Supplementary Materials

The PD-L1/TLR7 dual-targeting nanobody-drug conjugate mediates potent tumor regression by elevating tumor immunogenicity in a host-expressed PD-L1 bias-dependent manner

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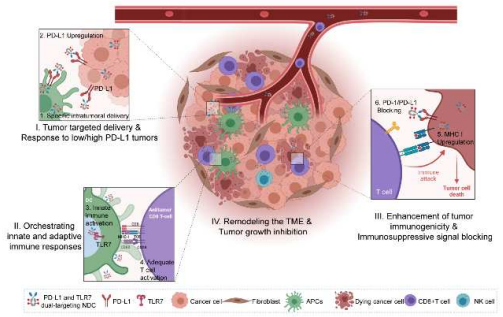
Graphical abstracts

The PD-L1/TLR7 dual-targeting nanobody-drug conjugate mediates potent tumor regression via elevating tumor immunogenicity in a host-expressed PD-L1 bias-dependent way



Authors
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In Brief
Relying on the complementarity of PD-L1 nanobody and TLR7 agonist, the PD-L1/TLR7 dual-targeting NDC was constructed and exerted potent antitumor efficacy. PD-L1 nanobodies with tumor-targeting properties can assist in the delivery of TLR7 agonists and release tumor immunosuppression. TLR7 agonists can enhance the immunogenicity of tumors and promote intratumoral PD-L1 expression. The NDC mediates tumor regression via a host-expressed PD-L1 bias-dependent way.

Supplemental methods

Mice

Female (six- to eight-week-old) BALB/c mice (Shanghai Sipp-BK), C57BL/6J mice (Shanghai Sipp-BK) and PD-L1-deficient C57BL/6J mice (Shanghai Model Organisms Center) were maintained under specific pathogen-free (SPF) conditions in the animal facility of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (SIMM). BALB/c-hPD1/hPDL1 mice (GemPharmatech) were housed in SPF conditions. Animal care and experiments were performed in accordance with GemPharmatech and SIMM using protocols approved by the Institutional Laboratory Animal Care and Use Committee (IACUC).

Cell lines

HEK 293F cells were purchased from Thermo Fisher and grown in FreeStyle™ 293 expression medium (Thermo Fisher). HEK 293T, Jurkat, RKO, CT26 and B16-F10 cell lines were purchased from the American Type Culture Collection (ATCC). NK-92 and LLC cell lines were purchased from Procell. CT26/hPD-L1 cell lines were

provided by GemPharmatech. All cells were cultured at 37°C in a 5% CO2 humidified atmosphere. The cell culture mediums were recommended by the reagent manufacturers above.

Plasmids

The vectors pFUSE-hIgG1-Fc2 and pFUSE-mIgG2b-Fc were purchased from InvivoGen. Vector pLVX-puro was purchased from Clontech. The vector pLV-NFAT-RE-Luci was purchased from Inovogen. LentiCRISPR v2, pVSV-G, pMDlg/pRRE and pRSV-Rev were purchased from Youbio. pECMV-mouse PD-1/PDCD1 was purchased from Miaolingbio.

Construction of stable transfected cell lines

The 293T/mPD-L1 cell line was generated by a lentiviral system. Briefly, the full murine PD-L1 gene was subcloned into the pLVX-puro vector (Clontech), which was then transfected into HEK 293T cells with the package plasmids (pVSV-G, pMDlg/pRRE and pRSV-Rev). Then, the virosomes were collected and transfected into HEK 293T cells. After transfection, the transfected HEK 293T cells were selected by puromycin, and murine PD-L1 expression in 293T cells was tested by flow cytometry (BD Biosciences). The PD-L1-deficient B16-F10 cell line was also constructed through lentivirus encoding sgRNA (5’ GTATGGCAGCAACGTCACGA 3’). Cells were selected by puromycin and tested by flow cytometry (BD Biosciences).

Quantitative biodistribution study

CT26 cells [induced with mouse IFN-γ (GenScript) for 24 h, 1×10^6] were injected subcutaneously into the right flank of BALB/c mice. Mice were treated intraperitoneally with 200 μg of Nb16 on day 10. Tumor tissues were collected on 0 h, 0.5 h, 1 h, 2 h, 4 h and 8 h after Nb16 treatment. The level of Nb16 in tumor tissues was measured by an anti-mFc-based ELISA.

Antibody conjugation and characterization

SZU-101 was connected to PEG to yield the intermediate compound SZU-107. 40 mg of SZU-107, 35 mg of HBTU, 21.34 mg of NHS, and 22 μL of DIPEA was added into 0.5 mL of DMF, and the reaction mixture was stirred at rt. After 30 mins, the HPLC indicated the completion. The solution was subjected to preparative HPLC and the fractions containing the products were collected and lyophilized to give a white powder as SZU-107-NHS. Subsequently, the activated ester was dissolved with DMSO. Antibodies and small molecules were reacted in a molar ratio of 1:10. Small molecules were added to Nb16, 4G11 or 10F.9G2 solution and the reaction was stirred at 4°C for 4 hours. After the reaction, the small molecules were removed by filtration with a 10 kD biofilter membrane and the novel compound Nb16-SZU-101, 4G11-SZU-101 or 10F.9G2-SZU-101 were obtained. To identify NDC, the antibodies were denatured to open the disulfide bond, after which the sample was identified by Xevo G2-XS QTOF mass spectrometer (Waters). The DAR value was calculated mainly based on the increase in molecular weight of NDC over the uncoupled antibody.

In *vitro* analysis of immune responses

Jurkat-NFAT effector cells reporter assay

Jurkat-NFAT-luciferase cells were constructed by stable transfection of pLV-NFAT-RE-Luci by lentivirus. Jurkat-NFAT-luciferase cells were activated with phorbol-12-myristate-13-acetate (PMA) and ionomycin. To test the activity of Nb16, CT26 cells were induced with mouse IFN-γ for 24 h. Then, 2 μg/mL Nb16 and activated Jurkat-NFAT-luciferase cells that were transfected with plasmids pECMV-mouse PD-1/PDCD1 were added. To test the blocking activity of 4G11, RKO cells were induced by human IFN-γ for 24 h. Then, activated Jurkat-NFAT-luciferase cells and 2 μg/mL 4G11 were added. Finally, Jurkat-NFAT-luciferase cells in the coculture system were collected and measured by luminescence activity.

SZU-101 promoted the proliferation and cytokine secretion of splenocytes

BALB/c mice were humanely sacrificed, and the spleen was collected. Single-cell suspensions of splenocytes were obtained by grinding spleen tissues. To detect cell proliferation, splenocytes were added to 96-well plates and stimulated with different concentrations of SZU-101 or SZU-107 for 72 h. The proliferation level of splenocytes was detected by Cell Proliferation Kit I (MTT, Roche). To detect cytokine secretion of splenocytes, the cells were added to 24-well plates and stimulated with SZU-101 for 24 h. TNF-α and IFN-γ in the supernatant were detected by the corresponding ELISA kits (Excell Bio).

SZU-101 acted on bone marrow-derived DCs

BALB/c mice were humanely sacrificed, and bone marrow cells were collected using a standard method. The cells were cultured with DMEM containing 25 ng/mL GM-CSF (GenScript) and 10 ng/mL IL-4 (GenScript) for 5 days. Subsequently, immature DCs were collected and stimulated with 1 μg/mL lipopolysaccharide (LPS)

or 50 μM SZU-101 for 24 h. Maturation markers in DCs were identified by flow cytometry.

SZU-101 acted on bone marrow-derived macrophages

Bone marrow cells were obtained as described above. Bone marrow cells were cultured with DMEM containing 20 ng/mL M-CSF for 4 days. Macrophage polarization was treated with 20 ng/mL IFN-γ (GenScript) and 100 ng/mL LPS for M1 polarization or 40 ng/mL IL-4 for M2 polarization. After one day, M2-type macrophages were stimulated with 50 μM SZU-101 for 24 h. M1 and M2 markers in macrophages were identified by flow cytometry.

SZU-101 acted on spleen-derived DCs and spleen-derived macrophages

Splenocytes were obtained as described above. Splenocytes (1×10^6 cells/mL) were added to 24-well plates at 500 μL/well. Mouse splenocytes were stimulated with SZU-101 (50 μM) for 24 h, and PD-L1 levels in DCs and macrophages were identified by flow cytometry.

SZU-101 acted on peritoneal macrophages

After 3 days of induction with Brewer thioglycolate medium, peritoneal macrophages were obtained from mice by peritoneal lavage. Macrophage polarization was treated with 20 ng/mL IFN-γ and 100 ng/mL LPS for M1 polarization or 40 ng/mL IL-4 for M2 polarization. After one day, M2-type macrophages were stimulated with 50 μM SZU-101 or imiquimod for 24 h. M1 and M2 markers in macrophages were identified by flow cytometry.

SZU-101 activated NK cells

NK-92 cells were treated with 50 μM SZU-101 for 24 h. After collection and blocking, NK-92 cells were stained with anti-human CD25 APC (BD Biosciences) and anti-human CD69 APC-Cy7 (BD Biosciences) and detected by flow cytometry.

SZU-101 did not promote PD-L1 expression in CT26 cells

CT26 cells were treated with 50 μM SZU-101 for 24 h. Then, the cells were collected and stained with Ms CD274 BV650 MIH5 (BD Biosciences) followed by detection with flow cytometry.

SZU-101 acted on CT26 cells and splenocytes coculture system

Splenocytes (300 μL, 5×10^5 cells/mL) and CT26 cells (300 μL, 5×10^5 cells/mL) were added to 24-well plates for coculture. Cells were stimulated with 50 μM SZU-101 for 24 h. For cytokine neutralization assays, 20 μg/mL of anti-IFN-γ antibodies (BioXcell) or 20 μg/mL of anti-TNF-α antibodies (BioXcell) were added with SZU-101. PD-L1 levels in CT26 cells were identified by flow cytometry.

In *vivo* treatment study

Antitumor activity of Nb16 or SZU-101 or combination or Nb16-SZU-101

CT26 cells (induced with mouse IFN-γ (GenScript) for 24 h) or noninducing CT26 cells (1×10^6 cells) were subcutaneously inoculated into female BALB/c mice. Mice were randomly grouped when the average tumor volume reached 40-60 mm^3 and injected with mFc, Nb16, SZU-101, combination or Nb16-SZU-101. To compare the efficacy of anti-PD-L1 antibody, 10F.9G2, 10F.9G2 combined with SZU-101, 10F.9G2-SZU-101 were administered. For survival studies, euthanasia endpoints were set as tumors larger than 1500 mm^3 or larger than 20 mm in length or mice with significant weight loss.

In the CT26 large tumor model, CT26 cells (1×10^6 cells) were subcutaneously inoculated into female BALB/c mice. Mice were randomly grouped when the average tumor volume reached 200 mm^3 and injected with mFc (200 μg/each) or Nb16-SZU-101 (200 μg/each) every two days for a total of four doses.

