Supplemental Materials For

**SUMOylation inhibitor subasumstat potentiates rituximab activity by IFN1-dependent macrophage and NK cell stimulation**

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Mouse macrophage generation and compound treatment

Femurs and tibias of 8–12 week old female C57BL/6 mice (Jackson Laboratory) were dissected from the surrounding muscle tissue and excess tissue was removed using sterile forceps and scissors, while the ends of the bone were kept intact. Any extra leftover muscle/tissue on the femur was removed by using lint-free tissue paper and gently cleaning the bones using 70% ethanol. Thereafter, intact bones were left in 70% ethanol for 10 minutes for disinfection and washed with PBS for another 5 minutes. Both ends were then cut with scissors and the bone marrow was flushed with approximately 5 ml RPMI 1640 media supplemented with 100 U/ml penicillin/streptomycin using a sterile syringe with a PrecisionGlide 25G × 7/8 inch (for femurs) or 27G × 1/2 inch (for tibias) needle. The bone marrow suspension cells were collected and centrifuged for 8 minutes at 350 x rcf at 4°C. Cells were then washed once with RPMI 1640 media supplemented with 100 U/ml penicillin/streptomycin. The cell pellets were resuspended in 3 ml of ammonium-chloride-potassium (ACK) lysing buffer (Thermo Fisher Scientific) and incubated for 5 minutes at room temperature to lyse red blood cells. Then 10 ml of RPMI 1640 media supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin/streptomycin was added, and cells were centrifuged for 8 minutes at 350 x rcf at 4°C. Cells were washed once with RPMI 1640 media supplemented with 100 U/ml penicillin/streptomycin and the pellets were resuspended in RPMI 1640 media supplemented with 20% heat-inactivated FBS, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 25 ng/ml mouse M-CSF (Peprotech) (mBMDM culture media) at 5 x 105 cells/ml. Cells were seeded on non-
tissue culture coated dishes and maintained at 37°C 5% CO₂. The date of cell plating was designated as Day 0 of culture. On Day 4, equal volume of mBMDM culture media was added. On Day 7, the cells were washed with PBS and treated with ice-cold 2 mM EDTA in PBS for 10 min. 4 ml of 0.5% BSA (Thermo Fisher Scientific) in PBS was added and the cells were pipetted up and down several times. The cell suspensions were collected and centrifuged for 8 minutes at 350 x rcf set at 4°C. The cell pellets were resuspended in mBMDM culture media and seeded on non-tissue culture coated plates at 5 x 10⁵ cells/ml. The cells were pretreated with 20 µg/ml anti-mouse IFNAR1 antibody (clone MAR1-5A3, BioXCell) for 1 h, and then treated with test compounds for 24-48 h [LPS at 100 ng/ml (InVivoGen), mouse IFNγ at 50 ng/ml, mouse IL-4 at 20 ng/ml (Peprotech), mouse IFNα1 at 10 kU/ml (PBL Assay Science), and TAK-981 at 1 µM unless otherwise mentioned]. Mouse IgG1 (clone MOPC-21, BioXCell) or DMSO was used as control. On Day 8-9, the cells were washed with PBS and treated with ice-cold 2 mM EDTA in PBS for 10 min. 4 ml of 0.5% BSA in PBS was added and the cells were pipetted up and down several times. The cell suspensions were collected and centrifuged for 8 minutes at 350 x rcf at 4°C. The cell pellets were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated FBS or Stain Buffer (BSA) (BD Biosciences) and proceeded with downstream assays. The differentiated mBMDM population was validated by flow cytometry as CD45⁺CD11b⁺F4/80⁺CD11c⁻CD14low (the purity was > 90%).
Human macrophage generation and compound treatment

