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Supplementary Information for

Anticancer efficacy of monotherapy with antibodies to SIRPα/SIRPβ1 mediated by induction of antitumorigenic macrophages.

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Supplementary Materials and Methods

Abs and reagents.

Rat mAbs to mouse SIRPα [MY-1 (rat IgG2a); P84 (rat IgG1), provided by C. F. Lagenaur (University of Pittsburgh, PA, USA)] and a mouse mAb to human SIRPα [SE12C3 (mouse IgG1) provided by H. J. Bühring (University Hospital Tübingen, Tübingen, Germany)] were generated and purified as described previously (1-4). A phycoerythrin (PE)–conjugated mAb to ARG1 (clone A1exF5), a PE-conjugated rat IgG2aκ isotype control (clone eBR2a), an allophycocyanin (APC)–conjugated mAb to mouse Ly6C (clone HK1.4), and a biotin-conjugated mAb to mouse CD80 (clone 16-10A1) were obtained from eBioscience (San Diego, CA). A PE-conjugated mAb to mouse MHCII (I-A/I-E, clone M5/114.15.2) was from BD Biosciences (San Jose, CA). An APC-conjugated mAb to mouse CD4 (clone RM4-5), a PE-conjugated mAb to mouse CD8α (clone 53-6.7), a Pacific Blue (PB)–conjugated mAb to mouse or human CD11b (clone M1/70), a peridinin chlorophyll protein (PerCP)/cyanine-5.5 (Cy5.5)–conjugated mAb to mouse CD45 (clone 30-F11), a PerCP/Cy5.5-conjugated mAb to mouse CD3ε (clone 145-2C11), a PB-conjugated mAb to mouse CD45 (clone 30-F11), an fluorescein isothiocyanate (FITC)-conjugated mAb to mouse CD206 (clone C068C2), an FITC-conjugated mAb to mouse CD49b (clone DX5), a biotin-conjugated mAb to mouse F4/80 (clone BM8), a biotin-conjugated mAb to mouse CD86 (clone GL-1), a biotin-conjugated mAb to mouse CD47 (clone miap301), a biotin-conjugated mAb to human CD47 (clone CC2C6), a biotin-conjugated mAb to mouse Ly6G (clone 1A8), a biotin-conjugated rat IgG1κ isotype control (clone RTK2071), a biotin-conjugated rat IgG2aκ isotype control (clone RTK2758), a PE-conjugated mAb to mouse TNFα (clone MP6-XT22), PE-conjugated mAbs to mouse MHCII (I-Aκ, clone 10-3.6), PE-conjugated mAbs to mouse Ly6G (clone 1A8), PE/Cy7-conjugated streptavidin, APC-conjugated streptavidin, an FITC-conjugated rat IgG2aκ isotype control (clone RTK2758), a PE-conjugated rat IgG2bκ isotype control (clone RTK4530), an FITC-conjugated rat IgG1κ isotype control (clone RTK2071), a PE-conjugated mouse IgG2aκ isotype control (clone MOPC-173), a PE/Cy7-conjugated mAb to human CD172a/b (clone SE5A5), a PE/Cy7-conjugated mouse IgG1κ isotype control (MOPC-21), a biotin-conjugated mouse IgG1κ isotype control (MOPC-21), a Brilliant Violet 421 (BV421)–conjugated mAb to human CD11b (clone ICRF44), a rat mAb to mouse CD16/32 (clone 93), Human TruStain FcX, and Zombie Aqua (Zombie Aqua Fixable Viability Kit) were from BioLegend (San Diego, CA). PB-conjugated streptavidin, Alexa Fluor 488–conjugated goat pAbs to rat IgG, and CFSE (CellTrace Cell Proliferation Kit) were from Thermo Fisher Scientific (Waltham, MA). Normal rat IgG, normal mouse IgG, and the F(ab')$_2$ fragment of goat pAbs to the Fc region of rat IgG [F(ab')$_2$ anti–rat IgG Fc] were from Jackson ImmunoResearch Laboratories (West Grove, PA). L-NMMA was obtained from Abcam (Cambridge, UK). Rat mAbs to mouse CSF1R (clone AFS98) and to mouse CD8α (clone YTS169.4), rat IgG2a isotype control (clone 2A3), rat IgG1 isotype control (clone BE0088), and mouse IgG1 isotype control (clone MOPC-21) were from Bio X Cell (Lebanon, NH). Rabbit pAbs to asialo-GM1 were from Wako (Osaka, Japan). Etanercept (Enbrel, p75 TNF receptor (TNFR)-Ig) and a rat mAb (IgG2a) to mouse SIRPβ1 (clone OX123) were obtained from Takeda Pharmaceuticals (Osaka, Japan) and Absolute Antibody (Redcar, UK), respectively. The F(ab')$_2$ fragment of MY-1 was prepared as described previously (4). Biotin-labeled P84 and OX123 were generated with the use of a Biotin Labeling Kit-NH$_2$ (Dojindo, Tokyo, Japan).

Animals.

Female C57BL/6J (B6), C3H/HeN (C3H), and BALB/c-*nu*/*nu* mice were obtained from SLC (Shizuoka, Japan) or CLEA Japan (Tokyo, Japan). *Sirpa*$^{f/f}$ mice were generated from C57BL/6J mice by Unitech (Chiba, Japan) as described previously (5). CMV-Cre (B6.C-Tg[CMV-cre]1Cgn/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were crossed with *Sirpa*$^{f/f}$ mice to generate *Sirpa*$^{f/f}$;CMV-Cre (*Sirpa*$^{-/-}$) descendants for study. All animals were maintained in the Institute for Experimental Animals at Kobe University Graduate School of Medicine under specific pathogen–free conditions and were housed in an air-conditioned room with a 12-h-light, 12-h-dark cycle. All animal experiments were performed according to the guidelines of the Animal Care and Experimentation Committee of Kobe University (Permit number: P190604-R2).

Cell culture.
The murine bladder cancer cell lines MBT2 and MB49 were obtained from the JCRB cell bank (National Institutes of Biomedical Innovation, Health, and Nutrition, Osaka, Japan) and Merck Millipore (Burlington, MA), respectively. The murine renal cancer cell line RENCA, the Lewis lung cancer cell line LL/2, the murine mammary cancer cell line FM3A, the human urinary bladder cancer cell lines T24 and HT1197, and the human renal cell cancer cell line ACHN were obtained from American Type Culture Collection (Manassas, VA). The murine osteosarcoma cell line LM8 was obtained from the RIKEN BRC cell bank (Riken BioResource Research Center, Ibaraki, Japan). MBT2, HT1197, ACHN, and LM8 cells were maintained in Eagle's minimal essential medium (Wako) supplemented with 10% fetal bovine serum (FBS); MB49 and LL/2 cells in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% FBS; RENCA and FM3A cells in RPMI 1640 medium (Wako) supplemented with 10% FBS; and T24 cells in McCoy's 5A medium (Wako) supplemented with 10% FBS.