In the B16-F10 model, C57BL/6 mice were inoculated subcutaneously with B16-F10 cells (2×10^5), Cas9 control B16-F10 cells (5×10^5) or PD-L1-deficient B16-F10 cells (5×10^5). Mice were randomly grouped and injected with mFc or Nb16-SZU-101.

Rechallenge

For the mice with complete tumor regression after Nb16-SZU-101 treatment, CT26 cells (5×10^5 cells) were injected subcutaneously into the opposite side flank one week after tumor regression, and tumor growth was observed and recorded.

LLC orthotopic lung tumor model

1×10^6 of LLC cells were injected into C57BL/6 mice through the tail vein. mFc and Nb16-SZU-101 were administered at the doses and times described above. After 20 days, the mice were sacrificed and the lungs were harvested for weighing and photographing. Lungs were embedded and stained with H&E and then examined microscopically.

B16F10 lung metastasis tumor model

1×10^6 of B16F10 cells were injected into C57BL/6 mice through the tail vein. mFc and Nb16-SZU-101 were administered at the doses and times described above. After 23 days, the mice were sacrificed and the lungs were harvested for weighing and photographing.

B16-F10 model in PD-L1-deficient mice

Six- to eight-week-old PD-L1-deficient C57BL/6J mice were inoculated subcutaneously with B16-F10 cells (5×10^5). Mice were randomly grouped when the average tumor volume reached 40-60 mm^3 and injected with mFc (200 μg/each) or Nb16-SZU-101 (200 μg/each) every two days for a total of four doses.

Evaluation of the antitumor activity of 4G11-SZU-101 in a CT26/hPD-L1 xenograft tumor model in PD-1/PD-L1 dual-humanized BALB/c mice

Six- to eight-week-old dual-humanized BALB/c mice were inoculated with CT26/hPD-L1 tumor cells (5×10^5 cells) subcutaneously. When the average tumor volume reached 80-120 mm^3, the mice were randomly divided into three groups [hIgG1 group (130 μg/each), 4G11 group (200 μg/each) and 4G11-SZU-101 group (200 μg/each)]. The dose was performed every three days for a total of six doses. Endpoint dissected tumor tissues were made into paraffin sections and characterized by immunohistochemical staining for CD8, NKR-P1C and GB expression.

Evaluation of PD-L1 expression induced by SZU-101 in vivo

CT26 cells (1×10^6) were injected subcutaneously into the right flank of BALB/c mice. Mice were treated everyday peritumorally with SZU-101 (3 mg/kg) from day 8 for total five doses. For cytokine neutralization experiments in vivo, anti-IFN-γ antibodies (BioXcell) or anti-TNF-α antibodies (BioXcell) were treated as 400 μg/each on day 8 and 200 μg/each on day 10 and day 12. Tumor tissues were collected. The level of PD-L1 of tumor cells and immune cells was measured by flow cytometry.

Evaluation of PD-L1 expression induced by NDC in vivo

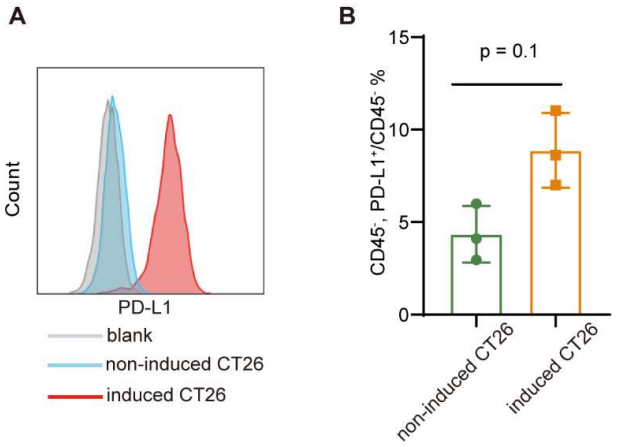
CT26 cells (1×10^6) were injected subcutaneously into the right flank of BALB/c mice. Mice were treated intraperitoneally with 200 μg of Nb16-SZU-101 on day 9, day 12, day 15, day 17 and day 19. Blood, spleen and tumor tissues were collected 48 h after the first dose and the fifth dose. The level of PD-L1 in different tissues was measured by flow cytometry.

TCGA cohorts analysis

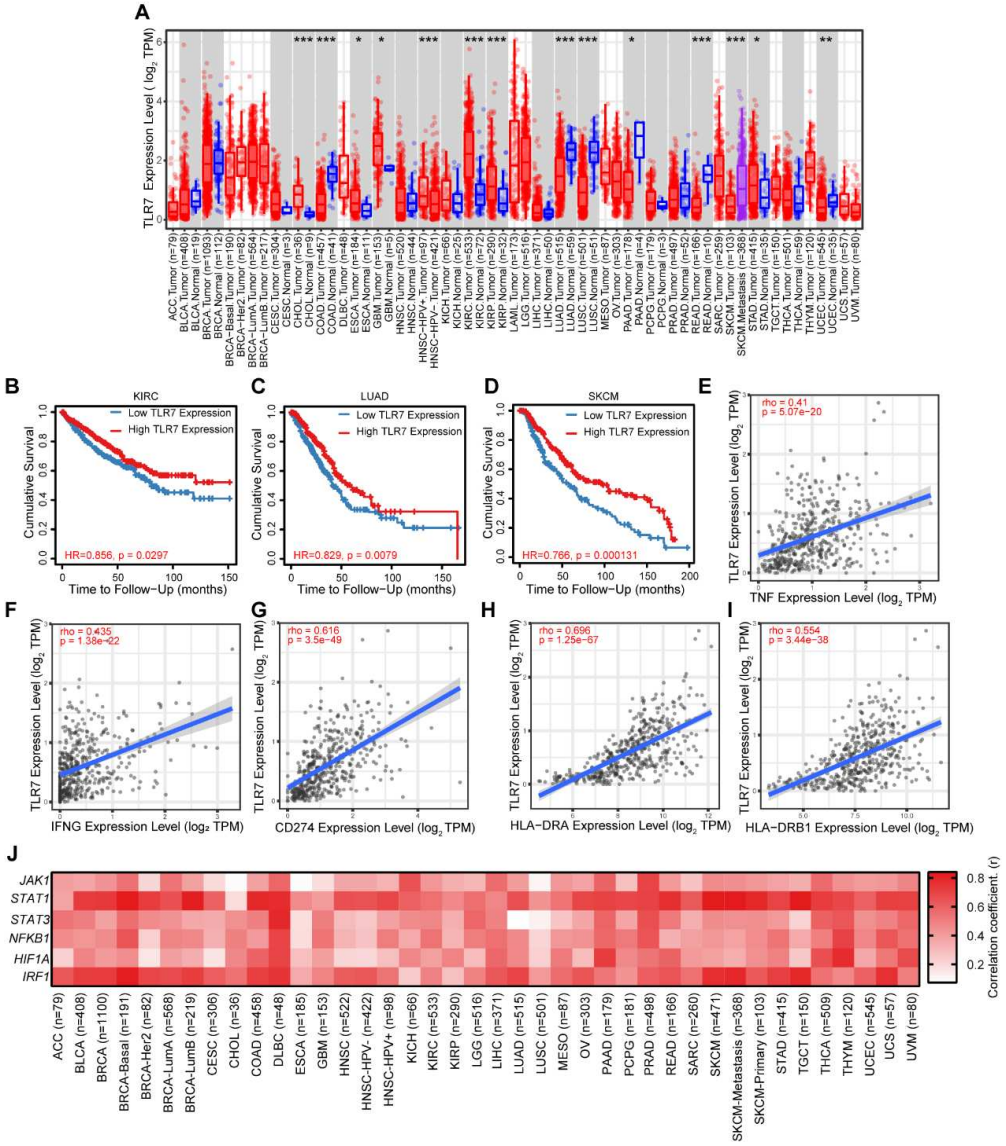
The TIMER2.0 online tool was used to analyze TLR7 expression levels in The Cancer Genome Atlas (TCGA) datasets. TIMER2.0 was used to explore the prognostic value of TLR7 in patients with different types of cancers and the correlation between TLR7 expression levels and intratumoral immune infiltration or other genes (TNF, IFNG, CD274, HLA-DRA and HLA-DRB1).

