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A designer self-assembled supramolecule amplifies macrophage immune responses against aggressive cancer

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Detailed Methods:

Materials: All chemical reagents were of analytical grade, used as supplied without further purification unless indicated. All reactions were performed under inert conditions unless otherwise indicated. Dichloromethane (DCM), anhydrous DCM, Methanol, Cholesterol, Dimethylamino Pyridine (DMAP), Succinic Anhydride, Sodium Sulfate, Pyridine, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), L- α -phosphatidylcholine and Sephadex G-25 were purchased from Sigma-Aldrich. BLZ-945 drug was purchased from Selleck Chem. 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] and the mini handheld Extruder kit (including 0.2 μ m Whatman Nucleopore Track-Etch Membrane, Whatman filter supports and 1.0 mL Hamiltonian syringes) were bought from Avanti Polar Lipids Inc. Analytical thin-layer chromatography (TLC) was performed using precoated silica gel Aluminium sheets 60 F₂₅₄ bought from EMD Laboratories. Spots on the TLC plates were visualized under UV light, and/or by treatment with alkaline permanganate solution followed by heating. MTS reagent was supplied by Promega. Column chromatography was conducted using silica gel (230-400 mesh) from Qualigens. ¹H NMR spectra were recorded on Bruker DPX 400MHz spectrometer. Chemical shifts are reported in δ (ppm) units using residual ¹H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-C Lite (Mestrelab Research) and/or XWinPlot (Bruker Biospin). Electrospray ionization mass spectra were recorded on a Micromass Q ToF 2 (Waters) and data were analyzed with MassLynx 4.0 (Waters) software. Mouse cytokine profiler kit (R&D systems), 6 well plates (Corning), DMEM, Fetal Bovine Serum (Gibco, Life Technologies). Arginase-1, iNOS Phospho CSF1R, Total CSF1R, Phospho AKT, Total AKT, Phospho NFkB, Total NFkB, Phospho ERK, Total ERK, SOCS3, SOCS1, Arg1, Phospho Ps670k, Total Ps670k, Phospho mTOR, Total mTOR and actin antibodies were purchased from Cell Signaling Technology. CD45, F4/80, CD206, CD11b, Ly6C, CD80, CD86, MHC II FACS antibodies were purchased from Biolegend Inc.

Synthesis of CSF1R inhibiting amphiphile: BLZ-945 (25 mg) was dissolved in 2 ml of anhydrous DCM followed by addition of 1.1 molar equivalent of cholesteryl hemisuccinate, EDC and DMAP. The reaction mixture was stirred at room temperature for 24h under argon. Upon completion of reaction as monitored by TLC, the solvent was evaporated under vacuum and the crude product was purified by using column chromatography, eluting with ethyl acetate:hexane gradient, to give CSF1R-inhibiting amphiphile as a light yellow solid. The obtained conjugate was analyzed by ¹H NMR and Mass spectrometry.

Quantum mechanical all-atomistic simulations: As the first step, we performed geometry optimization of the molecular subunits using quantum mechanical (QM) methods to obtain the lowest energy conformation of the molecular subunit. Following the QM optimization step, partial charges on each atom of the drug molecules were computed using Gaussian 09 with B3LYP exchange correlation functional and 6311G basis set, and electrostatic potentials were fitted with CHELPG scheme. Next we developed the force field parameters of the molecular subunit. Bond and angle potential parameters were taken from CHARMM force

field. Most of the dihedral potentials were taken from CHARMM force field, and those that were not present in the CHARMM force field, were calculated and parameterized. In terms of non-bonded potentials, Lennard-Jones (LJ) parameters were adapted from CHARMM force field, while for coulomb potential, partial charges on each atom obtained from QM calculations were used. Following the force field development step, the subunit was energy optimized using the developed force field using steepest descent algorithm. We next performed a short molecular dynamics (MD) simulation of the molecular subunit in vacuum using the developed force field, and the output structure obtained was matched with the structure obtained after QM optimization of the drug molecule. If the structures obtained did not match, we looped back to the force field development circle, and different parameters were tweaked, and this iteration continued till the structures obtained after QM optimization and MD in vacuum matched. All the MD simulations were performed using GROMACS-4.6.1 package. QM geometry-optimized structures of BLZ945 and CSF-1R inhibiting molecular subunit were considered as the starting structure for MD simulations. All the other bonded and non-bonded parameters were taken from CHARMM force field. All covalent bonds in SOPC and the drugs were constrained with LINCS algorithm. SETTLE method was used to constrain the covalent bonds in water. Time step of 2 fs was used for all the simulations. Isothermal and isobaric ensemble (NPT) and periodic boundary condition (PBC) were used. Temperature was kept constant at 300 K for all the systems using Nose-Hoover thermostat with a coupling constant of 0.5 ps. Pressure was kept constant at 1 atm using Parrinello-Rahman barostat with coupling constant of 10 ps. Semi-isotropic pressure coupling was used. Neighbor searching was done with Verlet algorithm as implemented in Gromacs 4.6.1. For long range electrostatic interactions, we used a particle mesh Ewald (PME) scheme with real space cut off of 1.4 nm. For van der Waals interactions 1.5 nm cut off was applied with a switching function at 1.4 nm. BLZ945 and CSF-1R inhibiting molecular subunit molecules were inserted inside the lipid bilayer structure and equilibrated till 10ns for all the systems. Models were constructed such that at the high drug concentration (20%), the system contained 256 molecules of SOPC and 64 molecules of geometry-optimized structure of drug molecules. The trajectories were saved for further analysis.

Synthesis and characterization of AK750 supramolecule: CSF1R-inhibiting amphiphile, L- α -phosphatidylcholine, and DSPE-PEG2000 at 0.2:0.5:0.3 molar ratios were dissolved in 1.0 mL DCM and solvent was evaporated to form thin film. The resulting thin film was hydrated with PBS at 60°C for 1.5 h to get supramolecules. The supramolecules were eluted through a Sephadex column and extruded through 0.4 μ m and then 0.2 μ m polycarbonate membrane using mini-extruder. The size was measured using Dynamic light Scattering (DLS), and drug loading was determined by UV spectroscopy. The incorporation efficiency was determined as the percentage of drug recovered from the supramolecule fractions as compared to the initial loading amount. The mean particle size of the AK750 was measured by Dynamic Light Scattering method using Zetasizer Nano ZS90 (Malvern, UK). 10 μ L of nanoparticles solution was diluted to 1ml using DI water and 3 sets of 10 measurements each were performed at 90 degree scattering angle to get the

average particle size. The zeta potential was measured using a Zetasizer ZS90 with the supramolecules diluted in water for measurement according to the manufacturer's manual. The physical stability of AK750 was evaluated by measuring changes in mean particle size and zeta potential during storage condition at 4°C.

Synthesis and characterization of anti-SIRP α -AK750 supramolecule: CSF-1R inhibiting amphiphile was synthesized by conjugating BLZ945 with Cholesteryl hemisuccinate using EDC and DMAP coupling reaction as described in the supplementary methods. The products were characterized by ^1H NMR spectroscopy and mass spectrometry. For supramolecule synthesis, 20 mol % of CSF-1R inhibiting amphiphile, 30 mol % 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] and 50 mol % L- α -phosphatidylcholine was dissolved in 1ml of DCM. The solvent was evaporated into a thin and uniform film using a rotary evaporator. The film was then hydrated with 1.0 ml H₂O for 1.5 h at 60°C. After hydration, 1 equivalent of EDC and NHS were each added and incubated at room temperature for 2 hours. To the samples, different concentrations (1 μl , 4 μl , 8 μl and 20 μl of 0.5mg/kg concentration) of either IgG (control) or anti-SIRP α antibodies were added and the samples were incubated at 4°C for 12h. After incubation, the samples were extruded at 60 °C using a 400 nm and then 200 nm PC membrane with 500 μl sample volume to obtain sub-200 nm particles. The samples were further passed to Sephadex G-25 column to remove free molecular subunits and non-conjugated antibodies. Drugs incorporated in the sample were determined using UV-vis spectroscopy. The incorporation efficiency was determined as the percentage of drug recovered from the nanoparticle fractions as compared to the initial loading amount. The mean particle size and zeta potential of the samples was measured using Zetasizer Nano ZS90 (Malvern, UK). The physical stability of the nanoparticles was evaluated by measuring changes in mean particle size and ζ potential during storage condition in 4°C.