Cancer cell engraftment and treatment.
MBT2 cells (1×10^5), MB49 cells (1×10^5), FM3A cells (2×10^6), LL/2 cells (1×10^6), or LM8 cells (1×10^6) were injected in 100 µL of phosphate-buffered saline (PBS) subcutaneously into the flank of female B6, C3H, BALB/c-*nu/nu*, or *Sirpa*$^{-/-}$ mice at 6 to 8 wk of age. Four or 7 days after cancer cell injection (when tumor volume had achieved an average of 30–100 mm^3), the mice were injected intraperitoneally with either normal rat IgG (200 µg), MY-1 (200 µg), or P84 (200 µg) every 4 days. For treatment with etanercept, C3H mice were injected intraperitoneally with the drug (30 mg/kg) at 5 days after injection of MBT2 cells as described above. The mice were then further injected with etanercept (30 mg/kg) in combination with either normal rat IgG (200 µg) or MY-1 (200 µg) at 7 days and every 4 days thereafter. Tumors were measured with digital calipers, and tumor volume was calculated as: $a \times b^2/2$ (where a is the largest diameter and b the smallest diameter).

Depletion of macrophages, CD8$^+$ T cells, or NK cells in vivo.
For depletion of macrophages in mice with established tumors, 6- to 8-wk-old female C3H mice were injected intravenously with a mAb to CSF1R (clone AFS98, 200 µg) at 5 and 7 days after injection of cancer cells and then every 4 days thereafter. Depletion of CD8$^+$ T cells or NK cells in tumor-bearing mice was performed as described previously (4), with minor modifications. Female C3H mice at 6 to 8 wk of age were injected intraperitoneally with a mAb to CD8α (clone YTS169.4, 400 µg) or pAbs to asialo-GM1 (50 µL) at 5 and 7 days after injection of cancer cells and then every 4 days thereafter. The effectiveness of macrophage, CD8$^+$ T cell, or NK cell depletion was determined by flow cytometric analysis of CD45$^+$CD11b$^+$Ly6C^{low}F4/80$^+$ cells (macrophages), CD45$^+$CD3ε$^+$CD49b$^-$CD8α$^+$ cells (CD8$^+$ T cells), or CD45$^+$CD3ε$^-$CD49b$^+$ cells (NK cells) among tumor-infiltrating cells or splenocytes from the treated animals.

Cell preparation.
For isolation of tumor-infiltrating cells, tumors were removed from mice, minced with forceps, and then digested with a solution consisting of collagenase IV (Worthington, Lakewood, NJ) at 1 mg/mL, DNase I (Sigma-Aldrich, St. Louis, MO) at 40 µg/mL, and 2% FBS in RPMI 1640 medium (Wako). The undigested material was removed by filtration through a 70-µm cell strainer (BD Biosciences), and the remaining cells were washed twice with PBS and subjected to flow cytometric analysis. For isolation of splenocytes, the mouse spleen was ground gently with autoclaved frosted-glass slides in PBS, fibrous material was removed by filtration through a 70-µm cell strainer, and RBCs in the filtrate were lysed with BD Pharm Lyse (BD Biosciences). The remaining cells were washed twice with PBS and then subjected to flow cytometric analysis.

Flow cytometry.
Flow cytometric analysis was performed as described previously (6). In brief, cells were incubated with a mAb to CD16/32 (for mouse cells) or human Trustain FcX (for human cells), stained with fluorochrome-conjugated mAbs, washed with FACS buffer (PBS supplemented with 2% FBS,

2mM ethylenediamine tetraacetic acid [EDTA], and 0.03% NaN$_3$), and stained with the use of a Zombie Aqua Fixable Viability Kit for discrimination between live and dead cells. For staining of ARG1 and TNFα, cells were stained for cell surface markers and then processed with the use of an Intracellular Fixation and Permeabilization Buffer Set (eBioscience) and labeled with a PE-conjugated mAb to mouse ARG1 or to mouse TNFα. For determination of the expression of SIRPα or SIRPβ1 on cancer cell lines, murine cell lines were incubated with MY-1 or a rat IgG2a isotype control and then stained with Alexa Fluor 488–conjugated pAbs to rat IgG, whereas human cell lines were stained with a PE/Cy7-conjugated mAb to human CD172a/b (SIRPα/β, clone SE5A5) or a PE/Cy7-conjugated isotype control IgG. Labeled cells were analyzed by flow cytometry with a FACSVerse instrument (BD Biosciences), and data were analyzed with FlowJo v10 software (BD Biosciences).

Isolation of mouse and human macrophages.
Mouse BMDMs were prepared as described previously (4), with minor modifications. In brief, bone-marrow cells were isolated from the femur and tibia and cultured in Iscove's modified Dulbecco's medium (IMDM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS and recombinant murine macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ) at 10 ng/mL. BMDMs obtained after 7 days of culture were used for assays. Bone-marrow cells from *Dap12$^{-/-}$* mice (7) were kindly provided by T. Suganami (Nagoya University, Aichi, Japan). For preparation of human macrophages, human cord blood was obtained from healthy volunteers who provided informed consent (The Hyogo Cord Blood Bank, Hyogo, Japan), and mononuclear cells were isolated from the buffy coat of the blood with the use of Lymphoprep (Alere Technologies, Oslo, Norway). The isolated mononuclear cells were washed twice with PBS containing 2 mM EDTA, transferred to culture dishes in IMDM supplemented with 10% FBS, and cultured overnight at 37°C in a humidified incubator containing 5% CO$_2$ to allow monocyte adhesion. Nonadherent cells were then removed, and the adherent monocytes were cultured for an additional 7 days in IMDM supplemented with 10% FBS and recombinant human M-CSF (BioLegend) at 50 ng/mL to obtain mature human macrophages. The experiments with human umbilical cord blood were approved by the Ethics Committees of Kobe University Graduate School of Medicine and the Hyogo Cord Blood Bank (No. 1820) and were performed in accordance with the tenets of the Declaration of Helsinki.

Isolation of mouse neutrophils.
Mouse neutrophils were isolated from bone-marrow cells of C3H mice using a magnetic cell separation system (Biolegend) with biotin-conjugated mAb to Ly6G (clone 1A8) and streptavidin nanobeads (Biolegend), according to the manufacturer's instructions. Isolated neutrophils were cultured for 16 h in RPMI 1640 medium supplemented with 10% FBS, recombinant murine granulocyte-colony stimulating factor (G-CSF) (BioLegend) at 10 ng/mL, and recombinant mouse interferon γ (IFNγ) (PeproTech) at 50 ng/mL. The purity of the cells was analyzed by flow cytometry was >95%.

Assays for TNFα and NO production.
For measurement of TNFα production, mouse BMDMs or human macrophages were seeded at a density of 1 × 10^5 per well in 96-well plates and cultured overnight in IMDM supplemented with 10% FBS and either recombinant murine M-CSF at 10 ng/mL or recombinant human M-CSF at 50 ng/mL plus recombinant human IFNγ at 100 ng/mL, respectively. The cells were then incubated for 24 or 48 h with either intact Abs (10 μg/mL), F(ab')$_2$ fragments (7.3 μg/mL), or Abs (10 μg/mL) cross-linked with F(ab')$_2$ anti–rat IgG Fc (3.8 μg/mL), and the culture supernatants were collected and assayed for mouse or human TNFα with ELISA kits (R&D Systems, Minneapolis, MN). Cross-linked Abs were prepared by incubation of intact Abs with F(ab')$_2$ anti–rat IgG Fc for 30 min at room temperature at a 2:1 molar ratio and were then added to the culture medium. For measurement of NO production, mouse BMDMs were incubated with control IgG (10 μg/mL) or MY-1 (10 μg/mL) for 48 h, after which the culture supernatants were collected and the production of NO was determined by measurement of nitrite (NO$_2^-$) generated from NO with the use of a colorimetric NO$_2$/NO$_3$ Assay Kit-C II (Dojindo).

