITF expression in splenocytes and tumor tissues obtained from 4T1-bearing mice. (A) The splenocytes were stained with CD11b, CD11c, F4/80, Gr1, and MITF antibodies and analyzed by flow cytometry (n = 3). (B) The tumor cells were stained with CD11b, CD11c, F4/80, Gr1, CD45, and MITF antibodies and analyzed by flow cytometry. The graphs represent MITF expression in myeloid cells from the tumor-bearing mice at various time points (n = 3). (C) Harvested tumor cells from the 4T1-bearing mice were stained with MITF antibody at the indicated time points. $MITF^+$ or $MITF^-$ cells were stained with CD11b and Gr1 antibodies and represented as a contour plot (n = 3-4). $*p < 0.05$, $**p < 0.01$.

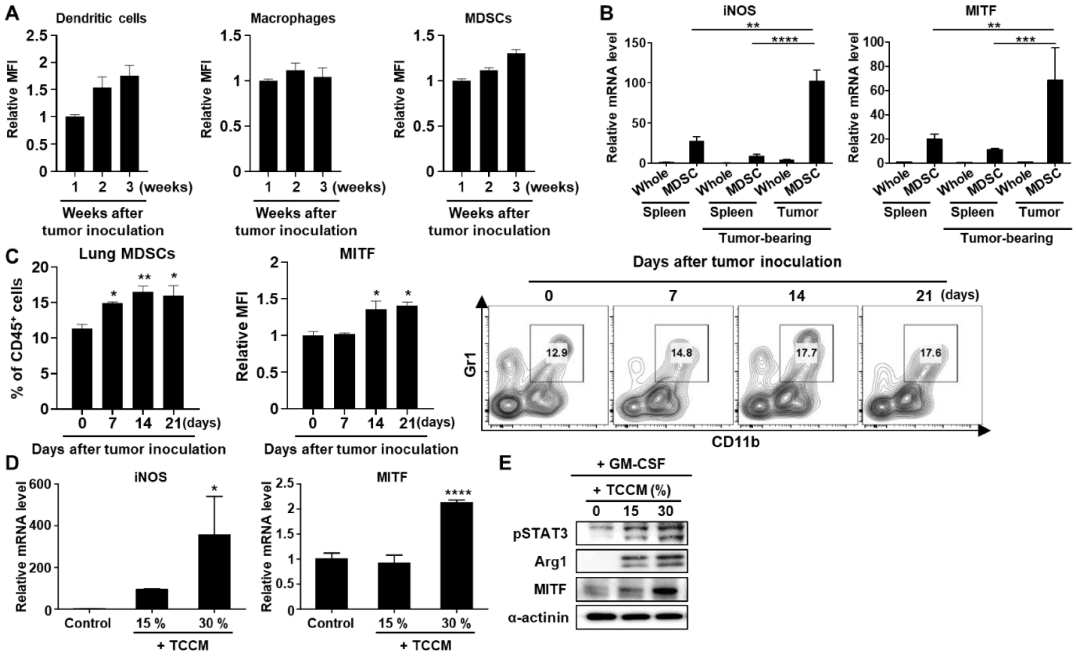


Supplementary figure 3. Effect of IL-18 and IL-10 on the expression of MITF and MDSC activation markers in MDSC-LCs. (A-I) Bone marrow-derived myeloid cells were cultured in the complete medium containing 10 ng/ml GM-CSF and treated with IL-18 (10 ng/ml or 50 ng/ml) or IL-10 (5 ng/ml). (A, E) The cells were collected at 24 h for cell analysis. (B, F) The mRNA expression levels of iNOS, IL-10, TGF-β, and MITF were measured by real-time

PCR. (C, G) The cells were collected at 96 h for cell analysis. (D, H) The mRNA expression levels of iNOS, IL-10, TGF-β, and MITF were measured by real-time PCR. (I) BM cells were treated with IL-18 for 96 h and then cultured in serum-free medium. After 48 h, the supernatant was collected for ELISA. (J) IL-10 secretion in the TCCM was measured by ELISA. All experiments were independently repeated at least 3 times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