Antibodies used in immunotyping

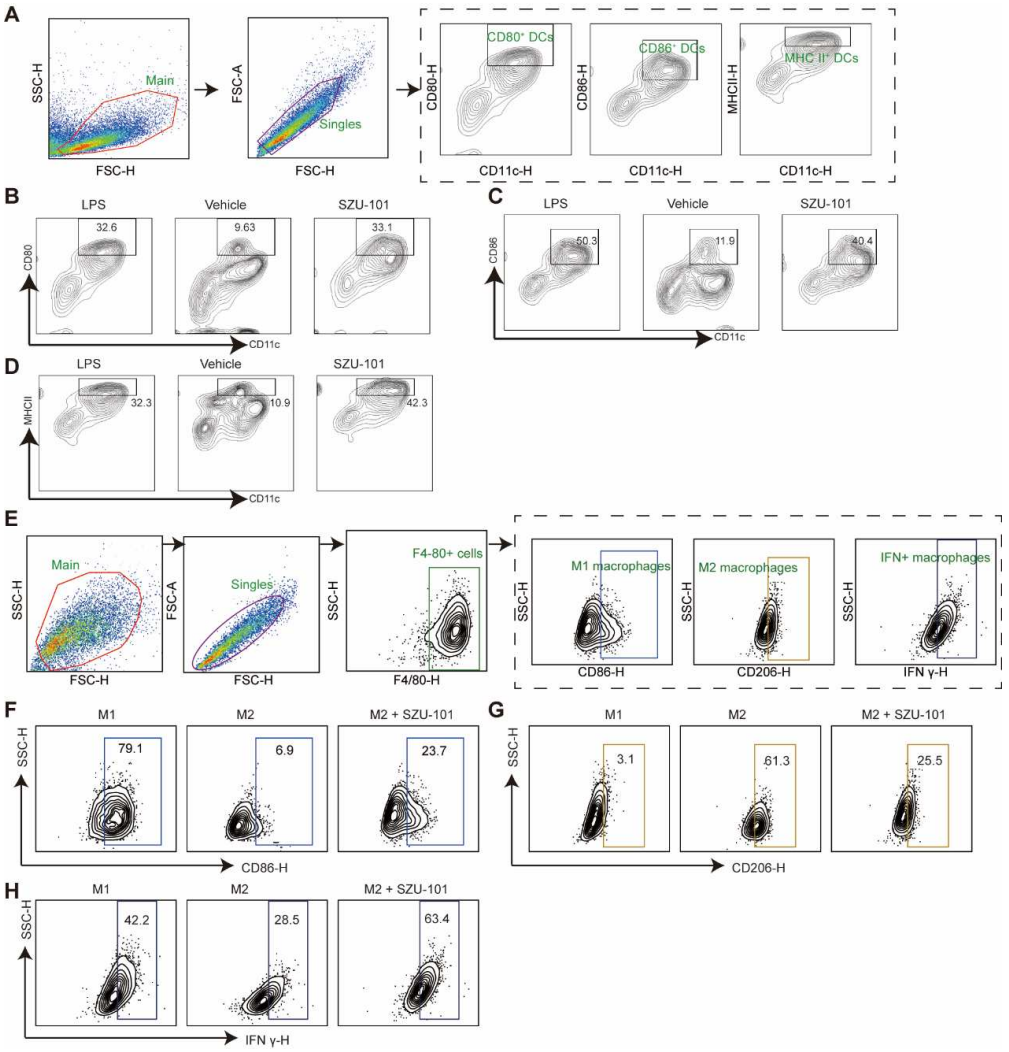
The antibodies used in immunotyping included FITC rat anti-mouse CD45 (BD Biosciences), PE-Cyanine7 rat anti-mouse F4/80 (BD Biosciences), APC rat anti-mouse CD86 (BD Biosciences), PE rat anti-mouse CD274 (BD Biosciences), Ms CD206 Alexa 647 MR5D3 (BD Biosciences), anti-mouse LAP (TW7-16B4) PE (Bioscience), FITC hamster anti-mouse CD3e (BD Biosciences), PerCP-Cy™5.5 Rat Anti-Mouse CD4 (BD Biosciences), APC Rat Anti-Mouse CD8a (BD Biosciences), PE Rat Anti-Mouse IFN-γ (BD Biosciences), anti-mouse granzyme B (NGZB) PE (eBioscience), PE Rat Anti-Mouse CD25 (BD Biosciences), anti-mouse/rat FOXP3 APC (eBioscience), PE-Cy™7 Hamster Anti-Mouse CD11c (BD Biosciences), BB515 Rat Anti-Mouse I-A/I-E (BD Biosciences), PE Hamster Anti-Mouse CD80 (BD Biosciences), APC Rat Anti-Mouse CD49b (BD Biosciences), Ms CD69 PE H1.2F3 (BD Biosciences), FITC anti-mouse CD206 (MMR), PE Rat IgG1 κ Isotype Control RUO (BD Biosciences), Rat IgG2a kappa Isotype Control (eBR2a), PE (eBioscience), PE Rat IgG2a λ Isotype Control RUO (BD Biosciences), RAT IGG2A K ISO CNTL (EBR2A) APC (eBioscience), Ms CD274 BV650 MIH5 (BD Biosciences) and FITC anti-mouse H-2Kd/H-2Dd (Biolegend).



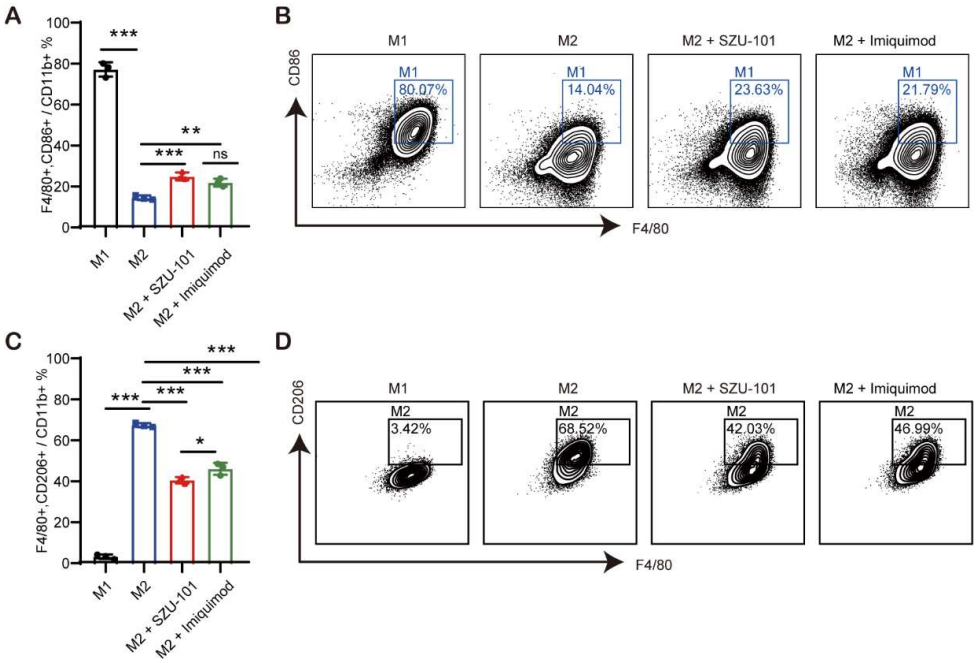
Supplemental figure S1 IFN-γ induced high PD-L1 expression in CT26 tumor models. (A) Flow cytometry histograms showing the high expression of PD-L1 in CT26 cells induced by IFN-γ (100 ng/mL) for 24 h. (B) BALB/c mice were subcutaneously inoculated with 1×10^6 CT26 cells (induced or not by IFN-γ). PD-L1 expression was detected by flow cytometry on day 10. The data are presented as the mean ± SEM.



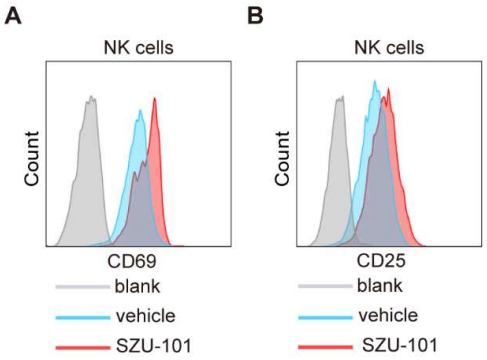
Supplemental figure S2 TLR7 expression correlates with prognosis and PD-L1-associated genes in TCGA cohorts. (A) Human TLR7 expression levels in different tumor types from the TCGA database were determined by TIMER2.0 (*P < 0.05, **P < 0.01, ***P < 0.001 by the Wilcoxon test). (B to D) Kaplan–Meier curves showing the OS in patients with different tumor types as indicated in the TCGA database and determined by TIMER2.0. (E to I) Correlation analysis between TLR7 and TNF (E), IFNG (F), CD274 (G), HLA-DRA (H) and HLA-DRB1 (I) in COAD from TCGA database were determined by TIMER2.0 and showed by scatter plots. (J) The expression association of CD274 with JAK1, STAT1, STAT3, NFKB1, HIF1A and IRF1 in different tumor types in the TCGA database as determined by TIMER2.0. Spearman's rank correlation coefficient is represented by r.



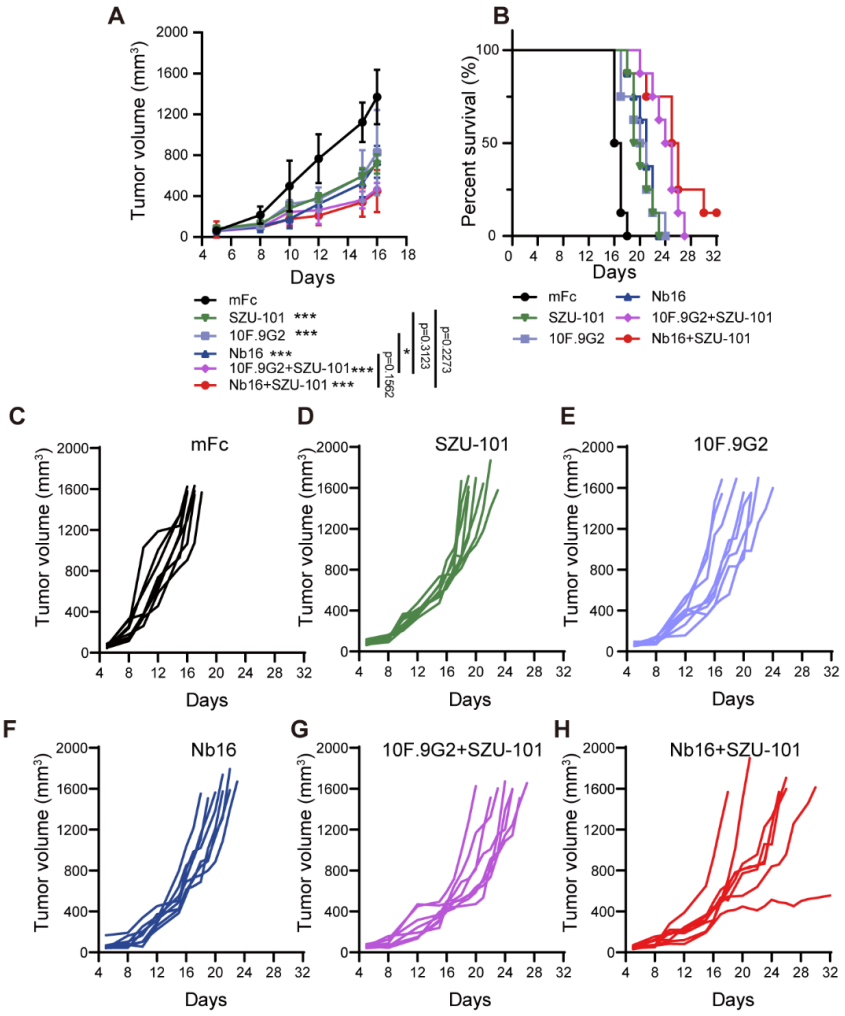
Supplemental figure S3 Gating strategy and raw data for SZU-101 induced BMDCs and BMDMs. (A) Representative flow-cytometry gating strategy for quantifying the BMDC activation markers. (B to D) Representative raw data for quantifying the BMDC activation markers. (E) Representative flow-cytometry gating strategy for quantifying the BMDMs. (F to H) Representative raw data for quantifying the BMDMs.