Cytotoxicity assay.
Mouse BMDMs or human macrophages were seeded at a density of 2×10^5 per well in six-well plates and cultured overnight in IMDM supplemented with 10% FBS and either recombinant murine M-CSF (10 ng/mL) or recombinant human M-CSF (50 ng/mL) plus recombinant human IFNγ (100 ng/mL), respectively. Target cells (1×10^4) labeled with CFSE (2.5 μM) were added to the macrophages (effector cells) and incubated in IMDM supplemented with or without 0.2% FBS in the presence of either intact Abs (10 μg/mL), F(ab')$_2$ fragments (7.3 μg/mL), or Abs (10 μg/mL) cross-linked with F(ab')$_2$ anti–rat IgG Fc (3.8 μg/mL). The cells were harvested, incubated with a mAb to mouse CD16/CD32 or with Human TruStain FcX and then either with a biotin-conjugated mAb to F4/80 and APC-conjugated streptavidin (for mouse BMDMs) or with a BV421-conjugated mAb to CD11b (for human macrophages). They were then stained for annexin V with the use of an IMMUNOCYTO Cytotoxicity Detection Kit (MBL, Aichi, Japan) and analyzed with a FACSVerse flow cytometer and FlowJo v10 software. The percentage cytotoxic activity of mouse or human macrophages toward CFSE-labeled target cells was calculated as: 100 × annexin V$^+$CFSE$^+$F4/80$^-$ cells/(annexin V$^+$CFSE$^+$F4/80$^-$ cells + annexin V$^-$CFSE$^+$F4/80$^-$ cells) or 100 × annexin V$^+$CFSE$^+$CD11b$^-$ cells/(annexin V$^+$CFSE$^+$CD11b$^-$ cells + annexin V$^-$CFSE$^+$CD11b$^-$ cells), respectively. For determination of cancer cell susceptibility to TNFα-induced cytotoxicity, cells (5×10^4) were plated in 24-well plates and incubated overnight in culture medium supplemented with 10% FBS. The cells were then incubated in serum-free culture medium together with vehicle or recombinant murine TNFα (PeproTech) for 48 h, stained for annexin V as described above, and analyzed with a FACSVerse flow cytometer and FlowJo v10 software. For measurement of neutrophil-mediated cytotoxicity toward cancer cells, DELFIA EuTDA cytotoxicity kit (PerkinElmer, Waltham, MA) was used, according to the manufacturer's instruction. In brief, MBT2 cells (target cells) labeled with TDA (2,2':6',2"-terpyridine-6,6"-dicarboxylate) were seeded in 96-well plates (5×10^4 per well) and incubated for 4 h with neutrophils in the presence of either control IgG (at 10 μg/mL), MY-1 (at 10 μg/mL), the F(ab')$_2$ fragment of MY-1 (at 7.3 μg/mL), or OX123 (at 10 μg/mL). The plates were then centrifuged, and 20 μl of supernatant was mixed with 200 μl of DELFIA Europium solution, after which fluorescence of Europium-TDA chelates was measured with a time-resolved fluorometer (Enspire multiplate reader; PerkinElmer). The percentage target cell lysis was calculated as: 100 × (experimental release – spontaneous release)/(maximal release – spontaneous release). Maximal release was determined by lysis of target cells with DELFIA lysis buffer; spontaneous release was measured by incubation of target cells in the absence of effector cells.

Phagocytosis assay.
Mouse BMDMs or human macrophages (1×10^5 each) were transferred to six-well plates and incubated overnight in IMDM supplemented with 10% FBS and either recombinant murine M-CSF (10 ng/mL) or recombinant human M-CSF (50 ng/mL) plus recombinant human IFNγ (100 ng/mL), respectively. Target cells (4×10^5) labeled with CFSE (2.5 μM) were added to the macrophages (effector cells) and incubated for 16 h in the presence of either intact Abs (10 μg/mL), F(ab')$_2$ fragments (7.3 μg/mL), or Abs (10 μg/mL) cross-linked with F(ab')$_2$ anti–rat IgG Fc (3.8 μg/mL) in IMDM. The cells were then harvested, incubated either with a mAb specific for mouse CD16/CD32 or in Human TruStain FcX, stained with a biotin-conjugated mAb to F4/80 and APC-conjugated streptavidin (for mouse macrophages) or with a BV421-conjugated mAb to human CD11b (for human macrophages), and analyzed with a FACSVerse flow cytometer and FlowJo v10 software. The percentage phagocytosis of CFSE-labeled target cells by mouse BMDMs or human macrophages was calculated as: 100 × F4/80$^+$CFSE$^+$ cells/(F4/80$^+$CFSE$^+$ cells + F4/80$^+$CFSE$^-$ cells) or 100 × CD11b$^+$CFSE$^+$ cells/(CD11b$^+$CFSE$^+$ cells + CD11b$^+$CFSE$^-$ cells), respectively.

Cell viability assay.
For determination of cell viability, a WST-8 assay (Nacalai Tesque) was performed as described previously (8). In brief, MB49 or MBT2 cells (1000/well) were seeded in 96-well plates, allowed to adhere overnight, and cultured for 1 to 2 days in culture medium supplemented with 10% FBS and either control IgG or MY-1. The cells were then incubated with the WST-8 labeling mixture

(Cell Count Reagent SF) for 2 h before measurement of absorbance at 450 nm with a microplate reader (2030 ARVO X4, PerkinElmer).

RNA interference.
For siRNA-mediated knockdown of SIRPβ1, mouse BMDMs were transfected with targeting or control siRNAs with the use of Lipofectamine RNAiMAX (Thermo Fisher Scientific). The sequences of the siRNAs targeting mouse SIRPβ1 (sense and antisense, respectively) were 5′-UAUCAAGAGUCGACUUUGUCC-3′ and ACAAAGUCGACUCUUGAUACA for siRNA #1, and 5′-UAGAACUUCUGUAUUUGGCAG-3′ and GCCAAAUACAGAAGUUCUAUC for siRNA #2. The MISSION siRNA universal negative control (Sigma-Aldrich) was used as the control siRNA. For validation of SIRPβ1 knockdown, mouse BMDMs transfected with siRNAs for 48 h were incubated with a mAb to CD16/32, stained with either biotin-conjugated isotype control IgG, a biotin-conjugated mAb to mouse SIRPβ1 (clone OX123), or a biotin-conjugated mAb to mouse SIRPα (clone P84), incubated with APC-conjugated streptavidin, and then analyzed by flow cytometry with the use of a FACSVerse instrument and FlowJo v10 software.