Human PBMC was purchased from STEMCELL Technologies. Primary monocytes were isolated from PBMC using human monocyte isolation Kit (STEMCELL Technologies) to remove non-monocytes, including T cells, B cells, NK cells, dendritic cells, granulocytes, and erythroid cells according to manufacturer’s instructions. Cells were resuspended with Immunocult-SF macrophage medium (STEMCELL Technologies) supplemented with 50 ng/ml human M-CSF (Peprotech) at 5 x 10^5 cells/ml. The cells were seeded onto non-tissue culture coated plates and maintained at 37°C 5% CO₂ incubator. The date of cell plating was designated as Day 0 of culture. On Day 4, half volume of Immunocult-SF macrophage medium supplemented with 50 ng/ml human M-CSF was added. On Day 6, the cells were pretreated with 50 ng/ml human IFNγ together with 20 µg/ml anti-human IFNAR2 antibody (clone MMHAR-2, Thermo Fisher Scientific) for 1 h, and then treated with test compounds for 24-48 h [human IFNγ at 50 ng/ml, human IL-4 at 20 ng/ml (Peprotech), human IFNα2 at 10 kU/ml (PBL Assay Science), and TAK-981 at 1 µM unless otherwise mentioned]. Mouse IgG2A (R&D Systems) or DMSO was used as control. On Day 7, the cells were washed once with PBS and treated with 2 ml of Accutase (STEMCELL Technologies) at 37°C 5% CO₂ for 15 minutes. Regarding samples for flow cytometry, the cells were treated with 2 ml of ice-cold 5 mM EDTA in PBS on ice for 40-50 minutes. 4 ml of 0.5% BSA in PBS was added and the cells were pipetted up and down several times. The cell suspensions were collected and centrifuged for 8 minutes at 350 x rcf set at 4°C. The cell pellets were resuspended in RPMI 1640 media.
supplemented with 10% heat-inactivated FBS or Stain Buffer (BSA) and proceeded with downstream assays. The differentiated hMDM population was validated by flow cytometry as CD45^+CD11b^+CD14^{low} (the purity was > 90%).

**Flow cytometry**

Cell suspensions were transferred to 96-well U-bottom plates and centrifuged for 5 minutes at 350 x rcf set at 4°C. Then the supernatant was removed and washed once with PBS. The cells were resuspended in PBS containing the LIVE/DEAD Fixable Dead Cell Staining solution (Thermo Fisher Scientific) diluted at 1:1000 on ice for 20 min. The cells were washed once with Stain Buffer (BSA) and suspended with anti-mouse CD16/CD32 antibody (BioXCell) and/or Human TruStain FcX Fc Receptor Blocking Solution (Biolegend) on ice for 10 min. Then, the cells were stained with the following fluorophore-conjugated antibodies for extracellular proteins on ice for 30 min while being protected from the light. After being stained, the cells were centrifuged for 5 min at 4°C. The cells were then washed with cell staining buffer and homogenized with BD Cytofix/Cytoper Buffer (BD Biosciences) on ice for 20 min. After being washed with 1x BD Perm/Wash Buffer (BD Biosciences), the cells were stained with the following fluorophore-conjugated antibodies for intracellular proteins on ice for 60 min while being protected from the light. After being washed with 1x BD Perm/Wash Buffer, the cells were resuspended with Stain Buffer (BSA). Samples were stored at 4°C in the dark until analyzed using flow cytometry (LSRFortessa, BD Biosciences) and
FACS Diva software, Version 8.0.1 (BD Biosciences). Data was analyzed with the FlowJo software v.10.1 (FlowJo LLC).


Antibodies from Biolegend; APC/Fire750 rat anti-mouse CD86, PerCP-Cy5.5 rat anti-mouse CD3, PerCP-Cy5.5 hamster anti-mouse CD11c, PerCP-Cy5.5 rat anti-mouse Ter119, APC-Cy7 rat anti-human/mouse CD11b, BV785 rat anti-mouse F4/80, FITC rat anti-mouse Ly6C, BV650 rat anti-mouse CD86, BV605 hamster anti-mouse CD69, BV421 hamster anti-mouse IFNγ, BV711 hamster anti-mouse FCGR4, PE-Cy7 mouse anti-human CD86, BV510 mouse anti-human CD206, and FITC mouse anti-human CD69. Antibodies from Thermo Fisher; PE-Cy7 rat anti-mouse iNOS, and PE rat anti-mouse CD206. Antibodies from Miltenyi; PE mouse anti-human CD56.