DAR Calculations for anti-SIRP α -AK750 supramolecule: To calculate the drug to anti-SIRP α antibody ratio (DAR), FITC-tagged anti-SIRP α antibody was conjugated to the supramolecules using EDC and NHS conjugation as described above. The amount of antibody conjugated to the supramolecule was determined by Fluorescence spectroscopy and the amount of CSF-1R inhibiting amphiphile was obtained as described previously. The number of supramolecules in the solution were analyzed by using Malvern NanoSight NS300 instrument according to the manufacturer's protocol. Average number of antibodies per supramolecule were calculated by taking a ratio total number of antibodies with total number of supramolecules in the solution. Similarly, average number of CSF-1R inhibiting amphiphile per supramolecule were calculated by using a ratio of total number of amphiphiles with total number of supramolecules in the solution. The DAR was calculated by taking a ratio of average number of amphiphiles per supramolecule to the average number of antibodies per supramolecule.

Release kinetics studies: Drug loaded AK750 (1mg drug/ml, 5ml) were suspended in PBS buffer (pH 7.4) or 4T1 cell lysate or Mouse Macrophage cell lysate and sealed in a dialysis tube (MWCO= 3500 Dalton, Spectrum Lab). The dialysis tube was suspended in 1L PBS pH 7.4 with gentle stirring to simulate the infinite sink tank condition. A 100 μ L portion of the aliquot was collected from the incubation medium at predetermined time intervals and replaced by equal volume of PBS buffer, and the released drug was quantified by UV-vis spectroscopy and plotted as cumulative drug release.

Stability in Human Serum: Drug Loaded antiSIRP α -AK750 supramolecule (1mg/mL) of different dilutions were prepared. Equal volumes of Human Serum were added to that and mixture was stirred gently at 4°C. The mean particle size of the Nanoparticle was measured by Dynamic Light Scattering method using Zetasizer Nano ZS90 (Malvern, UK) at regular intervals.

Cryo-Transmission Electron Microscopy for AK750: The sample was preserved in vitrified ice supported by holey carbon films on 400 mesh copper grids. The sample was prepared by applying 3 μ L of sample suspension to a cleaned grid, blotting away with filter paper and immediately proceeding with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using an FEI Tecnai Cryo-Bio 200KV FEG TEM, operating at 120KeV equipped with 2 Gatan Sirius CCD cameras one 2K*2K and one 4K*4K pixel vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below -170°C. Images of the grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnification, high magnification images were acquired at nominal magnification of 52,000x (0.21 nm/pixel), and 21,000x (0.50 nm/pixel). Images were acquired at a nominal under focus of -5 μ m (21,000x) and -4 μ m (52,000x) at electron doses of $\sim$ 10-15 e/ $\text{\AA}^2$.

AK750 Internalization Assay: RAW264.7 Macrophage cells (5×10^5 cells) were seeded in 10 ml petri dish. Then, free BLZ-945 or equivalent amount of AK750 (20 μ M) was added to the cells and incubated for 4h and 20h in 5% CO₂ atmosphere at 37°C. After incubation, cells were washed thrice with PBS and replenished with fresh media. After desired time of incubation, 2×10^6 cells were lysed from each sample, centrifuged and supernatant was collected. Amount of drug in the samples were measured by UV-Vis spectroscopy using drug free cells as control.

Western Blot Assay to study inhibition of CSF1R phosphorylation at different time points: 5×10^5 RAW264.7 Cells were seeded in each well of a 6 well plate. The cells were incubated with BLZ945 or equivalent amount of AK750 (67 nM) for different time intervals in 5% CO₂ atmosphere at 37°C. After incubation, the cells were stimulated with MCSF cytokine (10 ng/mL) for 20 minutes followed by washing with ice cold PBS. The lysate was collected by scraping using NP40 Lysis buffer supplemented with protease

and phosphatase inhibitor (Roche diagnostic). Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a 4-20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Then membranes were incubated in TBST with Phospho CSF1R (1:1000 dilution), CSF1R (1:1000 dilution), and actin (1:2000 dilution) antibodies (all antibodies from Cell Signalling Technology) overnight at 4°C. After appropriate amount of washing with TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

Western Blot Assay to study sustained inhibition of CSF1R phosphorylation: 7×10^5 RAW264.7 Cells were seeded in 5 ml plates. The cells were incubated with free drugs or equivalent amount of AK750 (67 nM). After 4 hours of transient exposure, the cells were washed with cold PBS thrice and replenished with fresh DMEM media. After 7 and 48 hours of incubation in 5% CO₂ atmosphere at 37°C, the cells were stimulated with MCSF (10 ng/mL) for 2 hours. Cells were washed once with ice cold PBS. Lysate was collected by scraping using NP40 Lysis buffer supplemented with protease and phosphatase inhibitor (Roche diagnostic). Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a 4-20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Then membranes were incubated in TBST with Phospho CSF1R (1:1000 dilution), CSF1R (1:1000 dilution), Phospho AKT (1:1000 dilution), Total AKT(1:1000 dilution), Phospho NFkB(1:1000 dilution), Total NFkB(1:1000 dilution), Phospho ERK(1:1000 dilution), Total ERK(1:1000 dilution), SOCS3(1:1000 dilution), SOCS1(1:1000 dilution), Arg1 (1:1000 dilution), Phospho Ps670k(1:1000 dilution), Total Ps670k(1:1000 dilution), Phospho mTOR(1:1000 dilution), Total mTOR(1:1000 dilution) and actin (1:2000 dilution) antibodies (all antibodies from Cell Signaling Technology) overnight at 4°C. After appropriate amount of washing with TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

Western Blot Assay to study inhibition of CSF1R phosphorylation during reprogramming of macrophages: 7×10^5 RAW264.7 Cells were seeded in 5 ml plates. The cells were then stimulated with 20ng/mL recombinant mouse IL-4 in basal DMEM media for 24hours. Following stimulation with IL-4 the cells were treated with 67nM of BLZ-945, PLX-3397 and AK750 for 12 hours respectively. After 12 hours of incubation in 5% CO₂ atmosphere at 37°C, cells were washed once with ice cold PBS. Lysate was collected by scraping using NP40 Lysis buffer supplemented with protease and phosphatase inhibitor (Roche diagnostic). Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a 4-20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Then membranes were incubated in TBST with Phospho CSF1R,

CSF1R, Phospho AKT, Total AKT, Phospho NFkB, Total NFkB, Phospho ERK, Total ERK, SOCS3, SOCS1, Arg1, iNOS, Phospho Ps670k, Total Ps670k, Phospho mTOR, Total mTOR and actin antibodies (all antibodies from Cell Signalling Technology) overnight at 4°C. After appropriate amount of washing with TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

Western Blotting to check for inhibition of CSF1R phosphorylation in tumor-conditioned media treated macrophages: Preparation of Tumor-conditioned media (TCM): 4T1 breast cancer cells and B16/F10 melanoma cells were seeded in a density of 50,000 cells/mL in 25mL plate and were allowed to achieve 80% sub confluency after which the media was aspirated and replaced with basal DMEM media. The cells were incubated in for 24hrs and the media was then passed through 0.2µm syringe filters and assayed immediately. RAW 264.7 cells were plated in six well plates in a density of 10⁵ cells per well and incubated for 24hrs. 2mL of freshly prepared Tumor-conditioned media was then added to each well and incubated for 24hrs. Following TCM treatment the RAW 264.7 cells were treated with BLZ-945 and AK750 (67nM) for the different time intervals in basal DMEM media. After treatment, the media was aspirated and the cells were washed with PBS and lysed in NP40 cell lysis buffer containing protease and phosphatase inhibitors. Protein estimation was done using Pierce BCA protein assay kit and western blotting was done to check for the expression of Phospho CSF1R, Arginase-1 and iNOS levels. Antibody dilutions and western blotting protocols were done according to manufacturer's protocols.