Statistics.
Data are presented as means ± standard error of the mean (SEM) and were analyzed with the two-tailed Welch's *t* test for comparisons of two groups, or with Welch and Brown-Forsythe one-way ANOVA followed by Dunnett's T3 test, with one-way ANOVA followed by Tukey's test, or with two-way repeated-measures ANOVA with the Greenhouse-Geisser correction followed by Šídák's or Tukey's test for multiple comparisons. Survival outcomes were compared with the log-rank test. A *P* value of <0.05 was considered statistically significant. All statistical analysis was performed with the use of GraphPad Prism 9.1 software (GraphPad, La Jolla, CA).
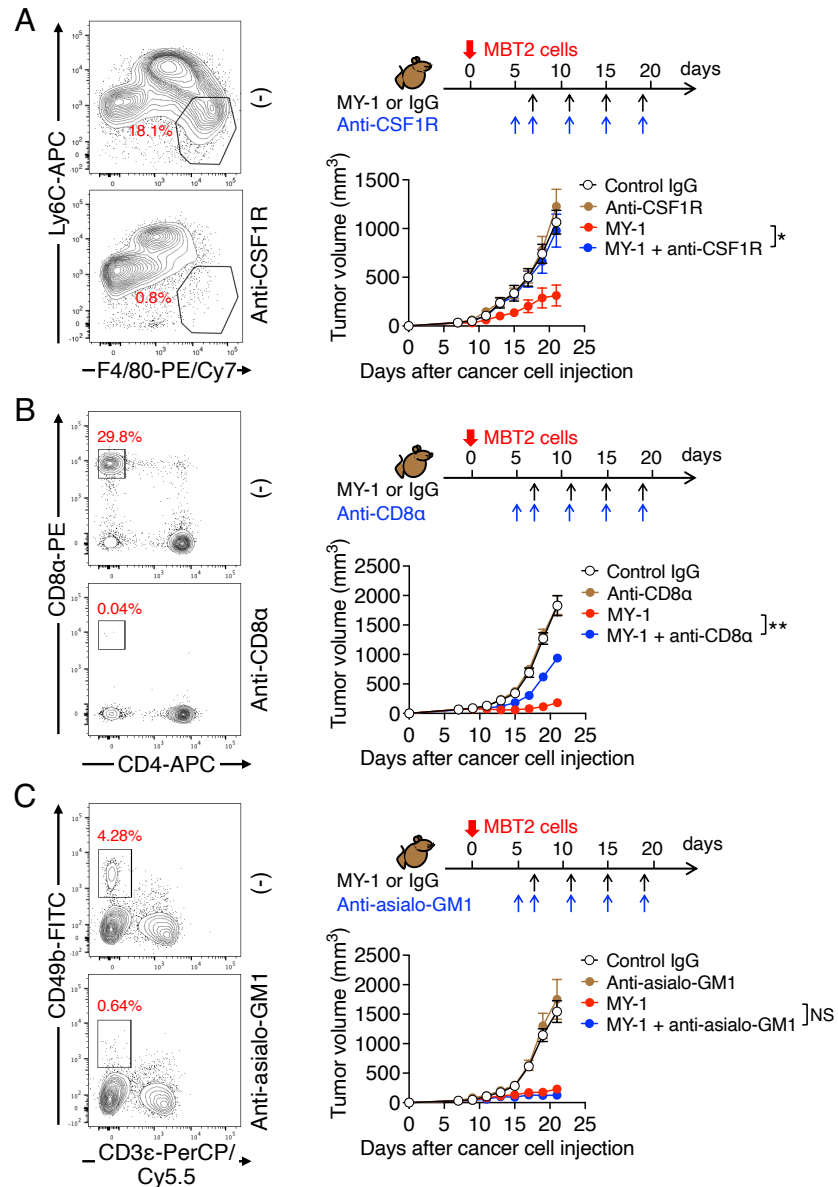
Fig. S1. Importance of macrophages and CD8+ T cells for the inhibitory effect of MY-1 on tumor formation by murine bladder cancer cells in syngeneic mice. C3H mice were injected subcutaneously with MBT2 cells, 5 days after which they were treated with the indicated Abs according to the indicated schedule. Four days after the onset of treatment with immune cell–depleting Abs alone, immune infiltrates of tumors (**A**) or splenocytes (**B** and **C**) were isolated and analyzed by flow cytometry to determine the frequency of $Ly6C^{low}F4/80^+$ macrophages among all viable $CD45^+CD11b^+$ cells (**A**), that of $CD8\alpha^+$ T cells among all viable T cells ($CD45^+CD3\varepsilon^+CD49b^-$ cells) (**B**), or that of $CD3\varepsilon^-CD49b^+$ NK cells among all viable $CD45^+$ splenocytes (**C**). Representative plots for three separate experiments are shown (left panels). Tumor volume was also determined at the indicated time points (right panels). The data are means ± SEM from two separate experiments [n = 8 (MY-1 or anti-CSF1R) or 9 (control IgG or MY-1 + anti-CSF1R) mice per group (**A**), n = 10 mice per group (**B**), or n = 9 (MY-1 or anti–asialo-GM1) or 10 (control IgG or MY-1 + anti–asialo-GM1) mice per group (**C**)]. *P < 0.05, **P < 0.01, NS (two-way repeated-measures ANOVA with the Greenhouse-Geisser correction and Tukey's multiple-comparison test). NS, not significant.
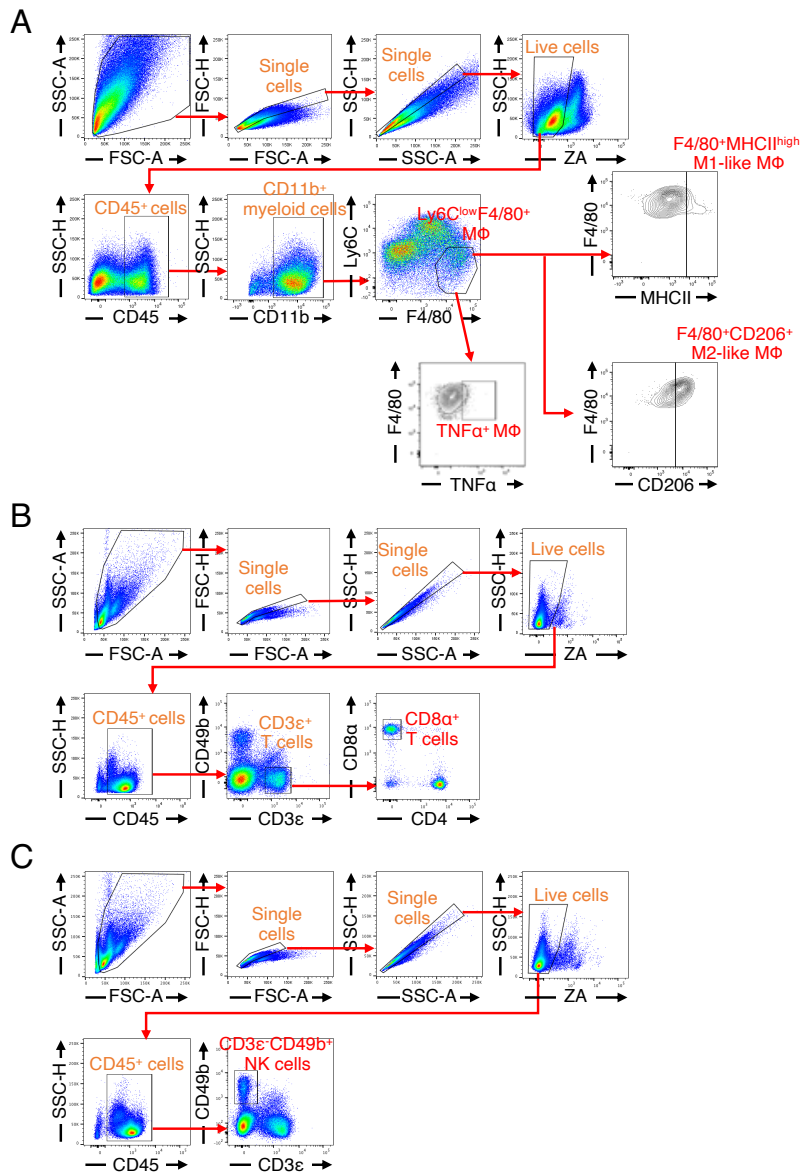
Fig. S2. Gating strategy for tumor-infiltrating macrophages, CD8⁺ T cells, and NK cells. Representative flow cytometric plots show the gating strategy for tumor-infiltrating macrophages (CD45$^+$CD11b$^+$Ly6C^{low}F4/80$^+$ cells), M1-like macrophages (CD45$^+$CD11b$^+$Ly6C^{low}F4/80$^+$MHCII^{high} cells), M2-like macrophages (CD45$^+$CD11b$^+$Ly6C^{low}F4/80$^+$CD206$^+$ cells), and TNFα$^+$ macrophages (CD45$^+$CD11b$^+$Ly6C^{low}F4/80$^+$TNFα$^+$ cells) (***A***) as well as for CD8$^+$ T cells (CD45$^+$CD3ε$^+$CD49b$^-$CD8α$^+$ cells) (***B***) and NK cells (CD45$^+$CD3ε$^-$CD49b$^+$ cells) (***C***). SSC, side scatter; FSC, forward scatter; ZA, Zombie Aqua; MΦ, macrophages.
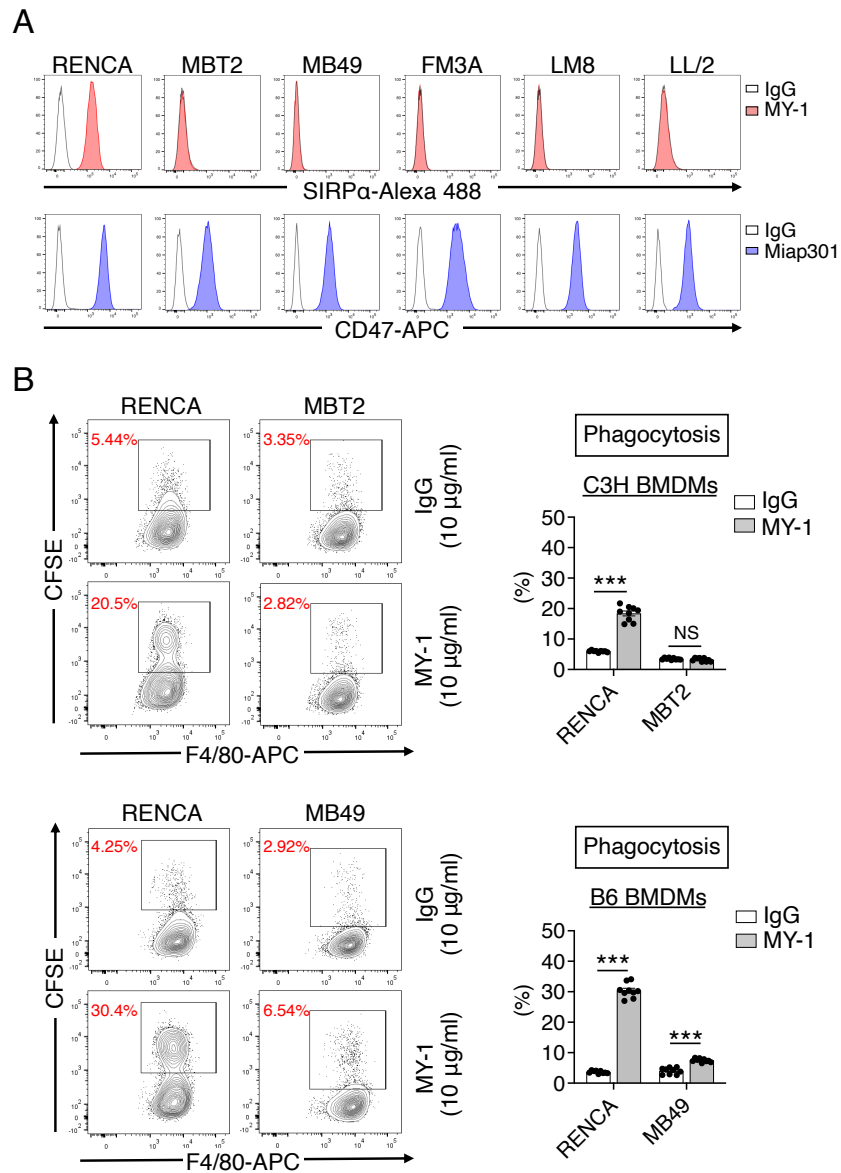