**Macrophage phagocytosis assays**
Daudi or Daudi-KILR cells were cultured in RPMI 1640 media supplemented with 10% FBS and at 0.2-2 x 10^6 cells/ml at 37°C 5% CO_2 incubator. Daudi or Raji cells were used for flow cytometry-based phagocytosis assays and Daudi-KILR cells were used for luminescence-based phagocytosis assay using PathHunter Prolable/Prolink (PL/PK) detection kit (DiscoverX) to enable a high-throughput assay format and to validate the results in different readouts. The cells were labeled with CellTrace Violet (ThermoFisher Scientific), according to manufacturer’s instructions. The cells were resuspended at 1.25 x 10^5 cells/ml in RPMI 1640 media supplemented with 10% heat-inactivated FBS and seeded in a 96 well clear or white round bottom plate (Thomas Scientific) at a density of 5 x 10^3 cells in 40 µL. After 10 µl of human control IgG1 (Biolegend), rituximab (Genentech), or daratumumab (Janssen Biotech) was added, the plate was placed at 37°C 5% CO_2 incubator for 1 h. For the flow cytometry-based assays, macrophages were labeled with CellTrace Red (ThermoFisher Scientific). The prepared cell suspension of macrophages at 1-4 x 10^5 cells/ml was added to the plate at a density of 0.5-2 x 10^4 cells in 50 µL. The cells were incubated at 37°C 5% CO_2 incubator for 24 h, washed with PBS, and stained with propidium iodide (PI). Phagocytosis of target cells by macrophages were assessed by flow cytometry and % phagocytosis was determined by the following equation; 100 x (the number of CellTrace Violet‘Red’ cells) / (the number of CellTrace Violet’ cells). Viability of non-phagocytosed Daudi cells was determined by the following equation; 100 x (the number of PI CellTrace Violet‘Red’ cells) / (the number of CellTrace Violet‘Red’ cells). Phagocytosis of Daudi-KILR cells by macrophages were assessed
using PathHunter Prolable/Prolink (PL/PK) detection kit (DiscoverX), according to manufacturer’s instructions.

**NK cell cytotoxicity assays**

Daudi, Daudi-KILR, or Raji cells were cultured in RPMI 1640 media supplemented with 10% FBS and at 0.2-2 x 10⁶ cells/ml at 37°C 5% CO₂ incubator. Daudi or Raji cells were used for flow cytometry-based phagocytosis assays and Daudi-KILR cells were used for luminescence-based phagocytosis assay using KILR detection kit (DiscoverX). The cells were resuspended at 1.25 x 10⁵ cells/ml in RPMI 1640 media supplemented with 10% heat-inactivated FBS and seeded in a 96 well white round bottom plate (Thomas Scientific) at a density of 5 x 10³ cells in 40 µL. After 10 µl of human control IgG1 (Biolegend) or rituximab (Genentech) was added, the plate was placed at 37°C 5% CO₂ incubator for 1 h. The prepared cell suspension of NK cells at 4 x 10⁵ cells/ml was added to the plate at a density of 2 x 10⁴ cells in 50 µL. The cells were incubated at 37°C 5% CO₂ incubator for 24 h, washed with PBS, and stained with PI. Cell lysis of Daudi or Raji cells by NK cells were assessed by flow cytometry % cell lysis was determined by the following equation; 100 x (the number of PI'CellTrace Violet’ cells) / (the number of CellTrace Violet’ cells). Cell lysis of Daudi-KILR cells by NK cells were assessed using KILR detection kit (DiscoverX), according to manufacturer’s instructions. Control cells were chemically lysed to detect maximum signal (100% cell lysis).
ELISA

The concentrations of proteins in culture supernatant or mouse serum were measured using the following kits according to manufacturer’s instruction. Mouse IFNβ; Verikine Mouse IFNβ ELISA (PBL Assay Science), mouse IP-10; IP-10 (CXCL10) Mouse ELISA Kit (Thermo Fisher Scientific), mouse complement C3; Mouse Complement C3 ELISA Kit (Abcam), Human IFNα; Human IFN Alpha All Subtype ELISA Kit High Sensitivity (TCM) (PBL Assay Science), Human IFNβ; Verikine HS Human IFNβ serum ELISA kit (PBL Assay Science), and Human IP-10; IP-10 (CXCL10) Human ELISA Kit (Thermo Fisher Scientific).