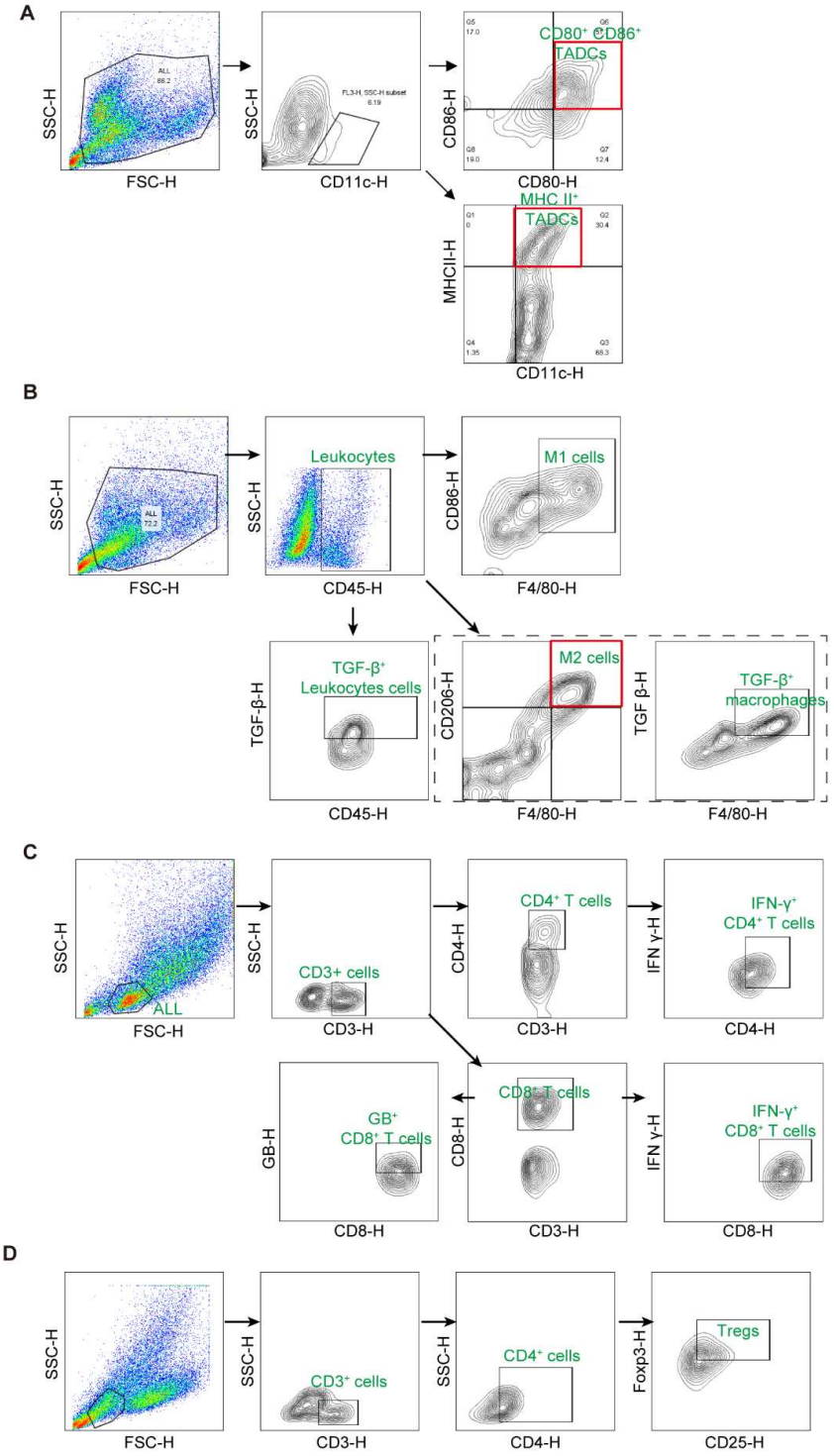
Supplemental figure S4 SZU-101 repolarized peritoneal macrophages. (A to D) BMDMs from BALB/c mice induced to differentiate into M2 macrophages were stimulated with SZU-101 or imiquimod for 24 h (n = 3). CD86 (A and B) and CD206 (C and D) expression in macrophages was determined by flow cytometry. The data are presented as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns not significant by one-way ANOVA followed by Tukey's multiple comparisons test.



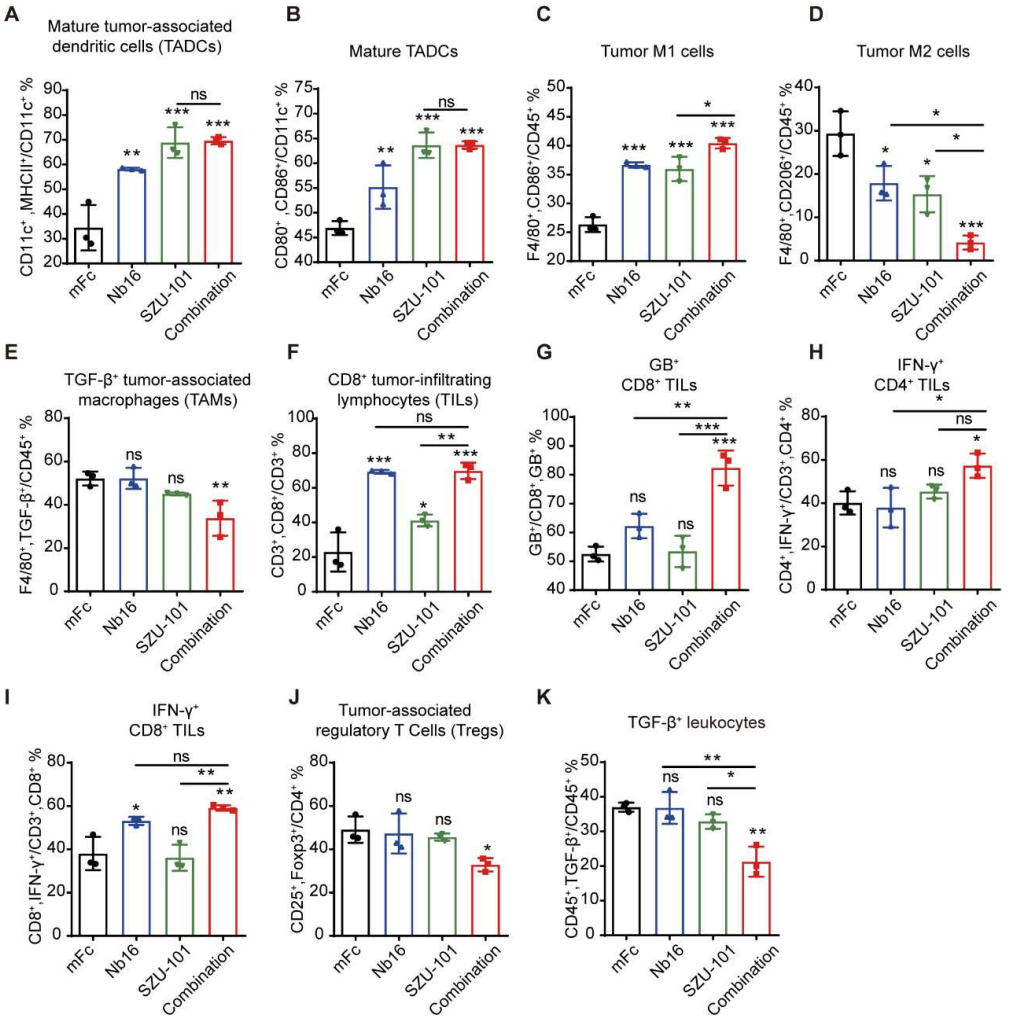
Supplemental figure S5 SZU-101 activated the NK cells. Flow cytometry histograms showing the expression of CD69 (A) and CD25 (B) on NK92 cells stimulated with 50 μM of SZU-101 for 24 h.



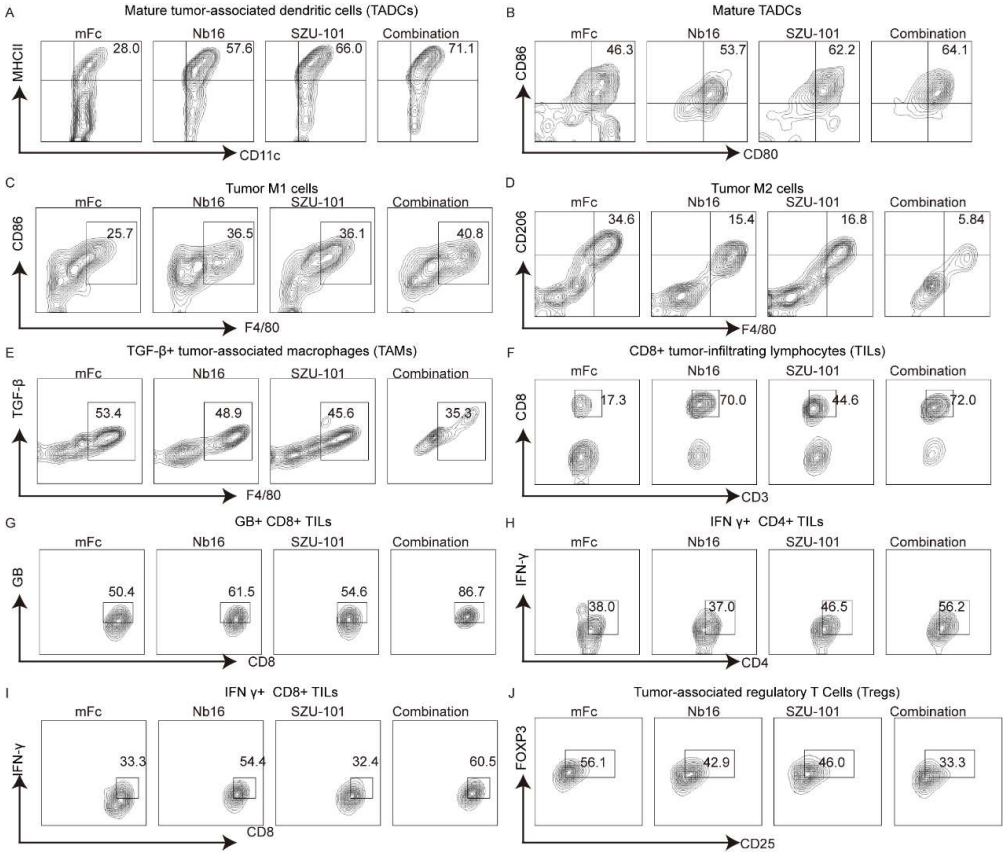
Supplemental figure S6 Comparison for antitumor activity of the anti-PD-L1 nanobody Nb16 combined with SZU-101 and the anti-PD-L1 monoclonal antibody 10F.9G2 combined with SZU-101. (A to H) BALB/c mice bearing CT26 tumors were treated with mFc, SZU-101, Nb16, 10F.9G2 or a combination of these compounds. Tumor growth (A, C to H) and survival curve (B) are shown (n = 8). The data are presented as the means ± SEM. *** $p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test.