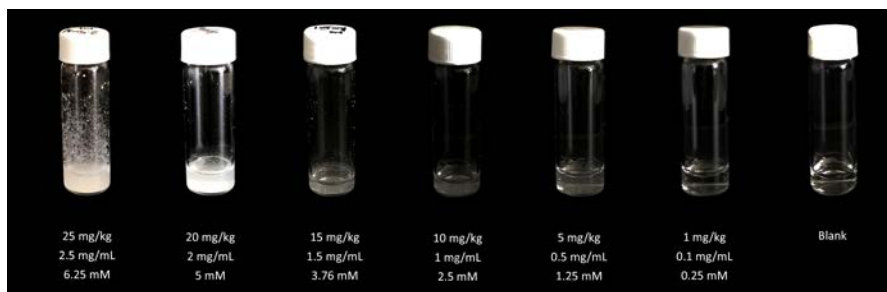
In-vitro Phagocytosis Assay with varying anti-SIRPα densities on anti-SIRPα-AK750: 5 × 10⁴ RAW264.7 cells were plated per well in an 8-well chamber slide. After 24h of IL-4 (20ng/mL) stimulation, anti-SIRPα-AK750 with varying densities of anti-SIRPα and IgG-AK750 were added at 67nM (equivalent to BLZ945 conc.) to macrophages and incubated for 3 hours at 37°C. The macrophages were labeled with APC anti- CD11b antibody. B16F10 melanoma cells (5×10⁴) were labeled with CFSE as per manufacturer's protocol and added to macrophages at 1:1 ratio and incubated for 8h. The imaging was performed with an Olympus Confocal microscope. The images were analyzed and quantification was performed using ImageJ software.

Flow cytometry assay to check for inhibition of autocrine CSF-1 signaling in RAW 264.7 macrophages: RAW 264.7 cells were seeded in a density of 1X10⁵ cells per well in 6 well plates and were allowed to reach 50% sub confluency. The cells were then stimulated with 20ng/mL recombinant mouse IL-4 in basal DMEM media for 24hours. Following stimulation with IL-4 the cells were treated with 67nM of BLZ-945 and AK750 for the different time intervals. On completion of the treatment, the media was aspirated and

the cells were collected after washing with cold PBS. The cells were stained with CD206, CD80, CD86, CD11b and MHC II antibodies and the results were analyzed using FlowJo software.

Inhibition of CSF-1R signaling in Bone marrow-derived macrophages (BMDM): BMDMs were generated as previously described (*Nature communications*, 2014, 5, 4696; *Leucocytes: Methods and Protocols*, 2011, 177-181). Briefly, bone marrow derived cells were isolated from the tibia and fibula of C57BL/6 mice (4-5 weeks). The cells were then cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) FBS supplemented with 10ng/mL of mouse MCSF for 7 days to obtain bone marrow derived macrophages. The macrophages were further stimulated with fresh medium 20 ng/mL of MCSF for 2 days to obtain M2 differentiated macrophages followed by treatment with 67nM of BLZ-945 or AK750 for 48hrs. After treatment, the cells were washed with cold PBS and collected for flow cytometric analysis. The macrophages phenotype was confirmed by staining with M2 markers (CD14 and CD206) and performing flow cytometric analysis. The results were analyzed using FlowJo software.

Efficacy study of AK750 in murine 4T1 breast cancer BALB/c model: 4T1 breast cancer cells (1×10^5) were implanted subcutaneously in the flanks of 4-week-old BALB/c mice (weighing 20g, Charles River Laboratories). The drug therapy was started on day 10. The drug therapy consisted of administration of Empty Vehicle (for control group), free BLZ945 (45mg/kg, i.p injection), AK750 (equivalent doses of free BLZ945 45mg/kg, tail-vein injection) every fourth day. 25 mg/kg of CSF-1 neutralizing antibody was used as a control. For an ideal comparison, the same route of administration should be used. We therefore performed a solubility test to establish the solubility-limited dose. As seen in the figure below, BLZ945 precipitates out at 1.25 mM, which means we can't even administer a dose of 5mg/kg (in 100 μ l volume of injection). As a result we used i.p. route, which allowed the higher doses to be administered. Solubility is therefore a major limitation of BLZ945, which limits bioavailability, and a major advantage of the AK750 supramolecule. The tumor volumes and body weights were monitored on every alternate day for 12 days. The tumor volume was calculated by using the formula, $L \times B^2/2$, where the longest diameter was considered as L and the shortest diameter as measured using a vernier caliper as B. The animals were sacrificed when either the average tumor volume of the control exceeded 2000 mm³ in the control group or the tumors were necrotic. The tumors and lungs were harvested immediately following sacrifice and processed for further analysis. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.



BLZ945 starts precipitating out in aq. sol. at concentrations >0.25 mM, which translates to a dose of 1mg/kg at a 100 μ l injection volume. The drug was dissolved in PBS at increasing concentration. The top numeric value depicts the dose achieved at 100 μ l injection volume.

FACS Analysis of *in vivo* tumor samples (4T1 breast cancer model): The harvested tumor tissues were thoroughly minced and suspended in separate 4 ml Type-I Collagenase solution (175U/mg). A single cell suspension was prepared for each tumor sample and incubated for 1 hour at 37°C and 5% CO₂ and finally passed through 40µm filter. Washing was carried out using RPMI-1640 media by centrifuging at 2000 rpm for 5 minutes (2 times). The single cell suspension was then used to identify the composition of total infiltrating leukocytes (CD45+/CD4+, CD45+/CD8+, CD45+/NK1.1+, CD45+/Ly6C+, CD45+/Ly6G+) The cell surface expression of CD11b, F4/80, Ly6C, CD206 was evaluated by surface staining in order to identify the percentage of M1 macrophages (CD11b+/CD80+, CD11b+/CD86+ and CD11b+/MHC II+) and M2 macrophages (CD11b+/CD206+) in the tumor bearing mice. The tumor single cell suspensions were analyzed and quantified using a BD J LSR II cytometer.

***In vivo* tumor distribution study:** For bio-distribution studies, DiR dye-labeled AK750 were synthesized by lipid-film hydration method. Briefly, 50 mol% of L-α-phosphatidylcholine, 19 mol% of CSF1R-inhibiting amphiphile, 1 mol% of DiR dye and 30 mol% of 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] (DSPE-PEG) were dissolved in 1.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film using a rotary evaporator. The lipid-drug film was then hydrated with 1.0 mL H₂O for 2 h at 60°C. B16/F10 melanoma cells (5 × 10⁵) were inoculated subcutaneously into the flank of each male C57B/L6 mice (4-6 weeks old, weight ~ 20 g). After the tumor reached ~ 500 mm³ volume, dye labeled supramolecules were injected through the tail vein of the tumor bearing mice. The imaging was performed at 3h, 12h and 24h post injection using IVIS filter set (excitation 710 nm and emission 760 nm). All the images were captured using Maestro (CRI) small animal *in vivo* fluorescence imaging system. For the purposes of this study, the exposure times for acquiring the data were kept the same. In addition, the stage settings (which influences the distance from the camera) and the fiber optic adjustable illuminator arms were also kept at the same settings for the duration of the study. Fluorescence signals were normalized and quantified using Maestro Software. The Maestro Software was used to conduct spectral unmixing, *i.e.* separate individual fluorophores from each other and background autofluorescence. This feature was used to produce the spectrally unmixed images. Using a control mouse, we assigned a spectrum for autofluorescence/background. A phantom containing the dye was used to assign a spectrum for signal. Using the Maestro Software, we established a “spectral library” which indicates a spectrum for in-vivo signal from the dye, minus autofluorescence. This spectral library, once established, was applied to all raw data to produce the “spectrally unmixed images”.