Quantitative PCR

RNA was extracted using the RNeasy kit (Qiagen). cDNA was synthesized using High-Capacity RNA to cDNA kit (Thermo Fisher Scientific). Real-time quantitative PCR was performed on a QuantiStudio 6 PCR system (Thermo Fisher Scientific) using TaqMan gene expression assays (Thermo Fisher Scientific). Relative gene expression was calculated using the ΔΔCt method following the manufacturer’s instructions. The expression ratios of the indicated genes were normalized by GAPDH expression.

RNA sequencing
RNA was isolated using RNeasy protocols (Qiagen). The integrity of total RNA was assessed by capillary electrophoresis with an Agilent Bioanalyzer 2100 (Agilent Technologies). mRNA was isolated from total RNA using magnetic beads with oligo-dT, and the captured mRNA was fragmented. The cDNA first strand and the second strand are then synthesized using reverse transcriptase, and the reverse transcription product is subjected to terminal repair, followed by addition of an A base at the 3' end. Subsequently, the fragment was ligated with a Illumina universal linker. The ligation product was purified to remove the incompletely attached product and the empty linker self-ligated product and then PCR amplified using a universal primer complementary to the linker sequence. PCR amplification products were purified to remove primer dimers, library sizes were determined using an Agilent Bioanalyzer 2100, library concentrations were determined using Qubit 2.0, and second generation sequencing was performed according to Illumina's standard protocol. FASTQ files were aligned and quantified with STAR (v2.4.2a)\textsuperscript{1} and RSEM (V1.2.29)\textsuperscript{2}. To obtain raw counts and fragments per kilobase per million reads (FPKM), RNA sequencing data was processed using the edgeR (v3.8.5)\textsuperscript{3}.

\textbf{Animal studies}

For cell line xenograft models, 8-10 week old female CB-17 severe combined immunodeficiency (SCID) mice were inoculated subcutaneously in the flank with 2 x 10\textsuperscript{6} Daudi, 4 x 10\textsuperscript{6} OCI-Ly10, 4 x 10\textsuperscript{6} TMD8 cells, or 5 x 10\textsuperscript{6} LP-1 cells. Non obese diabetes (NOD)-SCID and NOD SCID gamma
(NSG) mice were also used for the subcutaneous OCI-Ly10 xenograft study to compare the
difference between strains. For a patient-derived xenograft lymphoma model PHTX-166L, 8-10
week old female SCID mice were subcutaneously implanted in the flank with approximately 2 mm
x 2 mm tumor chunks under light isoflurane anesthesia. For a disseminated model, 8 week old
female SCID mice were inoculated intravenously from the tail vein with 2 x 10^6 OCI-Ly10 cells
stably expressing firefly luciferase. For a mouse A20-hCD38 syngeneic model, 8-10 week old
female BALB/c mice were inoculated subcutaneously in the flank with 5.0 x 10^6 A20-hCD38 cells.
Subcutaneous tumor growth was monitored twice per week using Vernier calipers and the mean
tumor volume was calculated using the formula; 0.5 x (length x width^2). When the mean tumor
volume reached approximately 100-200 mm^3, animals were randomized into treatment groups (n =
5-8/group). Disseminated tumor growth was monitored by bioluminescence imaging using the IVIS
Imaging System (PerkinElmer). 9 days after tumor inoculation, animals were randomized into
treatment groups with an average total flux around one million photon/second per group (n = 7-8/group). Mice were dosed intravenously with TAK-981 at 5-15 mg/kg once or twice per week,
rituximab at 1-10 mg/kg once per week, daratumumab at 2.5-20 mg/kg twice per week,
obinutuzumab at 1 mg/kg once per week, or the combination of agents for 2-5 weeks. An effector-
dead version of rituximab (LALA-PG) with abolished FcγR and C1q binding were generated by
introduction of Leu234Ala/Leu235Ala/Phe329Gly (LALA-PG) Fc mutations^4,5. To deplete
macrophages, mice were intravenously administered 200 µl (first dose) or 100 µl (all subsequent
doses) clodronate (ClodronateLiposomes) twice per week 24 hours prior to receiving TAK-981 or rituximab. To deplete NK cells, mice were intraperitoneally administered 0.25, 0.5, or 1.0 mg anti-asialo GM1 antisera (Fujifilm Wako Chemicals) once or twice weekly 24 hours prior to receiving TAK-981 or rituximab. To deplete complement, mice were intravenously administered 250 or 750 U/kg cobra venom factor (EMD Millipore) twice weekly 24 hours prior to receiving TAK-981 or rituximab. Blood was collected and red blood cells were lysed using ammonium-chloride-potassium (ACK) lysing buffer (Thermo Fisher Scientific) before antibody staining for flow cytometry analysis. Blood plasma or serum fraction was collected and stored at -80°C for ELISA analysis. For all antitumor activity studies, tumor size and body weight were measured once or twice weekly. Any animal with tumor volume larger than 2000 mm³ was sacrificed, and survival duration of each animal was recorded based on time each animal was removed. The survival rate was determined by the Kaplan-Meier analysis (Prism v7.0 and v8.0, GraphPad). For efficacy studies, we used rate-based analysis to compare the growth rates of different arms⁶. Two-sided unpaired t-test with unequal variances is used to determine the statistical significance of growth rate differences. The mice were housed and maintained in accordance with the institutional guidelines established by the Institutional Animal Care and Use Committee, in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee.
Supplemental References