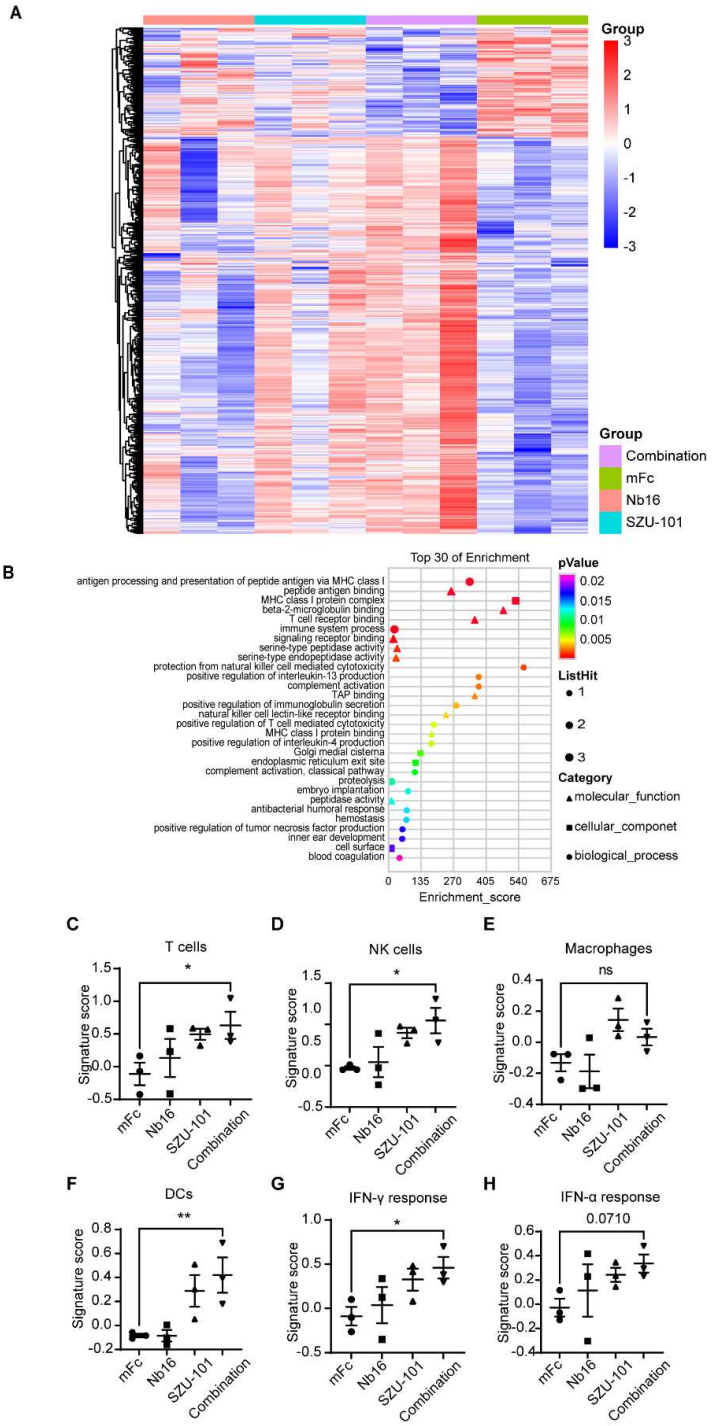
Supplemental figure S7 Gating strategy for intratumoral immune cells. (A-D) Representative flow-cytometry gating strategy for quantifying the proportion of various immune cell subsets in murine tumors.



Supplemental figure S8 Immunophenotyping for Nb16 and SZU-101 combination therapy. (A to K) Immune effector cells in tumors treated with mFc, Nb16, SZU-101 or their combination were quantified by flow cytometry (n = 3). Plots show the proportion of cells in a specific cell population. The data are presented as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns not significant by one-way ANOVA followed by Tukey's multiple comparisons test.

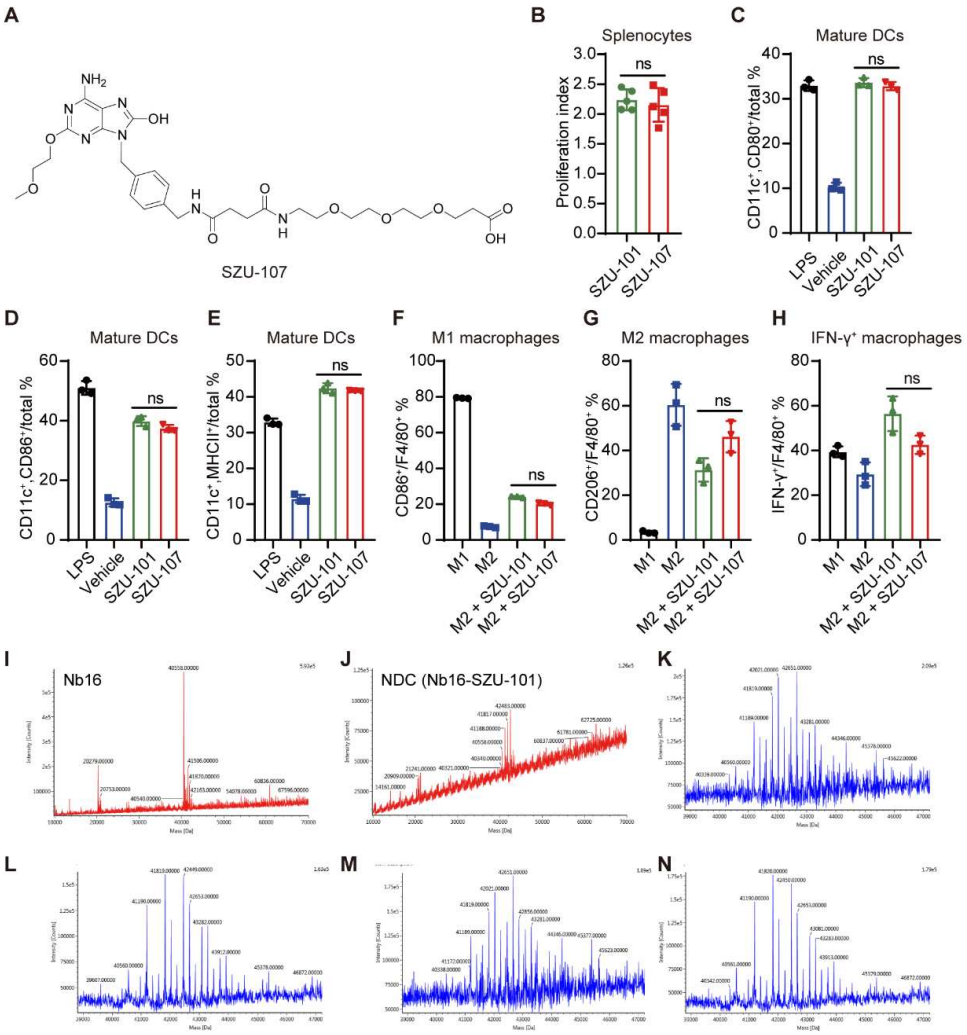


Supplemental figure S9 Representative raw data of the immunophenotyping for Nb16 and SZU-101 combination therapy. (A to J) Immune effector cells in tumors treated with mFc, Nb16, SZU-101 or their combination were quantified by flow cytometry (n = 3).

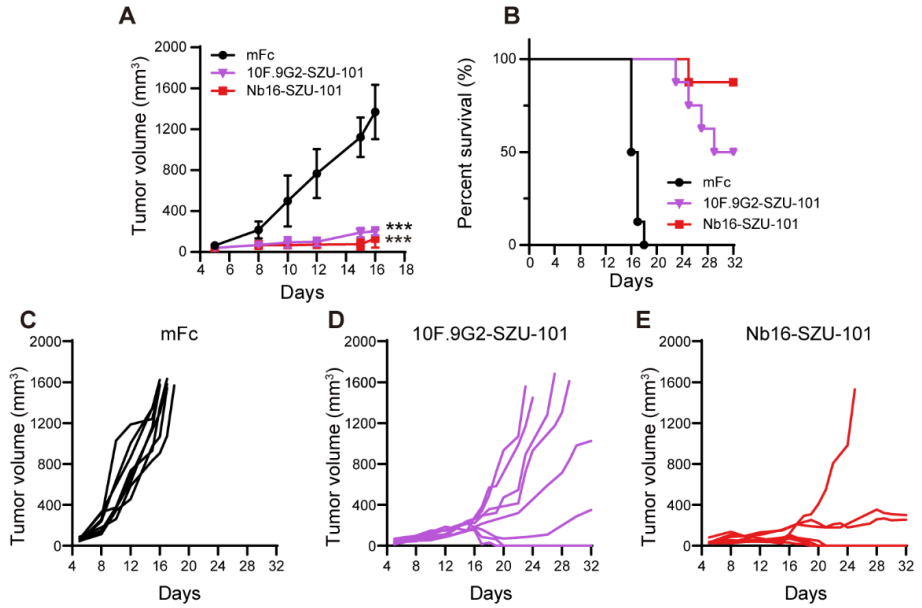


Supplemental figure S10 RNA-seq results for Nb16 and SZU-101 combination therapy. (A) Heatmap shows the gene expression for significantly differentially expressed genes from RNA-seq analysis. The colors in each box represent the $\log_2$(fold-change) in the expression of a gene in the Nb16, SZU-101 or combination group relative to the median mFc group; rows represent individual genes, and columns represent individual mice. (B) The bubble plot shows the GO enrichment analysis of differentially expressed genes upregulated by combination treatment

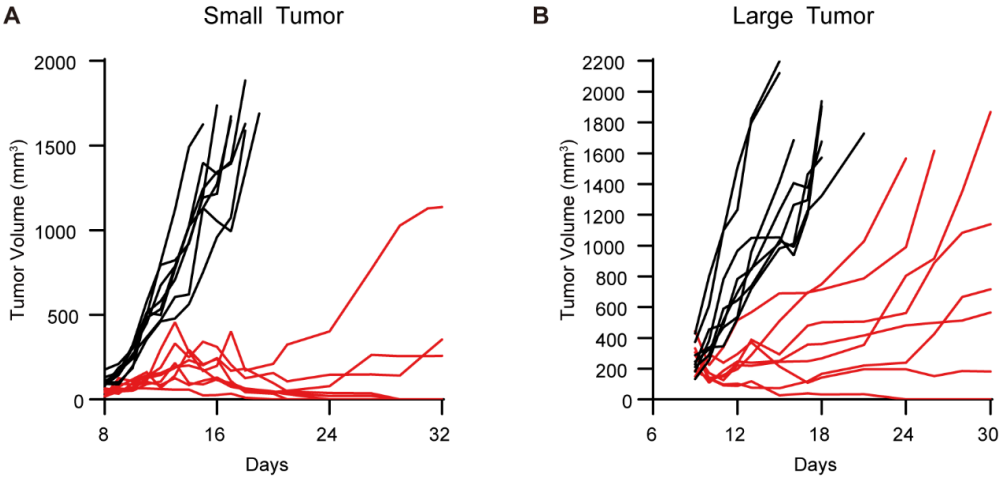
compared to mFc (C to H) Gene expression signatures associated with T cells (C), NK cells (D), macrophages (E), DCs (F), the IFN-γ response (G) and the IFN-α response (H). Signature scores [defined as the mean log2(fold change) among all genes in the signature] are shown as scatter plots. The data are presented as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; ns, not significant as determined by one-way ANOVA followed by Tukey's multiple comparisons test.