Fig. S3. Effect of MY-1 on phagocytosis of SIRPα-positive or SIRPα-negative cancer cells by macrophages. (***A***) Representative flow cytometry histograms showing the expression of SIRPα and CD47 on the cell surface for murine bladder cancer MBT2 and MB49 cells, mammary cancer FM3A cells, osteosarcoma LM8 cells, Lewis Lung cancer LL/2 cells, and renal cancer RENCA cells. Data are representative of three separate experiments. (***B***) CFSE-labeled RENCA cells, MBT2 cells (upper panels), or MB49 cells (lower panels) were inculcated for 4 h with BMDMs from C3H mice (upper panels) or B6 mice (lower panels) as well as in the presence of either MY-1 or control IgG (each at 10 μg/mL). Cells were then harvested for flow cytometric determination of the percentage of CFSE⁺F4/80⁺ BMDMs (BMDMs that had phagocytosed CFSE-labeled cancer cells) among all F4/80⁺ BMDMs. Representative flow cytometric plots (left panels) as well as quantitative data (means ± SEM, *n* = 9) from three separate experiments, each performed in triplicate (right panels), are shown. ***P < 0.001, NS (Welch and Brown-Forsythe ANOVA with Dunnett's T3 multiple-comparison test).
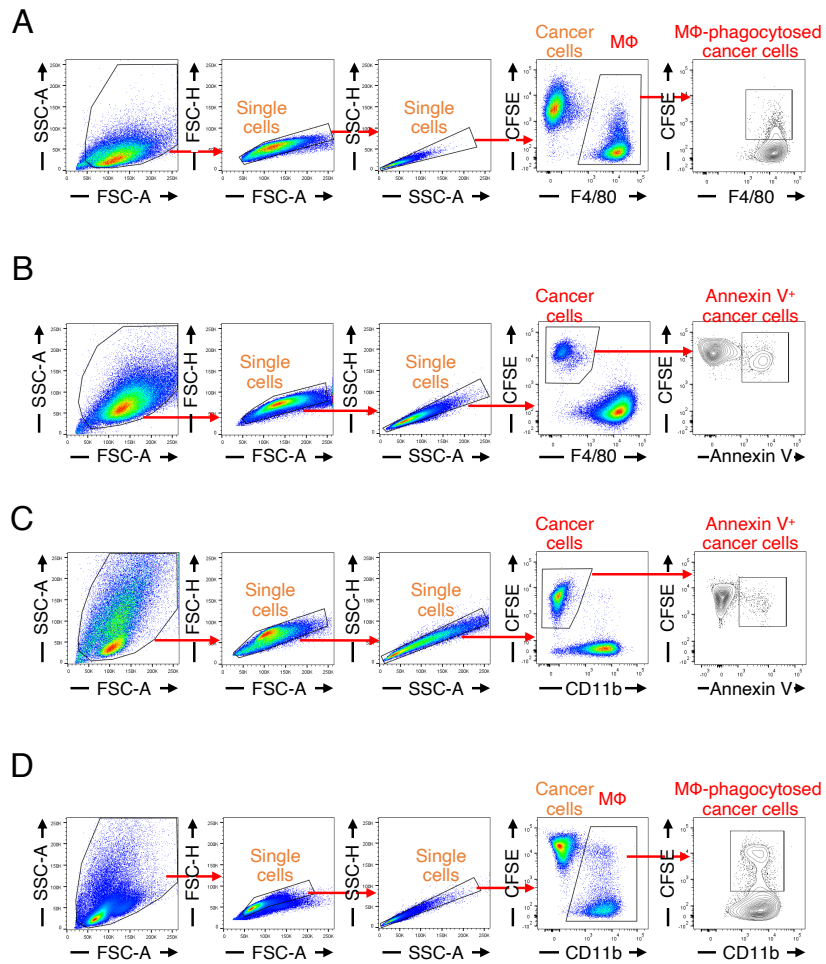
Fig. S4. Gating strategy for cancer cells and macrophages in phagocytosis and cytotoxicity assays. Representative flow cytometric plots show the gating strategy for murine BMDMs from C3H mice and CFSE-labeled cancer (MBT2) cells (*A* and *B*) or for human macrophages and CFSE-labeled cancer (T24) cells (*C* and *D*) in phagocytosis (*A* and *D*) or cytotoxicity (*B* and *C*) assays.
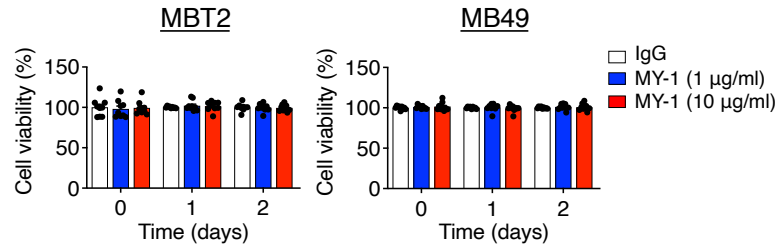
Fig. S5. Lack of effect of MY-1 on the viability of MBT2 or MB49 cells. MBT2 or MB49 cells were treated with MY-1 (1 or 10 μg/mL) or control IgG (10 μg/mL) for the indicated times and then assayed for cell viability. Data are means ± SEM from three separate experiments, each performed in triplicate (n = 9 for each group).
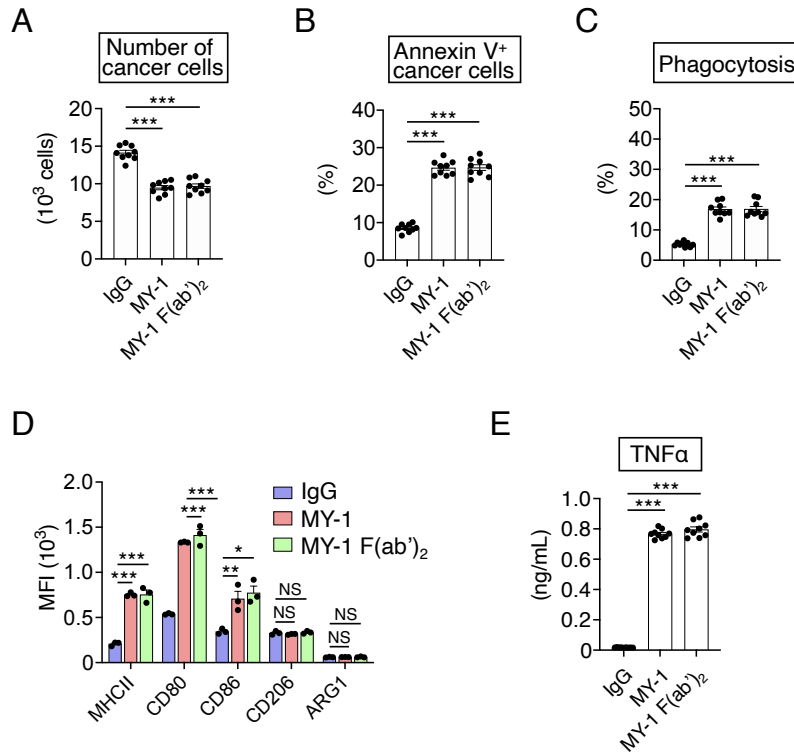
Fig. S6. The Fc portion of MY-1 is not required for promotion of the killing activity of macrophages against MBT2 cells as well as for polarization of macrophages toward a M1-like phenotype. CFSE-labeled MBT2 cells were incubated for 16 h with BMDMs from C3H mice and in the presence of control IgG (10 μg/mL), intact MY-1 (10 μg/mL), or the F(ab')$_2$ fragment of MY-1 (7.3 μg/mL). Cells were then harvested for flow cytometric determination of the number of cancer cells (**A**), the percentage of annexin V$^+$ cancer cells (**B**), and the percentage of BMDMs that had phagocytosed cancer cells (**C**) as in **Fig. 2A–C**. (**D**) MFI for MHCII, CD80, CD86, CD206, and ARG1 expression in C3H BMDMs in the presence of control IgG (10 μg/mL), intact MY-1 (10 μg/mL), or the F(ab')$_2$ fragment of MY-1 (7.3 μg/mL) for 24 h. (**E**) C3H BMDMs were treated with control IgG (10 μg/mL), intact MY-1 (10 μg/mL), or the F(ab')$_2$ fragment