Supplemental Figure 1. TAK-981 treatment enhances M1 polarization and suppresses M2 polarization in hMDM and mBMDM.
(A) Representative flow cytometry scatter plots indicating percentage of live cells in 1 μM TAK-981-treated hMDM from 2 donors at 24 h. (B) Amount of pan-IFNα in the supernatant of 1 μM TAK-981-treated hMDM at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (C) Representative flow cytometry scatter plots indicating percentage of live cells in 1 μM TAK-981-treated mBMDM at 24 h. (D) Amount of indicated proteins in the supernatant of 1 μM TAK-981- or 10 kU/mL IFNα1-treated mBMDM at the indicated time points (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (E) MFI of indicated proteins in 100 ng/ml LPS-, 50 ng/ml IFNγ-, 20 ng/ml IL-4-, and/or 1 μM TAK-981-treated mBMDM at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (F) mRNA expression of CD86 in TAK-981-treated hMDM at 24 h (mean; n = 2 biological replicates). (G) MFI of indicated proteins in 20 μg/ml aIFNAR1- and/or 1 μM TAK-981-treated mBMDM at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (H) mRNA expression of indicated genes in 1 μM TAK-981- or 10 kU/mL IFNα2-treated hMDM at the indicated time points (mean; n = 2 biological replicates). (I) FPKM of indicated mRNAs in 1 μM TAK-981-treated hMDM from 2 donors at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (J) mRNA expression of indicated genes in 1 μM TAK-981-treated mBMDM at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (K) MFI of CD206 in hMDM treated with 20 ng/ml IL-4 for 24 h followed by 1 μM TAK-981 treatment for 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test).
mRNA expression of indicated genes in 100 ng/ml LPS-, 20 ng/ml IL-4-, and/or 1 μM TAK-981-treated mBMDM at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. N.S., not significant (p > 0.05).
Supplemental Figure 2. TAK-981 treatment does not increase the expression of FCGRs on human NK cells.