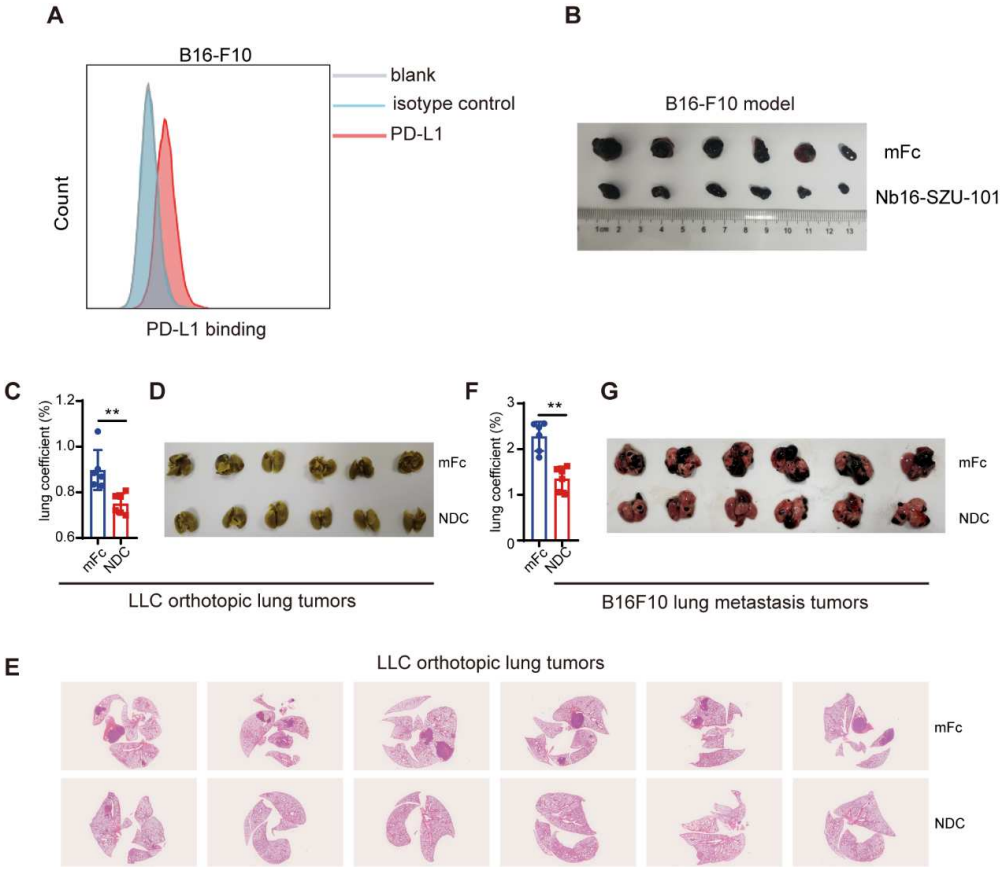
Supplemental figure S11 Identification of the PD-L1/TLR7 dual-targeting nanobody-drug conjugate. (A) The structure of SZU-107. (B) Spleen cells obtained from BALB/c mice were treated with either SZU-101 or SZU-107 (50 μM, n = 5). An MTT assay was performed to determine the cell proliferation activity, shown as the proliferation index. (C to E) BALB/c mouse BMDCs were treated with complete medium, LPS, 50 μM SZU-101 or 50 μM SZU-107 for 24 h (n = 3). CD80 (C), CD86 (D) and MHC Ⅱ (E) on DCs were detected by flow cytometry. (F to H) BALB/c mouse BMDMs induced to differentiate into M2 macrophages were stimulated with 50 μM SZU-101 or 50 μM SZU-107 for 24 h (n = 3). CD86 (F), CD206 (G) and IFN-γ (H) expression in macrophages was determined by flow cytometry. (I and J) Nb16 (I) and NDC (J) were identified by mass spectrometry, and the DAR of NDC was determined according to the change in the molecular weight of the antibody. (K to N) NDC identification results of different batches. Data indicate mean ± SEM. ns not significant as determined by one-way ANOVA followed by Tukey's multiple comparisons test or unpaired t test.



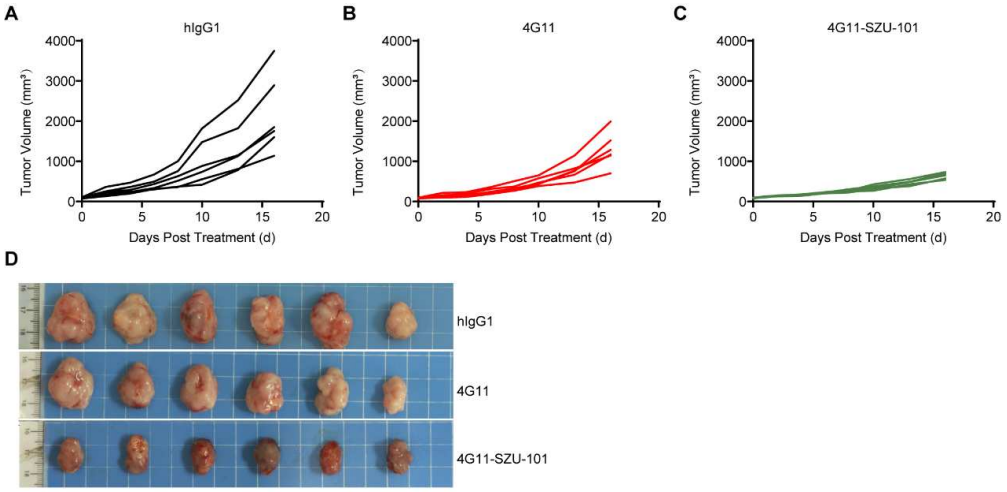
Supplemental figure S12 Comparison for antitumor activity of Nb16-SZU-101 and 10F.9G2-SZU-101. (A to E) BALB/c mice bearing CT26 tumors were treated with mFc, Nb16-SZU-101, and 10F.9G2-SZU-101. Tumor growth (A, C to E) and survival curve (B) are shown (n = 7). The data are presented as the mean ± SEM. *** $p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test.



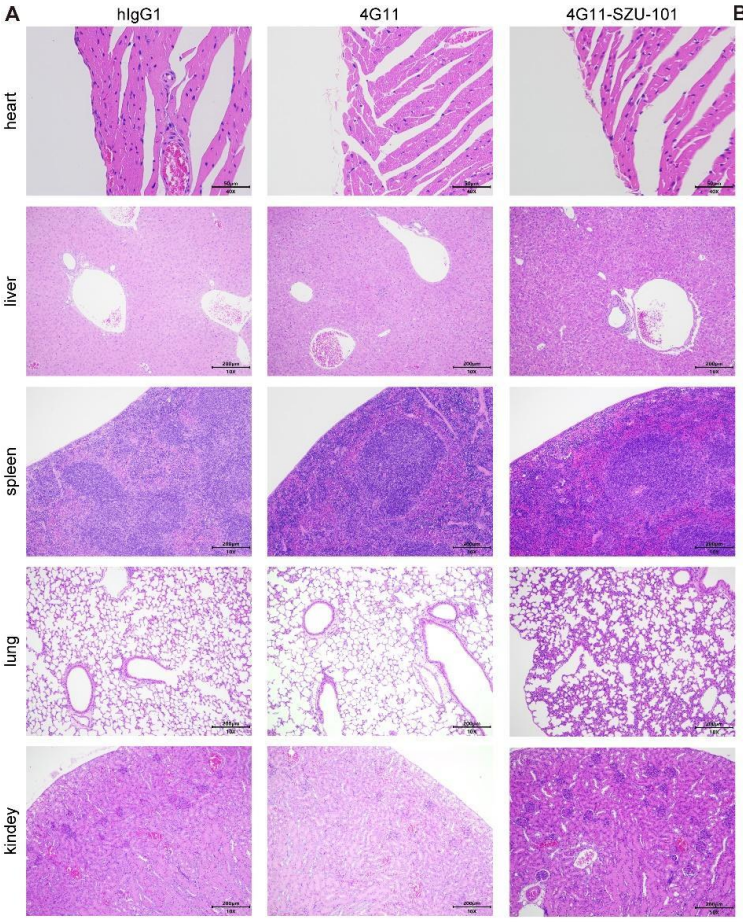
Supplemental figure S13 NDC inhibited small CT26 tumors and large CT26 tumors. BALB/c mice bearing CT26 tumors were treated with 200 μg of mFc or NDC when tumor volume was <50 mm^3 (small tumor, A) or >200 mm^3 (large tumor, B). Tumor growth in individual mice is shown (n = 8).



Supplemental figure S14 NDC inhibited the growth of B16F10 tumors, LLC orthotopic lung tumors and B16F10 lung metastasis tumors. (A) Flow cytometry histograms showing the expression levels of PD-L1 in B16-F10 cells. (B) Mice bearing B16-F10 tumors were treated with mFc or NDC. The photograph shows the size of the tumor at the end of the experiment. (C and D) Mice bearing LLC orthotopic lung tumors were treated with mFc or NDC. The scatter plot of the lung coefficient and photograph of the whole lung are shown (n = 6). (F and G) Mice bearing B16F10 lung metastasis tumors were treated with mFc or NDC. The scatter plot of the lung coefficient and photograph of the whole lung are shown (n = 6). (E) H&E staining photograph of LLC orthotopic lung tumors (n = 6). The data are presented as the mean ± SEM. ** $p < 0.01$ as determined by unpaired t test.



Supplemental figure S15 Antitumor activity of 4G11-SZU-101. PD-1/PD-L1 dual-humanized BALB/c mice were subcutaneously inoculated with CT26/hPD-L1 cells (5×10^5). Mice bearing tumors were treated with 130 μg of hIgG1, 200 μg of 4G11 or 200 μg of 4G11-SZU-101. Tumor growth (A to C) in individual mice is shown (n = 6). (D) The photograph shows the size of the tumor at the end of the experiment.