of MY-1 (7.3 μg/mL) for 48 h, after which culture supernatants were collected and assayed for TNFα. Data are means ± SEM for three separate experiments, each performed in triplicate (n = 9 for each group) (**A**-**C** and **E**) or for three separate experiments (n = 3 for each group) (**D**). *P < 0.05, **P < 0.01, ***P < 0.001, NS by Welch and Brown-Forsythe ANOVA with Dunnett's T3 multiple-comparison test (**A**-**C** and **E**) or by one-way ANOVA with the Tukey's multiple-comparison test (**D**).
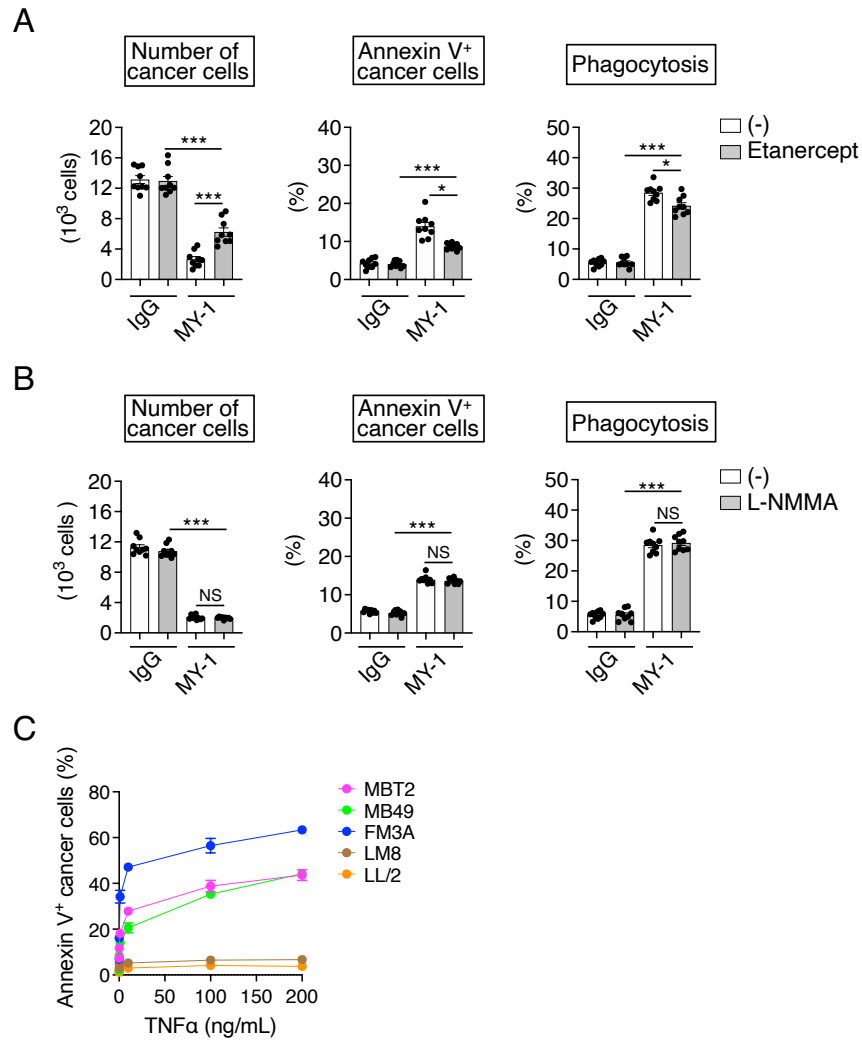
Fig. S7. Importance of TNFα for MY-1–induced killing of MB49 cells by macrophages as well as the sensitivity of murine cancer cell lines to TNFα-induced apoptosis or death. (*A* and *B*) CFSE-labeled MB49 cells were cocultured for 16 h with BMDMs from B6 mice in the presence of control IgG or MY-1 (each at 10 µg/mL) as well as of vehicle (–), etanercept at 10 µg/mL (*A*), or L-NMMA at 300 µM (*B*). The cells were then harvested for determination of the number of cancer cells (left panels), the percentage of annexin V$^+$ cancer cells (middle panels), and the percentage of BMDMs that had phagocytosed cancer cells (right panels) as in **Fig. 2*A–C***. (*C*) Murine bladder cancer MBT2 and MB49 cells, mammary cancer FM3A cells, osteosarcoma LM8 cells, and Lewis Lung cancer LL/2 cells were treated for 48 h with recombinant mouse TNFα at the indicated concentrations and then subjected to flow cytometric analysis for determination of the percentage of annexin V$^+$ cells. All data are means ± SEM for three separate experiments, each performed in triplicate (*n* = 9 for each group) (*A* and *B*), or for three separate experiments (*n* = 3 for each group) (*C*). *$P < 0.05$, ***$P < 0.001$, NS (Welch and Brown-Forsythe ANOVA with Dunnett's T3 multiple-comparison test).
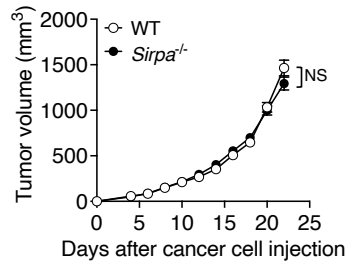
Fig. S8. Lack of effect of SIRPα deletion in mice on tumor formation by MB49 cells. Wild-type (WT) and *Sirpa*$^{-/-}$ mice were injected subcutaneously with MB49 cells, and then tumor volume was measured. The data are means ± SEM from two separate experiments (*n* = 9 mice per group. NS (two-way repeated-measures ANOVA with the Greenhouse-Geisser correction and Šídák's multiple-comparison test).
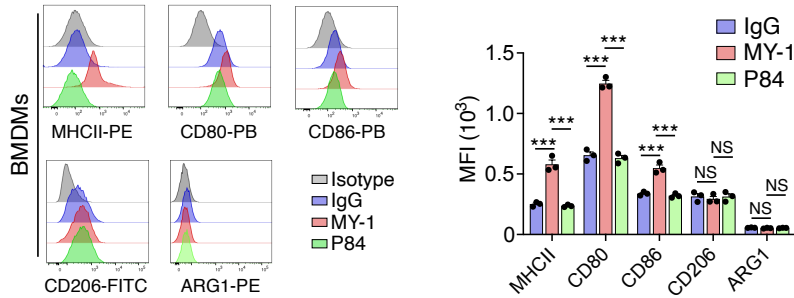
Fig. S9. Lack of effect of the P84 mAb to mouse SIRPα on the expression of MHCII, CD80, CD86, CD206, and ARG1 in macrophages. BMDMs from C3H mice were treated with control IgG, P84, or MY-1 (each at 10 μg/mL) for 48 h and then analyzed for the expression of MHCII, CD80, CD86, CD206, and ARG1 by flow cytometry. Representative overlaid flow cytometry histograms (left panel) as well as quantitative data for MFI (means ± SEM, n = 3 for each group) from three separate experiments (right panel) are shown. ***P < 0.001, NS (one-way ANOVA with the Tukey's multiple-comparison test).