(A) Representative flow cytometry scatter plots indicating percentage of live cells in 1 μM TAK-981-treated human NK cells at 24 h. (B) MFI of indicated proteins in 1 μM TAK-981- or 10 kU/ml IFNα2-treated human NK cells at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (C) Percentage of FCGR1+ or FCGR3+ cells in 1 μM TAK-981- or 10 kU/ml IFNα2-treated human NK cells at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (D) mRNA expression of indicated genes in 1 μM TAK-981- or 10 kU/ml IFNα2-treated human NK cells at 24 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). **p < 0.01 and ***p < 0.001. N.S., not significant (p > 0.05). Experiment was repeated using human NK cells generated from 3 healthy PBMC donors.
Supplemental Figure 3. Effects of TAK-981 on macrophage phagocytosis and NK cell cytotoxicity.
(A) Schematic diagram of macrophage phagocytosis/NK cell cytotoxicity assays. (B) Phagocytic activity of 1 μM TAK-981- or 10 kU/mL IFNα2-treated hMDM against Daudi-KILR cells at an effector to target (E:T) ratio of 4:1 (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (C) Viability of non-phagocytosed Daudi cells (defined as CellTrace Red Violet+ events) co-cultured with hMDM (pre-treated with 1 μM TAK-981) in the presence of 1 μg/ml hIgG1 or rituximab (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (D) Cell lysis of Daudi-KILR cells treated with the indicated concentrations of TAK-981 or IFNα2 for 24 h (mean with SD; n = 3 biological replicates). (E) Cell lysis of Daudi-KILR cells treated with 1 μg/mL rituximab for 1 h (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (F) Phagocytic activity of hMDM against Daudi-KILR cells (pretreated with 1 μM TAK-981 for 24 h) in the presence of rituximab at an E:T ratio of 4:1 (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (G) Phagocytic activity of 50 ng/ml IFNγ- and/or 1 μM TAK-981-treated mBMDM against Daudi-KILR cells in the presence of 1 ng/ml human IgG1 or 1 ng/ml rituximab at an E:T ratio of 4:1 (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (H) Representative of flow cytometry scatter plots. Daudi cells are defined as CellTrace Violet+ events. % of PI+CellTrace Violet+ in CellTrace Violet+ events corresponds to % of lysed Daudi cells in total Daudi cells. (I) Cytotoxicity of 1 μM TAK-981-treated human NK cells against Daudi or Raji cells in the presence of 1 μg/ml human IgG1 or 1 μg/ml rituximab at an E:T ratio of 4:1 (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). (J)
Phagocytic activity of 1 μM TAK-981-treated hMDM against Raji cells in the presence of 1 μg/ml human IgG1 or 1 μg/ml rituximab at an E:T ratio of 1:1 (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. N.S., not significant (p > 0.05).
Supplemental Figure 4. Antitumor activity of TAK-981/rituximab combination in the disseminated OCI-Ly10-luc model

OCI-Ly10-luc-bearing mice (n = 7-8 mice per group in a single experiment) were treated with TAK-981 (7.5 mg/kg, biweekly) and/or rituximab (10 mg/kg, biweekly) as indicated for 3 weeks. (A) Individual bioluminescence image (B) total flux; mean with SD, two-tailed unpaired Welch’s t-test. The p value of the synergy score was p < 0.001. (C) survival rates; log-rank test was performed for statistical analysis of the survival curves. *p < 0.05 and ***p < 0.001. N.S., not significant (p > 0.05).
Supplemental Figure 5. Survival benefit of TAK-981/rituximab or TAK-981/daratumumab combination in TMD8 and LP-1 xenograft models

Survival rates in (A) TMD8-bearing mice treated with TAK-981 (15 mg/kg, biweekly) and/or rituximab (3 mg/kg, weekly) as indicated for 2 weeks (n = 8 mice per group in the representative study). (B) LP-1-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly) and/or daratumumab (2.5 mg/kg, biweekly) as indicated for 5 weeks (n = 8 mice per group in the representative study).

Weibull regression hazard ratio was determined for statistical analysis of the survival curves. *p < 0.05, **p < 0.01 and ***p < 0.001. At least 2 similar experiments were performed.
Supplemental Figure 6. Antitumor activity of TAK-981/daratumumab combination in syngeneic mice bearing A20-hCD38 cells

(A) Individual tumor volumes (B) survival rates (C) individual body weight of A20-hCD38-bearing mice treated with TAK-981 (15 mg/kg, once in every other weeks) and/or daratumumab (20 mg/kg, biweekly) as indicated for 3 weeks (n = 8 mice per group in the representative study). Weibull regression hazard ratio was determined for statistical analysis of the survival curves. ***p < 0.001, N.S., not significant (p > 0.05). 2 similar experiments were performed.
Supplemental Figure 7. Treatment with TAK-981 as a single agent or as in combination with monoclonal antibodies does not cause marked body weight loss in mice.