Category	Organs
Cardiovascular system	Heart
	Aorta (thoracic)
	Bone marrow, sternum
Lymphohematopoietic system	Thymus
	Spleen
	Lymph node, mandibular
	Lymph node, mesenteric
	Gut-associated lymphoid tissue
Respiratory system	Lung
	Trachea
Digestive system	Esophagus
	Stomach
	Small intestine, duodenum
	Small intestine, ileum
	Small intestine, jejunum
	Large intestine, cecum
	Large intestine, colon
	Large intestine, rectum
	Salivary gland, mandibular gland
	Salivary gland, sublingual gland
	Pancreas
	Liver
Nervous system	Brain
	Spinal cord (cervical, thoracic and lumbar)
	Nerve, optic
	Nerve, sciatic
Special sense organs	Eye
	Harderian gland
Endocrine system	Pituitary gland
	Thyroid gland
	Parathyroid gland
	Adrenal gland
Urinary system	Kidney
	Urinary bladder
Musculoskeletal system	Skeletal muscle, biceps femoris
	Femur
	Sternum
Integumentary system	Skin (site of mammary gland)
Reproductive system	Ovary
	Uterus
	Cervix
	Vagina
	Oviduct
	Mammary gland
Others	Tail

Supplemental figure S16 Pathological analysis after NDC treatment. PD-1/PD-L1 dual-humanized BALB/c mice were subcutaneously inoculated with CT26/hPD-L1 cells. Mice bearing tumors were treated with hIgG1, 4G11 or 4G11-SZU-101 every three days for total five doses. Tissues and organs of mice were collected and H&E staining and pathological analysis were performed (B). H&E staining images of major organs are shown (A).

A

Group	Animal No.	wbc	ne#	ly#	mo#	eo#	ba#	ne%	ly%	mo%	eo%	ba%	rbc	hb	het	mcv	mch	mchc	rdw	plt	mpv
		K/uL	K/uL	K/uL	K/uL	K/uL	K/uL	%	%	%	%	%	M/uL	g/L	%	fL	Pg	g/L	%	K/uL	fL
hIgG1	897	10.30	1.21	7.91	1.16	0.02	0	11.70	76.82	11.29	0.18	0.02	5.01	92	33.0	65.8	18.4	279	27.0	947	4.8
	975	9.14	2.34	5.21	1.56	0.02	0	25.65	57.02	17.07	0.26	0	8.50	141	50.7	59.6	16.6	278	18.3	781	5.0
	993	6.52	1.91	3.78	0.81	0.02	0.01	29.34	57.90	12.44	0.25	0.08	7.65	123	43.5	56.9	16.1	283	16.3	604	5.2
	994	7.48	1.60	4.88	0.98	0.02	0	21.36	65.27	13.15	0.22	0	7.64	125	47.1	61.7	16.4	265	21.1	809	4.7
	982	15.18	2.10	11.64	1.42	0.02	0	13.85	76.66	9.36	0.11	0.03	5.07	90	34.1	67.2	17.8	264	25.1	1017	4.8
	999	13.12	2.89	8.78	1.41	0.04	0	22.02	66.95	10.71	0.29	0.03	8.03	132	46.5	57.9	16.4	284	17.5	1121	4.9
	Mean	**10.29**	**2.01**	**7.03**	**1.22**	**0.02**	**0.00**	**20.65**	**66.77**	**12.34**	**0.22**	**0.03**	**6.98**	**117**	**42.5**	**61.5**	**17.0**	**276**	**20.9**	**880**	**4.9**
	SD	**3.33**	**0.58**	**2.95**	**0.29**	**0.01**	**0.00**	**6.77**	**8.66**	**2.67**	**0.06**	**0.03**	**1.54**	**21.24**	**7.30**	**4.21**	**0.92**	**8.83**	**4.34**	**185.66**	**0.18**
4G11	995	5.54	0.50	4.30	0.73	0.01	0	9.06	77.62	13.14	0.18	0	6.81	110	42.7	62.7	16.2	258	16.1	772	5.2
	581	7.80	1.05	5.89	0.74	0.11	0.02	13.51	75.51	9.43	1.35	0.20	8.91	129	56.5	63.4	14.5	228	18.0	805	6.2
	979	3.22	0.47	2.31	0.41	0.03	0.01	14.61	71.64	12.77	0.79	0.20	6.43	107	44.0	68.5	16.6	243	16.4	623	5.2
	896	5.26	0.71	3.86	0.69	0	0	13.42	73.42	13.03	0.09	0.03	7.72	118	43.5	56.3	15.3	271	15.0	486	4.9
	970	6.70	1.35	4.46	0.87	0.01	0	20.20	66.61	13.05	0.14	0	6.94	107	39.6	57.0	15.4	270	23.1	901	4.8
	972	3.68	0.31	3.02	0.35	0	0	8.38	82.00	9.50	0.09	0.04	5.47	91	33.6	61.4	16.6	271	20.5	643	4.8
	Mean	**5.37**	**0.73**	**3.97**	**0.63**	**0.03**	**0.01**	**13.20**	**74.47**	**11.82**	**0.44**	**0.08**	**7.05**	**110**	**43.3**	**61.6**	**15.8**	**257**	**18.2**	**705**	**5.2**
	SD	**1.74**	**0.40**	**1.24**	**0.20**	**0.04**	**0.01**	**4.28**	**5.26**	**1.83**	**0.52**	**0.10**	**1.17**	**12.68**	**7.52**	**4.50**	**0.84**	**17.88**	**3.07**	**149.29**	**0.53**
4G11-SZU-101	992	13.56	1.77	10.06	1.68	0.04	0	13.08	74.20	12.37	0.33	0.03	8.18	126	43.3	52.9	15.4	291	19.0	792	5.3
	962	8.30	1.07	6.07	1.13	0.02	0.01	12.94	73.09	13.64	0.24	0.10	7.06	109	37.4	53.0	15.4	291	18.7	835	5.3
	990	8.82	1.30	6.24	1.22	0.05	0	14.74	70.80	13.86	0.60	0	7.22	109	38.5	53.3	15.1	283	18.7	407	6.1
	965	9.64	1.78	6.81	1.02	0.03	0	18.44	70.63	10.54	0.36	0.02	8.06	118	43.3	53.7	14.6	273	17.7	487	5.7
	984	5.74	0.75	4.51	0.45	0.02	0	13.15	78.59	7.82	0.38	0.06	8.82	130	47.5	53.9	14.7	274	18.0	515	5.1
	891	8.90	1.19	6.90	0.77	0.04	0	13.38	77.48	8.66	0.42	0.05	8.08	115	40.8	50.5	14.2	282	19.2	492	5.5
	Mean	**9.16**	**1.31**	**6.77**	**1.05**	**0.03**	**0.00**	**14.29**	**74.13**	**11.15**	**0.39**	**0.04**	**7.90**	**118**	**41.8**	**52.9**	**14.9**	**282**	**18.6**	**588**	**5.5**
	SD	**2.54**	**0.40**	**1.83**	**0.42**	**0.01**	**0.00**	**2.14**	**3.33**	**2.56**	**0.12**	**0.04**	**0.66**	**8.70**	**3.69**	**1.23**	**0.48**	**7.84**	**0.58**	**178.96**	**0.36**

Index	Full name	Unit
wbc	white blood cell(leukocyte) count	K/uL
rbc	red blood cell(erythrocyte) count	M/uL
hb	hemoglobin	g/L
het	hematocrit	%
mcv	mean corpuscular(erythrocyte) volume	fL
mch	mean corpuscular(erythrocyte) hemoglobin	Pg
mchc	mean corpuscular(erythrocyte) hemoglobin concentration	g/L
rdw	red cell (erythrocyte volume) distribution width	%
plt	platelet(thrombocyte) count	K/uL
mpv	mean platelet(thrombocyte) volume	fL
ne#	neutrophils count	K/uL
ne%	percent of neutrophils	%
ly#	lymphocytes count	K/uL
ly%	percent of lymphocytes	%
eo#	eosinophils count	K/uL
eo%	percent of eosinophils	%
mo#	monocytes count	K/uL
mo%	percent of monocytes	%
ba#	basophils count	K/uL
ba%	percent of basophils	%

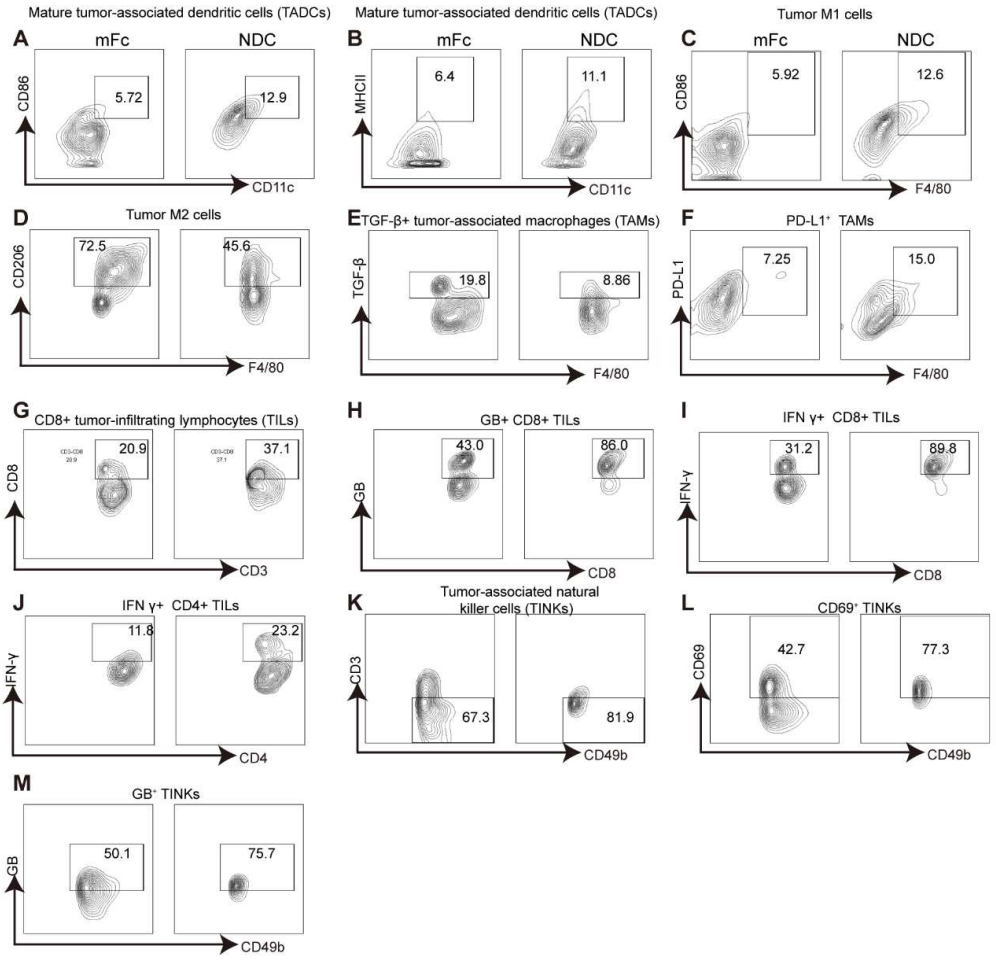
Supplemental figure S17 Hematology analysis after NDC treatment. Mice bearing tumors were treated with 130 μg of hIgG1, 200 μg of 4G11 or 200 μg of 4G11-SZU-101 every three days for total five doses. Blood was collected and hematology was measured by blood counters (Hemavet 950 FS).