The amount of CSF1R inhibitor accumulated in the tumor was further analyzed using LC/MS/MS. Briefly, the tumor tissue samples were homogenized in PBS (3 ml per gram tumor), and the homogenates were extracted

with 3 volume of methanol containing internal standard (propranolol). The supernatant was analyzed by LC-MS/MS using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). All samples were compared to a calibration curve prepared with blank homogenates of the respective tissues. After separation on a C18 reverse phase HPLC column (Agilent Zorbax StableBond 3.5 μ m, 2.1 x 30 mm) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

Efficacy study of AK750 in murine B16/F10 Melanoma C57BL/6 model: B16/F10 Melanoma cells (1×10^5) were implanted subcutaneously in the flanks of 4-6 weeks old C57BL/6 mice (weighing 20g, Charles River Laboratories). The drug therapy was started when the tumor volume reached $\sim 50 \text{ mm}^3$. The date of start of drug therapy was considered as day 0. The drug therapy consisted of administration of Empty Vehicle (for control group), free BLZ (45mg/kg, i.p injection), AK750 (Equivalent doses of free BLZ945 45mg/kg, tail-vein injection) every alternate day for total 3 dosages. The tumor volumes and body weights were monitored on every alternate day for 10 days after injection. The tumor volume was calculated as described previously. The animals were sacrificed when either the average tumor volume of the control exceeded 4000 mm^3 in the control group or the tumors were necrotic. The tumors were harvested immediately following sacrifice and processed for further analysis. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

Western blot analysis of *in vivo* tumor Samples: Tumor samples were immediately flash froze in liquid N_2 upon resection and homogenized and then lysed in ice-cold NP40 cell lysis buffer containing protease and phosphatase inhibitors. The cellular debris was removed by centrifugation at 15000 RPM and the supernatant was used for the experiment. Protein estimation was done by BCA protein assay kit. 20 μ g protein was loaded in each well and probed for Phospho-CSF1R, Total CSF1R, and β -actin. Detection was done using G-box from Syngene and densitometric quantification was done by image J software.

FACS Analysis of *in vivo* tumor samples (B16/F10 melanoma model): The harvested tumor tissues were thoroughly minced and suspended in separate 4 ml Type-I Collagenase solution (175U/mg). A single cell suspension was prepared for each tumor sample and incubated for 1 hour at 37°C and 5% CO_2 and finally passed through 40 μ m filter. Washing was carried out using RPMI-1640 media by centrifuging at 2000 rpm for 5 minutes (2 times). The pellet was re-suspended in 1ml of recommended media for CD11b+ macrophage isolation. The isolation was carried out using EasySep Mouse CD 11b positive selection kit II according to the manufacturer's protocol (Stem cell technologies). The phenotype of the isolated CD11b + macrophage-cells from the tumor single cell suspension was quantified using a BD J LSR II cytometer. The cell surface expression of CD11b, F4/80, Ly6C, CD206 was evaluated by surface staining in order to identify the percentage of M1 macrophages (CD11b+/CD80+, CD11b+/CD86+ and CD11b+/MHC II+) and M2 macrophages (CD11b+/CD206+) in the tumor bearing mice.

Anti-SIRP α -Supramolecule Internalization Assay: The FITC-tagged-supramolecule was synthesized by lipid-film hydration method using 5 mol% of FITC-cholesterol conjugate, 30 mol % 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] and 65 mol % L- α -phosphatidylcholine as described previously. Anti-IgG (control) or anti-SIRP α antibodies were conjugated using EDC and NHS conjugation reaction. The amount of FITC-cholesterol present was determined using a fluorescence spectrometer. RAW 264.7 cells were seeded (50×10^3) onto an 8-well chamber slide and were labeled with either APC anti-CD11b & APC anti-SIRP α antibody. M2 phenotype was induced through IL-4 stimulation (20ng/ml). FITC-tagged supramolecules (non-targeted; IgG-conjugated & anti-SIRP α conjugated) were then added (10 μ M of dye conc) for 4h. After 4h incubation, the cells were washed with ice-cold PBS. The cells were later fixed using 4% paraformaldehyde and were mounted using VectaMount reagent and were imaged using Nikon Fluorescence microscope.

Western Blot Assay to study inhibition of CSF1R phosphorylation using anti-SIRP α -AK750: For western blot 4×10^5 RAW264.7 Cells were seeded in a 5ml culture plate. The cells were then stimulated with 20ng/mL recombinant mouse IL-4 in basal DMEM media for 24 hours. Following stimulation with IL-4 the cells were treated with 67nM (BLZ-945 equivalent) of IgG-AK750 and anti-SIRP α -AK750 for 48h hours respectively. On completion of the treatment, the media was aspirated and the cells were collected after washing with cold PBS. Lysate was collected by using RIPA Lysis buffer supplemented with protease and phosphatase inhibitor (Roche diagnostic). Amount of protein was measured by BCA assay and equal amount of protein lysates were electrophoresed on a 4-20% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Then membranes were incubated in TBST with Phospho CSF1R (1:1000 dilution), CSF1R (1:1000 dilution), and β -actin antibodies overnight at 4°C. After appropriate amount of washing with TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:10000) for 1 hour. Detection was done using G-box from Syngene.

In-vitro Phagocytosis Assay: 5×10^4 RAW264.7 cells were plated per well in an 8-well chamber slide. After 24h of IL-4 (20ng/mL) stimulation, anti-SIRP α -AK750, AK750, IgG-AK750, and BLZ945 free drug were added at 67nM (equivalent to BLZ-945 conc.) to macrophages and incubated for 3 hours at 37°C. The macrophages were labeled with APC anti- CD11b antibody. B16/F10 melanoma cells (5×10^4) were labeled with CFSE as per manufacturer's protocol and added to macrophages at 1:1 ratio and incubated for 8h. The imaging was performed with an Olympus Confocal microscope. The images were analyzed and quantification was performed using ImageJ software.

MTS Cell Proliferation Colorimetric Assay: 5×10^3 cells were seeded into 96-well flat-bottomed plates. BLZ945, AK750, anti-SIRP α -AK750 were added at appropriate concentrations (0.0001, 0.01, 0.1, 1, 10, 100 μ M equivalent to BLZ945 conc). The plates were then incubated for 72 h in a 5% CO₂ atmosphere at 37 °C.

The cells were washed and incubated with 100 μ L phenol red free medium (without FBS) containing 20 μ L of the Cell-Titer 96 Aqueous One Solution reagent (Promega). After 2 h incubation in 5% CO₂ atmosphere at 37 °C, the absorbance in each well was recorded at 490 nm using an Epoch (BioTek) plate reader. Results were quantified by subtracting the blank value from each value then normalizing against the control values and results were analyzed by using Prism software (GraphPad). Data shown are mean $\pm$ SE of n = 3.

***In vitro* Drug Toxicity assay:** The cells were grown in six-well plates (3 x 10⁵ cells per well) incubated in the presence of AK750 at varying concentrations concentration at 37 °C for 72 h. After 72 h, the cells were washed with PBS and collected. The cells were then treated with APC Annexin-V (Bio Legend) and Propidium iodide (Bio legend) and incubated in the dark, at room temperature, for 15 min. The cells were then washed with PBS. The cell suspension were then transferred to FACS tubes and analyzed for Annexin-V and PI staining on a BD DUAL LSR Fortessa. Data were analyzed using a FlowJo software.