(A) Body weight in Daudi-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly), rituximab (3 mg/kg, weekly), and/or daratumumab (7.5 mg/kg, biweekly) as indicated for 3 weeks (mean with SD; n = 8 mice per group in the representative study). (B) Body weight in OCI-Ly10-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly) and/or rituximab (1 mg/kg, weekly) as indicated for 2
weeks (mean with $SD$; $n = 8$ mice per group in the representative study). (C) Body weight in OCI-Ly10-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly), obinutuzumab (1 mg/kg, weekly), and/or rituximab (1 mg/kg, weekly) as indicated for 2 weeks (mean with $SD$; $n = 8$ mice per group in a single experiment). (D) Body weight in TMD8-bearing mice treated with TAK-981 (15 mg/kg, biweekly) and/or rituximab (3 mg/kg, weekly) as indicated for 2 weeks (mean with $SD$; $n = 8$ mice per group in the representative study). (E) Body weight in PHTX-166L-bearing mice treated with TAK-981 (5 mg/kg, weekly) and/or rituximab (5 mg/kg, weekly) as indicated for 2 weeks (mean with $SD$; $n = 6$ mice per group in the representative study). (F) Body weight in LP-1-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly) and/or daratumumab (2.5 mg/kg, biweekly) as indicated for 5 weeks (mean with $SD$; $n = 8$ mice per group in the representative study). (G) Body weight in disseminated OCI-Ly10-luc-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly) and/or rituximab (10 mg/kg, weekly) as indicated for 3 weeks (mean with $SD$; $n = 7$-8 mice per group in a single experiment). At least 2 similar experiments were performed unless otherwise specified.
**Supplemental Figure 8.** In vitro combination of TAK-981 with rituximab does not show combination benefit.

Daudi, OCI-Ly19, or TMD8 cells were treated with indicated concentrations of TAK-981 and/or rituximab for 72 h. Cell viability was measured by Celltiter-Glo (mean with SD; n = 3 biological replicates).
Supplemental Figure 9. In vitro validation of rituximab LALA-PG mutant.

(A) Representative flow cytometry histograms indicating binding to Daudi cells. (B) Cytotoxicity of human NK cells against Daudi cells in the presence of the indicated concentrations of rituximab or rituximab LALA-PG (mean with SD; n = 3 biological replicates, two-tailed unpaired Welch’s t-test). *p < 0.05, ***p < 0.001, and ****p < 0.0001.
Supplemental Figure 10. Treatment with clodronate or anti-asialo GM1 depletes macrophages or NK cells in SCID mice.

(A) The number of NKp46⁺ NK cells in 1 x 10⁶ total cells in blood from anti-asialo GM1 (aASGM1; administered at 0.25, 0.5, or 1.0 mg on day 0)-treated SCID mice (mean with SD; n = 3 mice per group in the representative study).

(B) The number of CD11b⁺Ly6G⁻Ly6C<low>F4/80⁺ TAM and NKp46⁺ NK cells in tumors from clodronate (administered at 200 μl on day 0 and 100 μl on day 3, 6, 10, 13)-treated OCI-Ly10 bearing SCID mice (mean with SD; n = 5 mice per group in the representative study, two-tailed unpaired Welch’s t-test) on day 16. **p < 0.01, N.S., not significant (p > 0.05). At least 2 similar experiments were performed.
Supplemental Figure 11. Co-treatment with clodronate and anti-asialo GM1 abrogates antitumor activity of TAK-981/rituximab combination