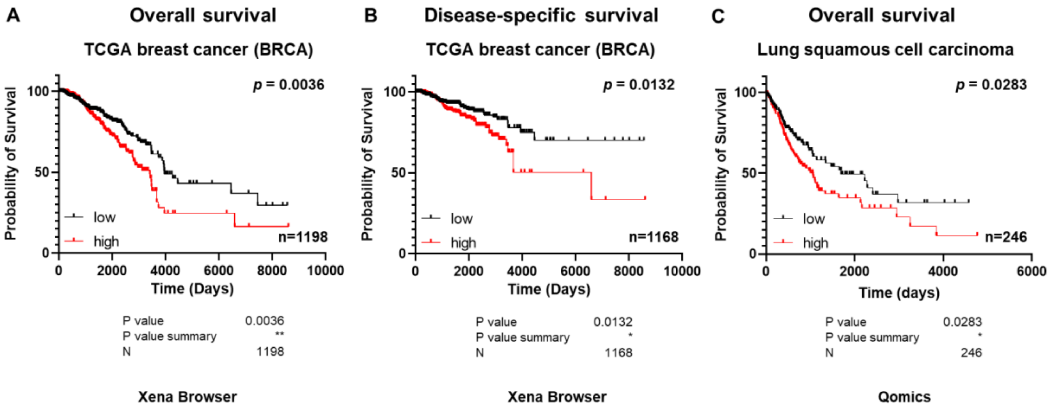


Supplementary figure 4. MITF upregulates the expression of activation markers in MDSC-LCs via HIF-1α. Bone marrow-derived myeloid cells were cultured in complete medium containing 10 ng/ml GM-CSF and TCCM or GM-CSF only. (A, B) HIF-1α expression was measured by real-time PCR (A) and western blot analysis (B). (C) Bone marrow-derived myeloid cells were treated with IBMX. HIF-1α expression was measured by western blot analysis. (D, E) Bone marrow-derived myeloid cells were treated with ML-329. HIF-1α expression was measured by real-time PCR and western blot analysis. (F) Myeloid cells were treated with 10 μM IBMX and 500 nM 2-ME2. The cells were harvested on Day 5 and the expression levels of MITF, HIF-1α, and Arg1 were measured by western blot analysis. (G) Splenic $CD3^+$ T cells were labeled with 2.5 μM CFSE, and CFSE-labeled T cells were stimulated with 3 μg/ml plate-bound anti-CD3 mAb and 1 μg/ml soluble anti-CD28 mAb for 2 h. The MDSC-LCs were cocultured with CFSE-labeled $CD3^+$ T cells for 72 h, and then, $CD8^+$ T cell proliferation was measured by flow cytometry at an MDSC-LC/T cell ratio of 1:1. All experiments were independently repeated at least 3 times. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.



Supplementary figure 5. MITF expression in tumor tissues obtained from LLC1-bearing mice. (A) The tumor cells obtained from mice after subcutaneous injection were stained with CD11b, CD11c, F4/80, Gr1, CD45, and MITF antibodies and analyzed by flow cytometry. The graphs represent MITF expression in myeloid cells from the tumor-bearing mice at various time points (n = 3-5). (B) The mRNA expression levels of iNOS and MITF in splenic MDSCs from the control mice and splenic MDSCs or tumor-associated MDSCs from the LLC1-bearing mice were detected by real-time PCR. (C) The lung tumor cells obtained from mice after tail vein injection were stained with CD11b, Gr1, CD45, and MITF antibodies and analyzed by flow cytometry. The graphs represent MITF expression in lung or lung tumor cells from the tumor-free or tumor-bearing mice at various time points. (D, E) Bone marrow-derived myeloid cells were cultured in complete medium containing 10 ng/ml GM-CSF and LLC1-TCCM or GM-CSF only for 96 h. The expression levels of the activation markers and MITF were measured by real-time PCR (D) and western blot analysis (E). The experiments were independently repeated at least 3 times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <$

0.0001.



Supplementary figure 6. A negative correlation between MITF expression and survival of breast and lung cancer patients. (A, B) The overall survival plot (n=1198) and disease-specific survival plot (n=1168) were analyzed by using the Xena Browser according to MITF expression in breast cancer patients using the TCGA TARGET GTEx dataset. (C) The overall survival plot (n=246) in stage II, III, and IV patients with lung squamous cell carcinoma was analyzed via the Q-omics software program. The red and black lines indicate the top 50% and the bottom 50% of MITF expression, respectively.