Repolarization of Macrophages differentiated from THP-1 cells: Human THP-1 cells were seeded in a 6 well plate at a concentration 100,000 cells/mL in RPMI. The cells were incubated in 150nM of PMA for 24h then incubated in basal media for another 24 h. The Thp-1 cells would have differentiated into M0 Macrophages. Cells were then treated with 20ng/mL of Human IL-4 and Human IL-13 for 48h in basal media to obtain M2 polarized Macrophages. These cells were then incubated With AK750 for 48h. After treatment, the cells were collected and were stained for M1 and M2 Markers and analyzed on a BD DUAK LSR Fortessa, Data Analysis was done using a FlowJo software

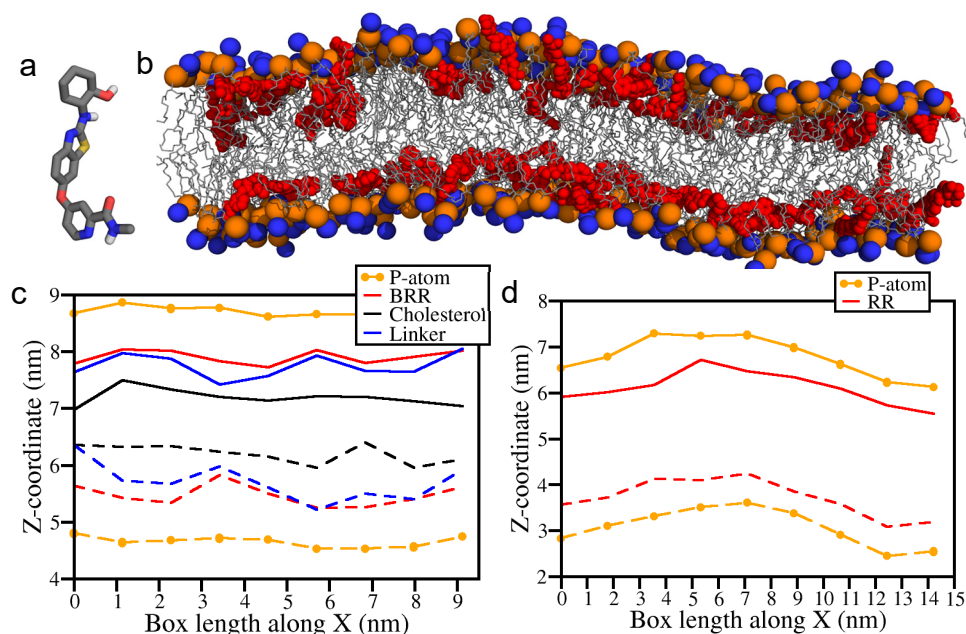
Biodistribution Studies in B16/F10 melanoma model: B16/F10 Melanoma cells (1 X10⁶) were implanted subcutaneously in the flanks of 4-6 weeks old C57BL/6 mice (weighing 20g, Charles River Laboratories). The drug administration was started when the tumor volume reached $\sim$ 200 mm³. The drug therapy consisted of administration of a single dose of free BLZ945, AK750 and anti-SIRP α -AK750 (Equivalent doses of BLZ945 15mg/kg, tail-vain injection). Organs were isolated from the mouse at different points. All tissues were homogenized in PBS, and homogenates were crashed with equal volumes of Dichloromethane, the aqueous phase containing PBS was carefully discarded using a Pasteur Pipette, the organic phase containing the drug was evaporated using a rotary evaporator and dissolved in a solution containing Methanol and Chloroform in a ratio of 1:1. Samples were analyzed by LC/MS/MS using an Waters Xevo TQ mass spectrometer coupled with an Acuity HPLC and a CTC PAL chilled autosampler, all controlled by MassLynx software (Waters). After separation on a C18 reverse phase HPLC column (Waters Acuity HSS T3 2.1x50mm 1.8 μ M) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

Pharmacokinetic studies in B16/F10 melanoma model: B16/F10 Melanoma cells (1 X10⁶) were implanted subcutaneously in the flanks of 4-6 weeks old C57BL/6 mice (weighing 20g, Charles River Laboratories). The drug administration was started when the tumor volume reached $\sim$ 200 mm³. The drug therapy consisted

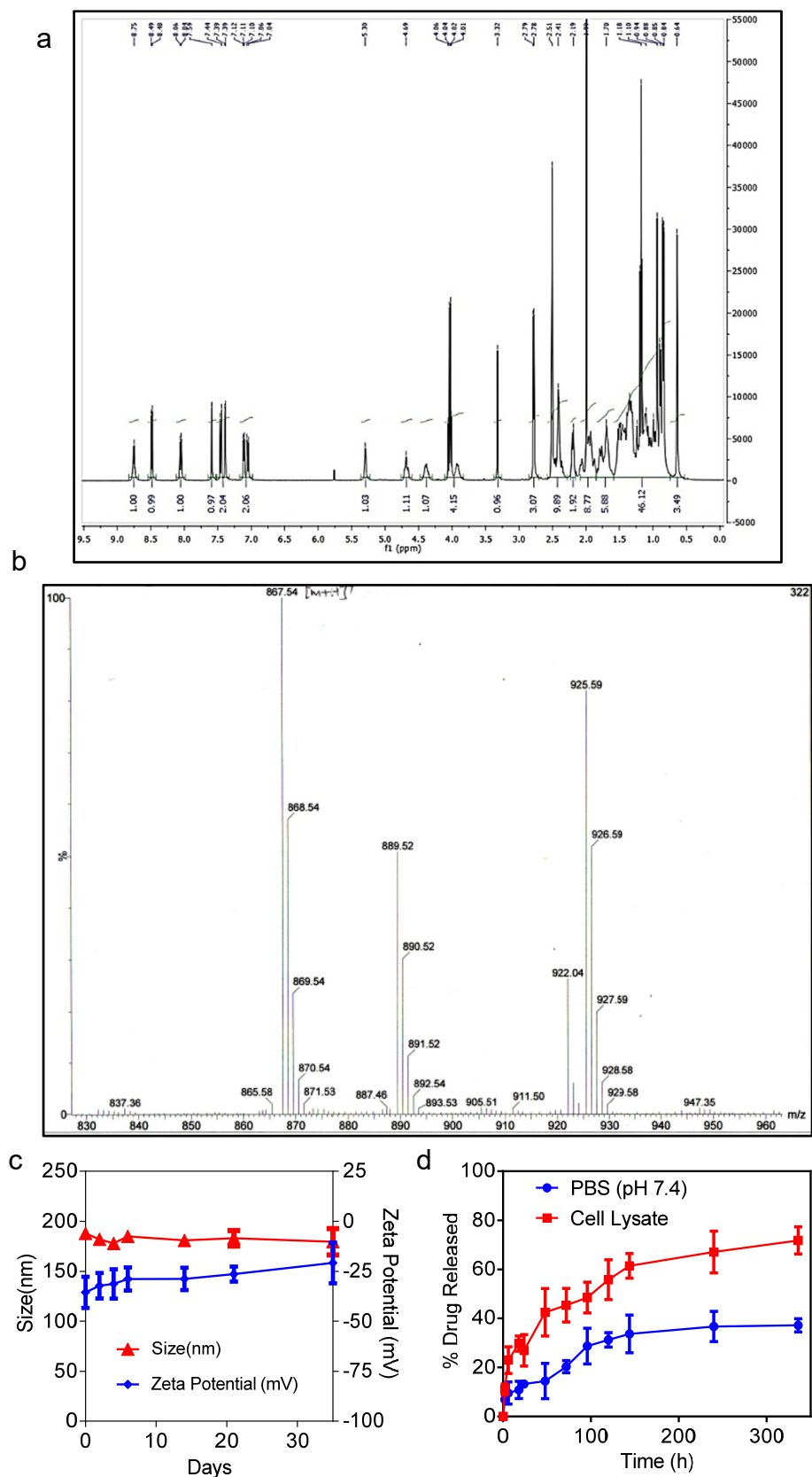
of administration of a single dose of free BLZ945, AK750 and anti-SIRP α -AK750 (Equivalent doses of BLZ945 15mg/kg, tail-vein injection). Blood was collected in heparin coated tubes (BD Vacutainer®) through cardiac puncture. Dichloromethane (1mL) was added to blood (100 μ L), and thoroughly mixed. The organic phase containing the drug was isolated and evaporated using a rotary evaporator and dissolved in a solution containing Methanol and Chloroform in a ratio of 1:1. Samples were analyzed by LC/MS/MS using a Waters Xevo TQ mass spectrometer coupled with an Acuity HPLC and a CTC PAL chilled autosampler, all controlled by MassLynx software (Waters). After separation on a C18 reverse phase HPLC column (Waters Acuity HSS T3 2.1x50mm 1.8 μ M) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