(A) Tumor volumes in OCI-Ly10-bearing mice were treated with TAK-981 (7.5 mg/kg, biweekly), rituximab (3 mg/kg, weekly), anti-asialo GM1 (aASGM1, 250 µg/mice, biweekly), and/or clodronate (200 µl/mouse at a first dose and 100 µl/mouse at subsequent doses, biweekly) as indicated for 2 weeks (mean with SD; n = 8 mice per group in a single experiment, two-tailed unpaired Welch’s t-test). (B) The number of CD11b⁺Ly6G⁻Ly6C⁻F4/80⁺macrophages per total 1 x 10⁵ cells in spleen from clodronate (administered 200 µl at day 1 and 100 µl at day 3)- and/or anti-
asialo GM1 (aASGM1, administered 0.25 mg or 1 mg at day 1 and 3)-treated SCID mice (mean with SD; n = 4-5 mice per group in a single experiment, two-tailed unpaired Welch’s t-test) on day 4. (C) The number of NKp46+ NK cells per total 1 x 10^5 cells in blood from clodronate (administered 200 μl at day 1 and 100 μl at day 3)- and/or anti-asialo GM1 (aASGM1, administered 0.25 mg or 1 mg at day 1 and 3)-treated SCID mice (mean with SD; n = 4-5 mice per group in a single experiment, two-tailed unpaired Welch’s t-test) on day 4. (D) TMD8-bearing mice were treated with TAK-981 (15 mg/kg, biweekly), rituximab (3 mg/kg, weekly), anti-asialo GM1 (aASGM1, 250 μg/mice, biweekly), and/or clodronate (CLO, 200 μl/mouse at a first dose and 100 μl/mouse at subsequent doses, biweekly) as indicated for 2 weeks. Dot plots show % change in tumor volumes from baseline on the indicated day of measurement (mean with SD; n = 7-8 mice per group in a single experiment, two-tailed unpaired Welch’s t-test). *p < 0.05, **p < 0.01, and ***p < 0.001.
Supplemental Figure 12. Antitumor activity of TAK-981/rituximab combination in NOD-SCID and NSG mice.

Tumor volumes in OCI-Ly10-bearing mice treated with TAK-981 (7.5 mg/kg, biweekly) and/or rituximab (1 mg/kg, weekly) as indicated for 2 weeks in NOD-SCID or NSG mice (mean with SD; n = 8 mice per group in a single experiment, two-tailed unpaired Welch’s t-test). N.S., not significant (p > 0.05).
Supplemental Figure 13. Treatment with cobra venom factor depletes complement but does not abrogate the antitumor activity of TAK-981/rituximab combination.

(A) Amount of complement C3 in serum from cobra venom factor (CVF; administered at 250 or 750 U/kg on day 0)-treated SCID mice (mean with SD; n = 3 mice per group in a single experiment) at indicated time points. (B) OCI-Ly10-bearing mice were treated with TAK-981 (7.5 mg/kg, biweekly), rituximab (3 mg/kg, weekly), cobra venom factor (CVF, 250 U/kg, biweekly), and/or clodronate liposome (CLO: 200 µl/mouse at a first dose and 100 µl/mouse at subsequent doses, biweekly) as indicated for 2 weeks. Dot plots show % change in tumor volumes from baseline on the indicated day of measurement (mean with SD; n = 5-8 mice per group in a single experiment). The number of mice achieved CR per the number of total mice in the group is shown below the bar graphs. **p < 0.01 and ***p < 0.001, N.S., not significant (p > 0.05).
Supplemental Figure 14. Gating strategy for the flow cytometry analysis performed in tumor samples from OCI-Ly10 bearing mice.
Supplemental Figure 15. Immunophenotyping analysis of OCI-Ly10 xenografts.

The number of \( \text{CD11b}^+ \text{Ly6G}^- \text{Ly6C}^{\text{low/F4/80}^+} \) macrophages, \( \text{NKp46}^+ \) NK cells, \( \text{CD11b}^+ \text{Ly6G}^- \text{Ly6C}^{\text{high}} \) monocytes, or \( \text{CD11b}^+ \text{Ly6G}^+ \text{Ly6C}^{\text{int}} \) neutrophils per gram of tumors from 7.5 mg/kg TAK-981 (administered on day 1, 4, and 8) and/or 1 mg/kg rituximab (administered on day 1 and 8) treated OCI-Ly10 tumor bearing mice on day 9 (mean with SD; \( n = 5 \) mice per group in the representative study, two-tailed unpaired Welch’s \( t \)-test). 2 similar experiments were performed.