A

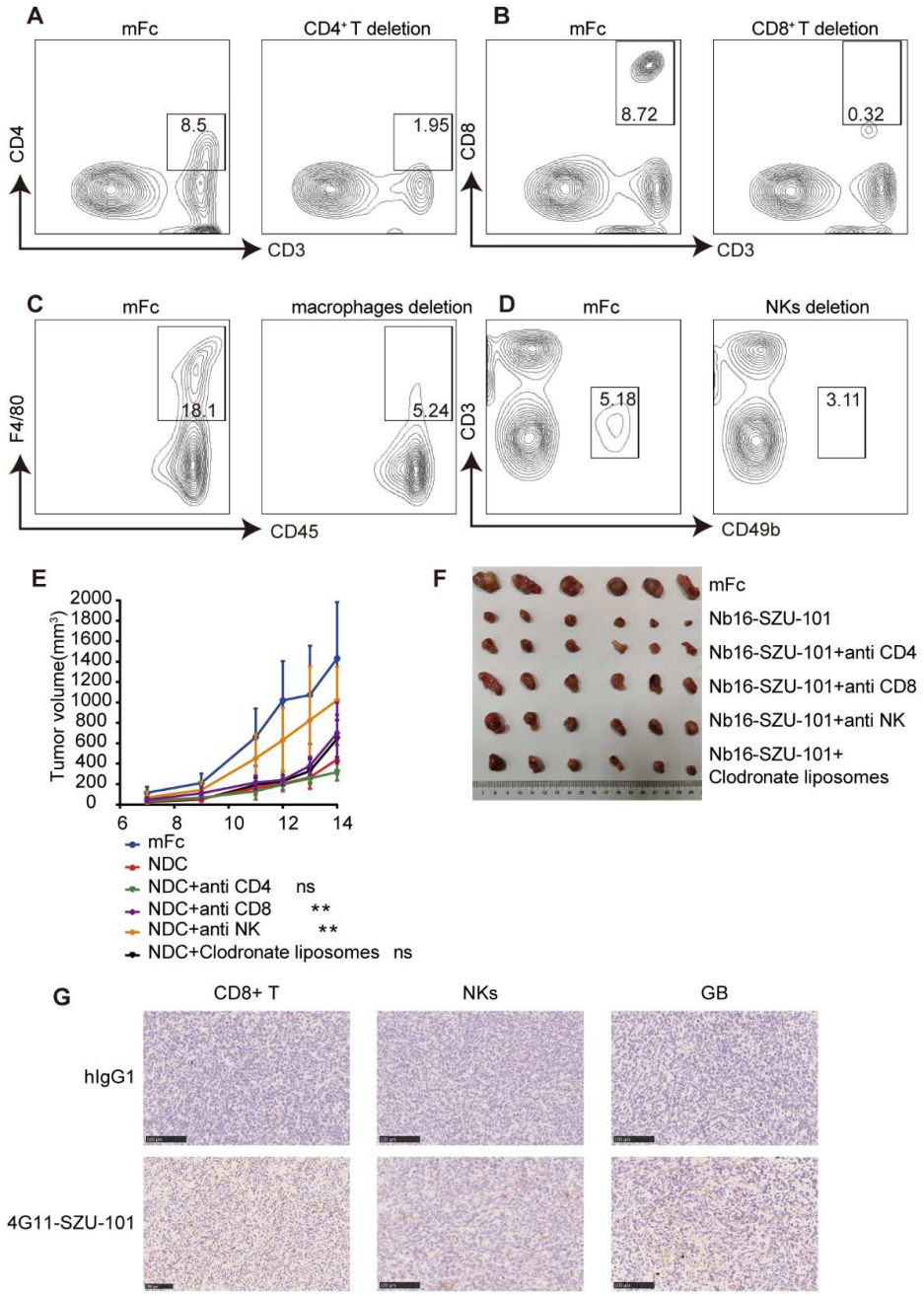
Group	Animal No.	ALT U/L	ALB g/L	TP g/L	AST U/L	ALP U/L	CREA μmol/L	TC μmol/L	TG μmol/L	UREA μmol/L	GLU μmol/L	TBIL μmol/L	CK U/L	Na mmol/L	K mmol/L	Cl mmol/L	GLOB g/L	A/G /
hIgG1	897	20.7	31.9	44.6	94.8	95	25	2.06	1.54	4.4	6.45	1	239	155	4.78	113.2	12.7	2.5
	975	17.4	29.5	43.7	181.2	64	33	2.36	2.59	6	6.51	0.8	312	156	4.86	112.4	14.2	2.1
	993	26.1	28.7	43.6	200.1	75	33	2.43	2.01	10.6	6.92	0.5	286	151	4.64	106.4	14.9	1.9
	994	45.1	29.7	45.7	162.6	72	31	2.45	3.3	5.6	7.19	0.8	255	153	4.56	110.3	16	1.9
	982	20.2	25.9	43.3	121.8	76	36	2.25	3.51	7.6	5.33	0.9	316	157	5.16	115.1	17.4	1.5
	999	14.2	29.8	47.8	96.7	67	27	3.35	5.71	5	5.74	0.7	318	155	4.07	113	18	1.7
Mean		**23.95**	**29.25**	**44.78**	**142.87**	**74.83**	**30.83**	**2.48**	**3.11**	**6.53**	**6.36**	**0.78**	**287.67**	**155**	**4.7**	**111.7**	**15.5**	**2**
SD		**11.09**	**1.96**	**1.72**	**44.77**	**10.91**	**4.12**	**0.45**	**1.48**	**2.27**	**0.70**	**0.17**	**33.92**	**2.17**	**0.36**	**3.03**	**2.00**	**0.34**
4G11	995	25	31.9	45.4	187.4	109	34	1.88	0.87	7.1	5.96	0.3	241	158	5.39	117.7	13.5	2.4
	581	38.5	30.5	44.2	224	84	30	2.07	1.08	6.5	9.71	0.8	263	154	5.26	112.6	13.7	2.2
	979	29.7	31.5	45.8	191.7	74	30	2.31	1.1	4.2	9.45	1.2	213	154	4.84	110.6	14.3	2.2
	896	18.9	28.1	40.5	150.8	80	30	1.89	0.74	5.6	8.66	1.1	149	160	4.89	117.4	12.4	2.3
	970	21.4	22.7	43.9	159.5	69	29	3.15	6.02	3.9	7.78	1.2	252	152	4.74	109.3	21.2	1.1
	972	15.1	26.9	42	145.9	64	29	1.82	1.72	4.9	8.79	0.7	278	155	4.69	113.1	15.1	1.8
Mean		**24.77**	**28.60**	**43.63**	**176.55**	**80.00**	**30.33**	**2.19**	**1.92**	**5.37**	**8.39**	**0.88**	**232.67**	**156**	**5.0**	**113.5**	**15.0**	**2**
SD		**8.39**	**3.49**	**2.03**	**29.97**	**15.94**	**1.86**	**0.50**	**2.04**	**1.27**	**1.37**	**0.35**	**46.49**	**2.95**	**0.29**	**3.46**	**3.15**	**0.49**
4G11-SZU-101	992	19	26.5	43.2	113.9	67	36	2.25	2.39	6.9	4.92	1.5	79	154	6.18	116.4	16.7	1.6
	962	40.9	24.2	38.3	193.4	74	43	0.68	1.58	4.7	4.75	2.1	437	157	6.53	116.3	14.1	1.7
	990	46	28.7	46	229.8	74	51	1.69	1.66	4.5	5.98	1.7	332	158	6.21	117.5	17.3	1.7
	965	21.3	26.1	41.5	164.2	75	38	1.57	1.97	3.2	6.66	1.8	316	154	5.46	111.7	15.4	1.7
	984	21.2	27.9	42.7	205.5	66	47	1.35	1.83	6.9	5.47	1.4	114	156	5.29	118	14.8	1.9
	891	20.3	27.6	43.3	155.4	62	37	1.69	1.49	5.8	6.2	1.5	227	153	5.87	114.4	15.7	1.8
Mean		**28.12**	**26.83**	**42.50**	**177.03**	**69.67**	**42.00**	**1.54**	**1.82**	**5.33**	**5.66**	**1.67**	**250.83**	**155**	**5.9**	**115.7**	**15.7**	**2**
SD		**12.01**	**1.60**	**2.53**	**41.19**	**5.39**	**6.07**	**0.51**	**0.33**	**1.47**	**0.75**	**0.26**	**137.34**	**1.97**	**0.48**	**2.33**	**1.18**	**0.10**

Index	Full name	Unit
ALT	Alanine aminotransferase	U/L
AST	Aspartate aminotransferase	U/L
GGT	Gamma glutamyltransferase	U/L
ALP	Alkaline phosphatase	U/L
CK	Creatine kinase	U/L
TBIL	Total bilirubin	μmol/L
UREA	Urea	mmol/L
CREA	Creatinine	μmol/L
GLU	Glucose	mmol/L
CHOL	Total cholesterol	mmol/L
TG	Triglycerides	mmol/L
TP	Total protein	g/L
ALB	Albumin	g/L
GLOB	Globulin	g/L
A/G	Albumin/Globulin ratio	/
Cl	Chloride	mmol/L
K	Potassium	mmol/L
Na	Sodium	mmol/L

Supplemental figure S18 Plasma chemistry analysis after NDC treatment. Mice bearing tumors were treated with 130 μg of hIgG1, 200 μg of 4G11 or 200 μg of 4G11-SZU-101 every three days for total five doses. Blood was collected plasma chemistry was measured by a biochemical analyzer (Cobas C501, Roche).



Supplemental figure S19 Representative raw data of the immunophenotyping for NDC therapy. (A to M) Immune effector cells in tumors treated with mFc and NDC were quantified by flow cytometry (n = 3).



Supplemental figure S20 Immune cell deletion study and immunohistochemical analysis of tumor treated with NDC. (A to D) Mice bearing CT26 tumors treated with anti-murine CD4, anti-murine CD8, anti-murine NK1.1, or clodronate liposomes. Deletion of specific cell populations was identified by flow cytometry. Contour maps show the proportion of cells in a specific cell population. The percentages of $CD4^+$ T cells (A), $CD8^+$ T cells (B), macrophages (C) and NK cells (D) are shown based on their respective markers. (E and F) Mice bearing CT26 tumors treated with mFc or Nb16-SZU-101 dosed with anti-murine CD4, anti-murine CD8, anti-murine NK1.1, or clodronate liposomes. Tumor growth (E) in mice and tumor photos (F) are shown (n = 6). (G) PD-1/PD-L1 dual-humanized BALB/c mice bearing CT26/hPD-L1

tumors were treated with hIgG1 and 4G11-SZU-101. CD8+ T-cell and NK cell infiltration and GB expression were analyzed by immunohistochemistry. The data are presented as the mean ± SEM. ** $p < 0.01$; ns, not significant as determined by one-way ANOVA followed by Tukey's multiple comparisons test.