Efficacy study of AK750 and AK750-SIRP α in murine B16/F10 Melanoma C57BL/6 model: B16/F10 Melanoma cells (1×10^6) were implanted subcutaneously in the flanks of 4-6 weeks old C57BL/6 mice (weighing 20g, Charles River Laboratories). The drug therapy was started when the tumor volume reached $\sim 100 \text{ mm}^3$. The date of start of drug therapy was considered as day 0. The drug therapy consisted of administration of free BLZ (45mg/kg, i.p injection), AK750 and anti-SIRP α -AK750 (Molar equivalent doses of BLZ945 45mg/kg, tail-vein injection). The tumor volumes and body weights were monitored on every day for 7 days after injection. The tumor volume was calculated by using the formula, $L \times B^2/2$, where the longest diameter was considered as L and the shortest diameter as measured using a Vernier caliper as B. The tumors were harvested immediately following sacrifice and processed for further analysis. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

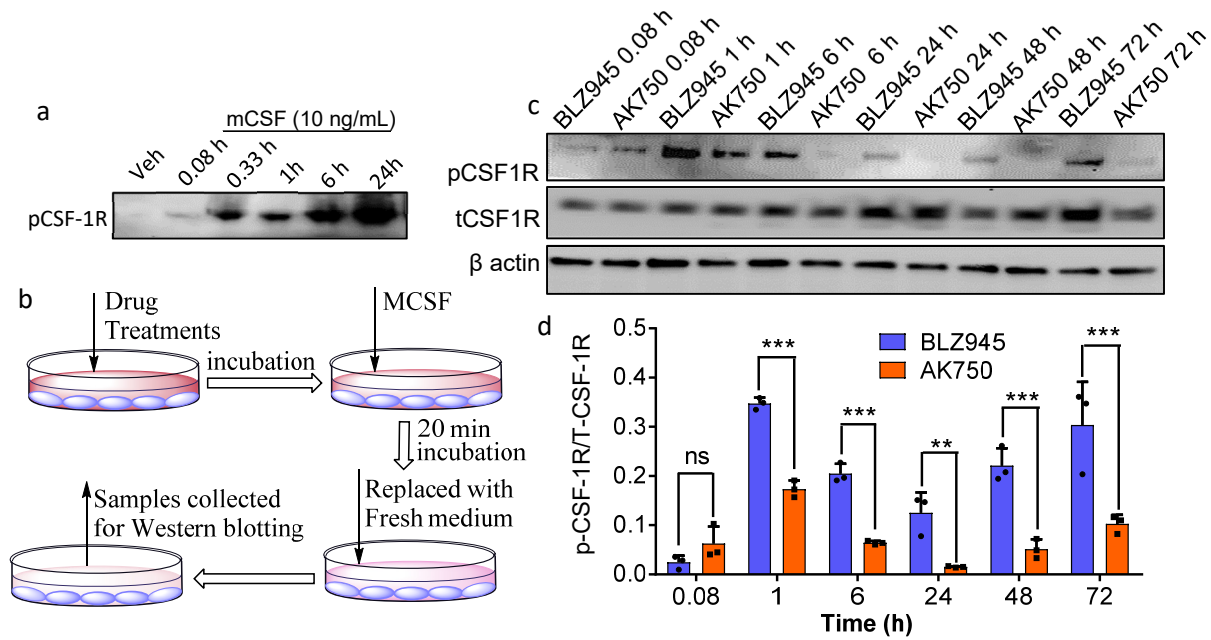
Histopathology and TUNEL Assay (Apoptotic Assay): The tissues were flash frozen, embedded in OCT (Tissue Tek) and sectioned. The sections were then stained with Alexa Fluor 488 Click-iT™ Plus TUNEL Assay kit (Thermo Fisher) following the manufacturer's protocol. The imaging was performed with an Olympus Confocal microscope. The images were analyzed and quantification was performed using ImageJ software.



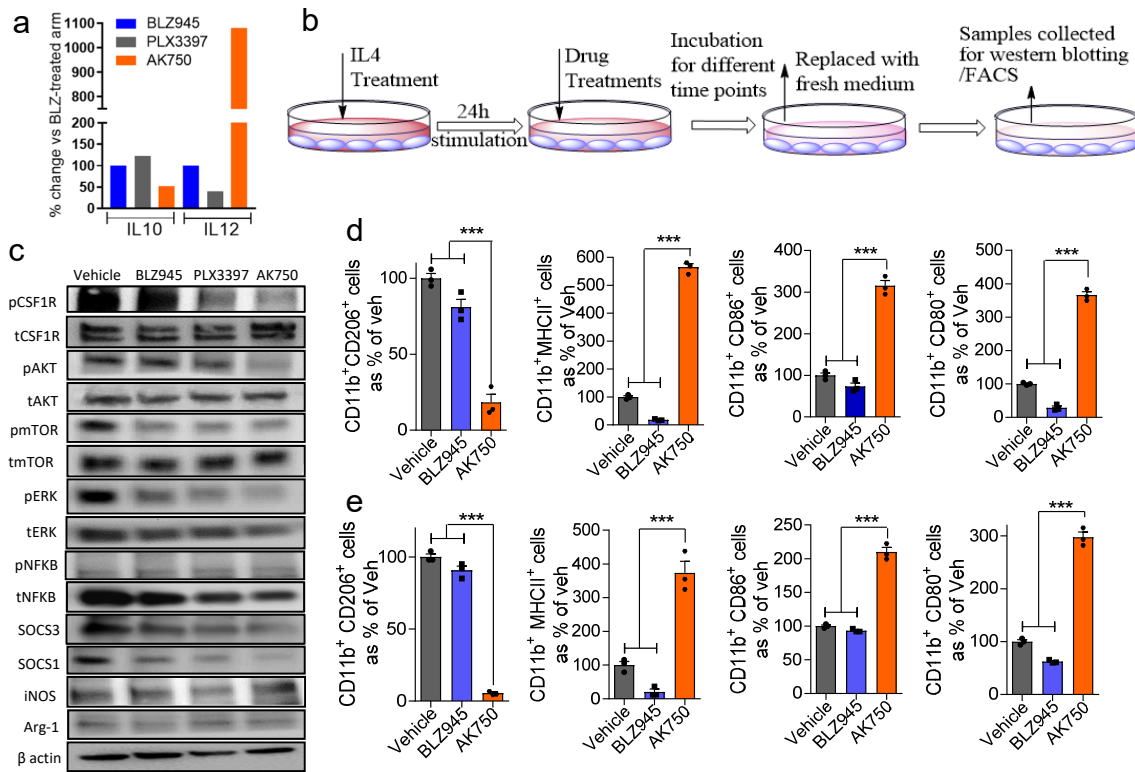
Suppl. Figure 1. Simulating the interactions of BLZ945 in a lipid bilayer. (a) QM optimized structure of BLZ-945; (b) Snapshot of all atomistic simulation of BLZ-945 interacting with SOPC lipid bilayer shows aggregates of BLZ-945 at the interface of hydrophilic lipid head and water interface; Lipid hydrophilic heads are shown in orange and blue spheres and lipid tails are shown in grey color. BLZ-945 is shown in red color; Graph showing positions of different groups present in the (c) BLZ-945 and (d) AK750 incorporated SOPC lipid bilayer system as mapped into XZ-plane (the plane is indicated in the schematics at the top left). Solid and dotted lines indicate two leaflets in the bilayer with hydrophobic core between the orange lines. Above and below orange lines there is water layer. Position of the P-atoms (orange) on SOPC appear outermost of the hydrophobic bilayer. The drug moiety in BLZ-945 and AK750 appears to be interacting similarly with lipid components. BLZ-945 and AK750 lie below P-atoms (orange) and cholesterol moiety (black) appears innermost in the bilayer in the hydrophobic region.