A

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            1         10        20        30        40        50
SIRPα       KELKVTQPEKSVSVAAGDSTVLNCTLTSLLPVGPIRWYRGVGPSRLLIYSFAGEYVPRI
SIRPβ1A     RELKVIQPVKSFFVGAGGSATLNCTVTSLLPVGPIRWYRGVGQSRLLIYPFTGEHSPRI
SIRPβ1B     RELKVIQPVKSFFVGAGGSATLNCTVTSLLPVGPIRWYRGVGQSRLLIQSFTGEHFPRI
SIRPβ1C     RELKVIQPVKSFFVGAGGSATLNCTVTSLLPVGPIRWFKGVGQSRLLIYSFTGERFPRI

            60        70        80        90        100       110
SIRPα       RNVSDTTKRNNMDFSIRISNVTPADAGIYYCVKFQKGSSEPDTEIQSGGGTEVYVL
SIRPβ1A     TNVSDVTKRNNMDFSIRISNVTPADSGTYYCVKFQRGSSEPDIEIQSGGGTELLVL
SIRPβ1B     TSVSDVTKRNNLDFSIRISNVTPADSGTYYCVKFQRGSSESDREIQSGGGTELSVL
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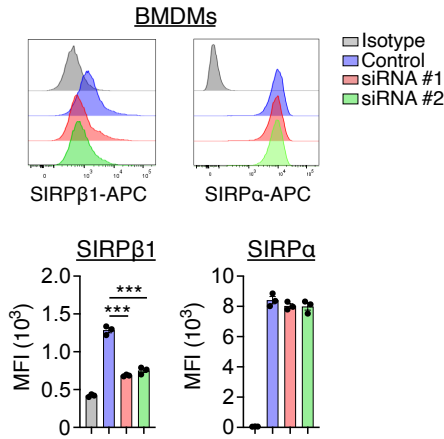
B

Fig. S10. Amino acid sequence alignment for the Ig-V–like domains of mouse SIRP proteins as well as siRNA-mediated knockdown of SIRPβ1 in mouse BMDMs. (**A**) Amino acid sequence alignment of the Ig-V–like domains of mouse SIRPα, SIRPβ1A, SIRPβ1B, and SIRPβ1C. Residues that differ between SIRPα and SIRPβ1 paralogs are shown in red. (**B**) Flow cytometric analysis of the expression of SIRPβ1 and SIRPα on C3H BMDMs transfected with control or mouse SIRPβ1–specific (#1 or #2) siRNAs for 48 h. Representative overlaid flow cytometry histograms (upper panel) and quantitative data for MFI (means ± SEM, n = 3 for each group) from three separate experiments (lower panel) are shown. ***P < 0.001 (one-way ANOVA with the Tukey's multiple-comparison test).
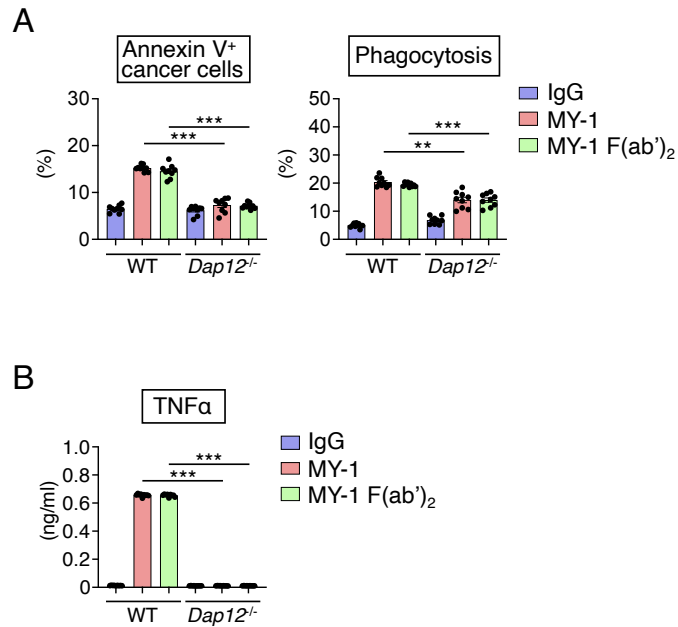
16