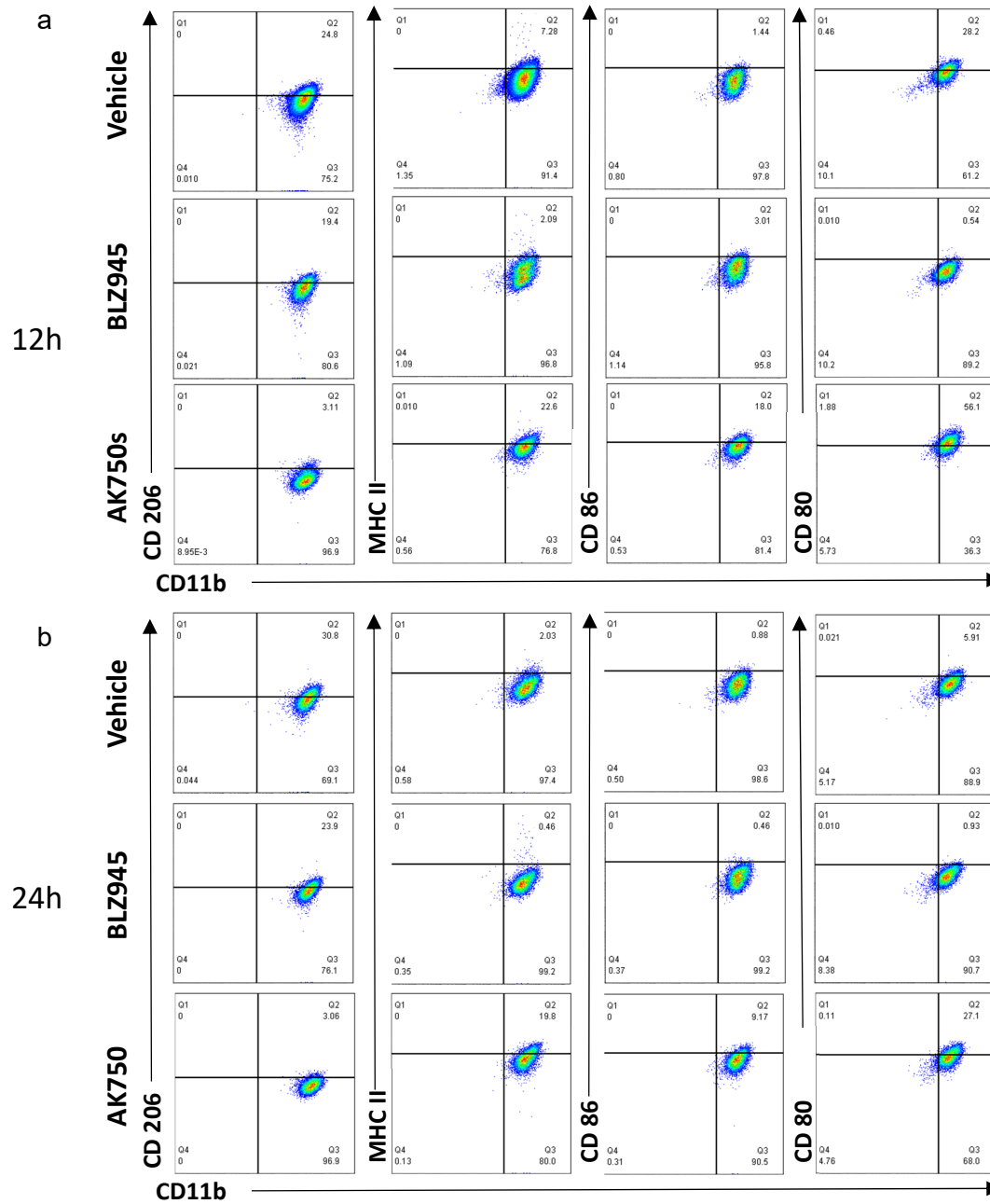
Suppl. Figure 2. Synthesis and characterization of CSF1R-inhibiting Supramolecule (AK750): (a) ^1H NMR spectra of CSF1R-inhibiting molecular subunit; (b) Mass Spectra of CSF1R-inhibiting molecular subunit; (c) Graph shows the stability of the AK750 as a function of the change in size distribution and zeta potential, measured by Dynamic Light Scattering. Data shows mean $\pm$ SD (n=3); (d) Graph shows the release of free drug from the AK750 in PBS (pH 7.4) or when incubated with 4T1 cell lysate. Data shows mean $\pm$ SD (n=3).