A

Annexin V⁺ cancer cells

Phagocytosis

IgG
MY-1
MY-1 F(ab')$_2$

B

TNFα

IgG
MY-1
MY-1 F(ab')$_2$

Fig. S11. Importance of DAP12 for the MY-1–dependent killing of MB49 cells by macrophages. (***A***) CFSE-labeled MB49 cells were incubated for 16 h with WT BMDMs or *Dap12*$^{-/-}$ BMDMs as well as with MY-1 (at 10 µg/mL), the F(ab')$_2$ fragment of MY-1 (7.3 µg/mL), or control IgG (at 10 µg/mL). The cells were then harvested for flow cytometric determination of the percentage of apoptotic or dead cancer cells (annexin V⁺CFSE⁺F4/80⁻ cells) among all cancer cells (CFSE⁺F4/80⁻ cells) (left panel) and the percentage of CFSE⁺F4/80⁺ BMDMs (BMDMs that had phagocytosed CFSE-labeled cancer cells) among all F4/80⁺ BMDMs (right panel). (***B***) WT BMDMs or *Dap12*$^{-/-}$ BMDMs were also treated with control IgG (at 10 µg/mL), MY-1 (at 10 µg/mL), or the F(ab')$_2$ fragment of MY-1 (7.3 µg/mL) for 48 h, after which culture supernatants were collected and assayed for TNFα. All data are means ± SEM for three separate experiments, each performed in triplicate (*n* = 9 for each group). **P < 0.01, ***P < 0.001 (Welch and Brown-Forsythe ANOVA with Dunnett's T3 multiple-comparison test).
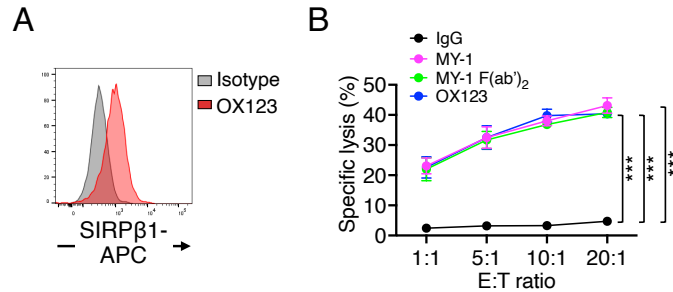
Fig. S12. Promotion by MY-1 of the neutrophil-mediated killing of murine bladder cancer cells. (*A*) A representative flow cytometric histogram for mouse SIRPβ1 expression on neutrophils isolated from bone-marrow cells of C3H mice. Data are representative of three separate experiments. (*B*) TDA (2,2':6',2"-terpyridine-6,6"-dicarboxylate)-labeled MBT2 cells (target cells) were incubated for 4 h with at the indicated E (effector):T (target) ratios with neutrophils (effector cells) from C3H mice in the presence of either control IgG (at 10 μg/mL), MY-1 (at 10 μg/mL), the F(ab')$_2$ fragment of MY-1 (at 7.3 μg/mL), or OX123 (at 10 μg/mL). The extent of target cell lysis was then determined on the basis of the amount of TDA released into the medium. Quantitative data are means ± SEM for three separate experiments (*n* = 3 for each group). ***P < 0.001 for indicated comparison (one-way ANOVA with the Tukey's multiple-comparison test).
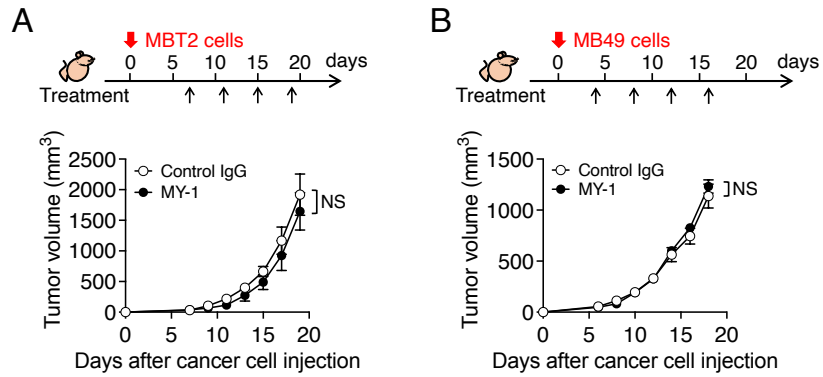
Fig. S13. Lack of effect of MY-1 on the growth of tumors formed by MBT2 or MB49 cells in BALB/c-*nu*/*nu* mice. Tumor volume was determined for BALB/c-*nu*/*nu* mice injected subcutaneously with MBT2 (**A**) or MB49 (**B**) cells and treated intraperitoneally with control IgG or MY-1 (each at 200 µg) according to the indicated schedule. Data are means ± SEM of *n* = 5 (**A**) or *n* = 10 (**B**) mice per group. NS (two-way repeated-measures ANOVA with the Greenhouse-Geisser correction and Šídák's multiple-comparison test).

SI References

1. W. Chuang, C. F. Lagenaur, Central nervous system antigen P84 can serve as a substrate for neurite outgrowth. *Dev Biol* **137**, 219-232 (1990).
2. M. Seiffert *et al.*, Signal-regulatory protein α (SIRPα) but not SIRPβ is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34$^+$CD38$^-$ hematopoietic cells. *Blood* **97**, 2741-2749 (2001).
3. N. Verjan Garcia *et al.*, SIRPα/CD172a regulates eosinophil homeostasis. *J Immunol* **187**, 2268-2277 (2011).
4. T. Yanagita *et al.*, Anti-SIRPα antibodies as a potential new tool for cancer immunotherapy. *JCI Insight* **2**, e89140 (2017).
5. K. Washio *et al.*, Dendritic cell SIRPα regulates homeostasis of dendritic cells in lymphoid organs. *Genes Cells* **20**, 451-463 (2015).
6. T. Nishimura *et al.*, SIRPα on CD11c$^+$ cells induces Th17 cell differentiation and subsequent inflammation in the CNS in experimental autoimmune encephalomyelitis. *Eur J Immunol* **50**, 1560-1570 (2020).
7. T. Kaifu *et al.*, Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice. *J Clin Invest* **111**, 323-332 (2003).
8. D. Hazama *et al.*, Macrocyclic peptide-mediated blockade of the CD47-SIRPα interaction as a potential cancer immunotherapy. *Cell Chem Biol* **27**, 1181-1191 e1187 (2020).