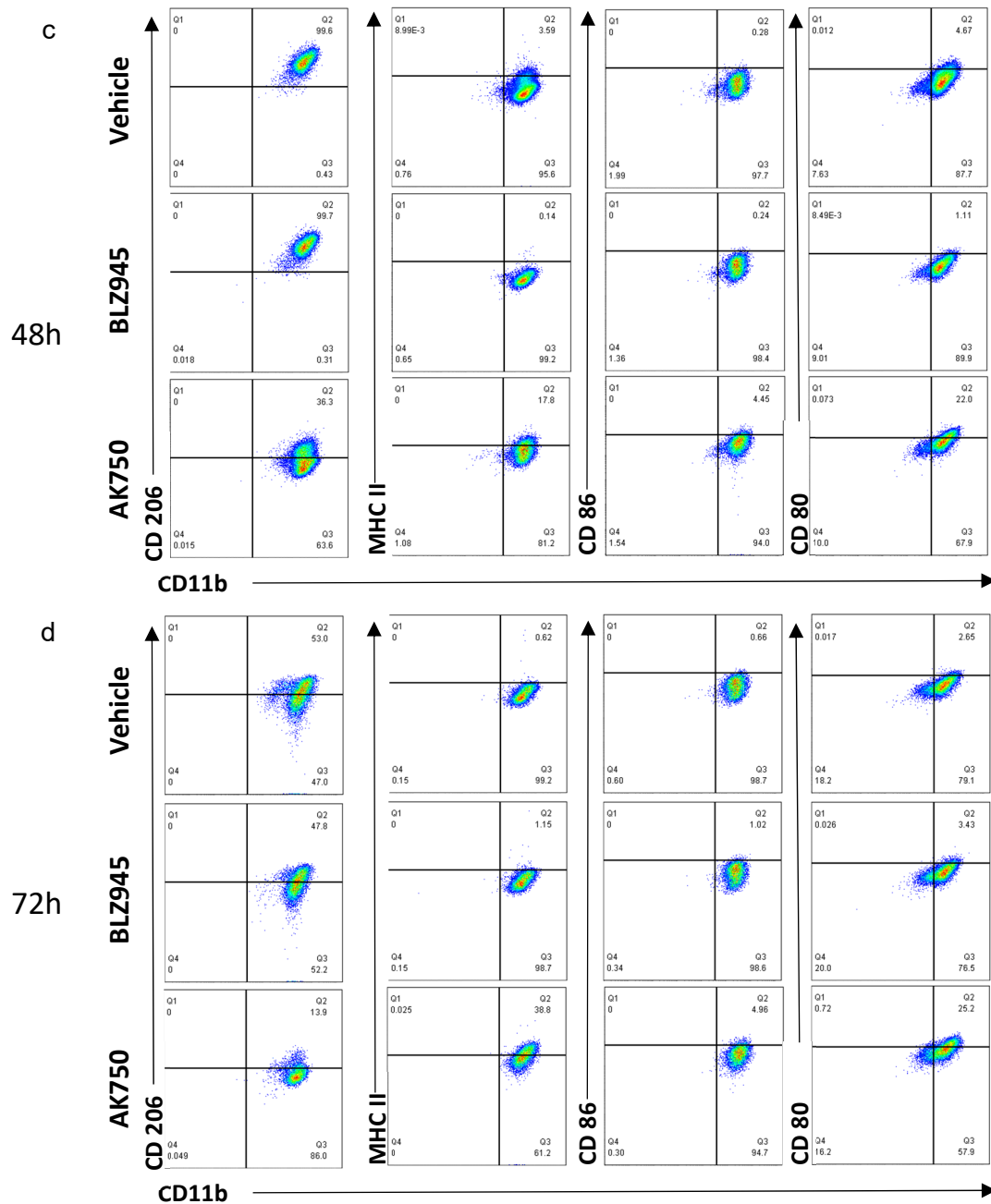
Suppl. Figure 3. AK750 inhibits CSF1R in a sustained manner. (a) Representative Western blot shows expression of phospho-CSF1R in RAW264.7 macrophages at 5 min, 30 min, 1 hour, 6 hours and 24 hours after treatment with 10ng/mL MCSF. (b) Cartoon representation showing the effect of BLZ945 and AK750 on macrophages. The cropped blot is used in the figure, and full-length blot is presented in Supplementary Fig. 16; (c) Expression of phospho-CSF1R in RAW264.7 cells at 5 min, 1 hour, 6 hours, 24 hours, 48 hours and 72 hours after treatment with either BLZ945 or AK750 (67nM) across different time points after treatment with B16F10 tumor conditioned media. Effect of BLZ-945 and AK750 on the inhibition of CSF1R phosphorylation when the drug or AK750 are already present in the cells prior to stimulation using MCSF was studied. After their respective drug or AK750 exposure times, the cells were washed with cold PBS twice to remove dead cells and drug or AK750 present outside the cells, followed by which they were stimulated with MCSF for 20 minutes. After stimulation the cells were washed with cold PBS and the cell lysates were collected. The cropped blots are used in the figure, and full-length blots are presented in Supplementary Fig. 17; (d) Graph showing the quantification of the CSF-1R phosphorylation inhibition in RAW264.7 cells after treatment with either BLZ945 or AK750. Statistical analysis was performed with student t-test. Data shows mean $\pm$ SEM (3 independent experiments); n.s., not significant; ** $p < 0.01$; *** $p < 0.001$.



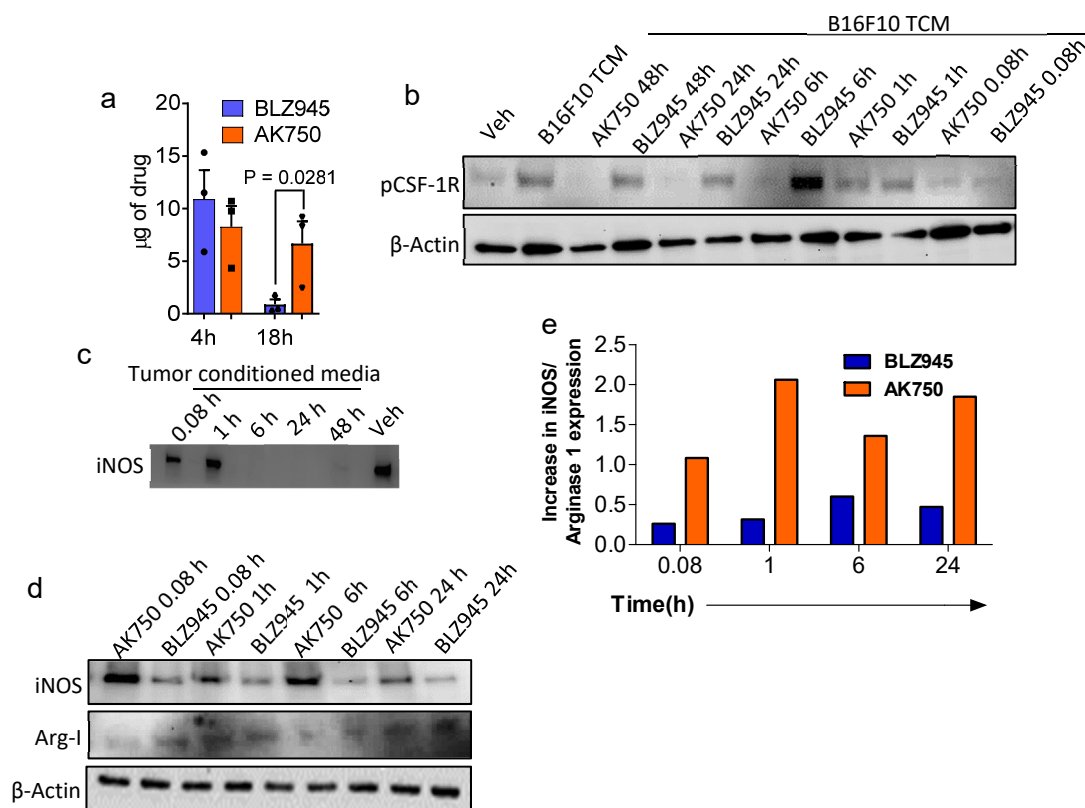
Suppl. Figure 4. AK750 efficiently repolarizes M2 macrophages to M1 phenotype. (a) RTPCR-analysis of macrophages treated with different CSF1R inhibitors show that treatment with AK750 results in the maximal increase in IL12, associated with a M1 response, and decrease in IL10, associated with a M2 response. Data is expressed as % of BLZ945-treated arm (n=2). (b) A schematic representation of macrophage repolarization assay after treatments with CSF-1R inhibitors. RAW264.7 cells were stimulated with IL4 for 24h and then treated with either BLZ945 or PLX3397 or AK750 for 4h, which was then replaced with fresh medium. The cell lysates were collected at different time points for Western blotting and FACS; (c) Representative Western blot shows expression of phospho CSF-1R, total CSF-1R and downstream signaling pathways after treatment with either BLZ945 or PLX 3397 or AK750. The cells were first stimulated with IL-4 for 24 h followed by treatment with either BLZ945 or PLX3397 or AK750 for 4h. After 4 hours of exposure to drug, the cells were washed with cold PBS to remove additional drug outside cells and then incubated with fresh media for 12h. The cropped blots are used in the figure, and full-length blots are presented in Supplementary Fig. 18; Effect of BLZ945 and AK750 in repolarization of the M2-macrophage phenotype formed upon stimulation with IL-4 to the M1-macrophage phenotype across different time points was studied. RAW264.7 cells were treated with IL-4 for 24 hours, followed by which they were treated with BLZ945 or AK750 in basal DMEM media and cells were collected at different time points (d) 24h and (e) 48h. M1 and M2 markers expression on the macrophages and their isotype control was evaluated separately by FACS (Representative FACS plots are shown in Supp. Fig. 5). Statistical analysis was performed with One-way ANOVA with Newman-Keuls post Test. Data shows mean ± SEM (n = 3); *p < 0.05; **p < 0.01; ***p < 0.001.



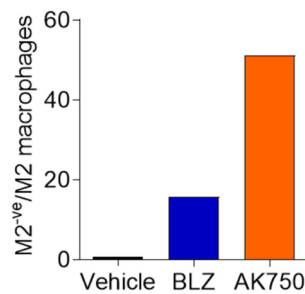
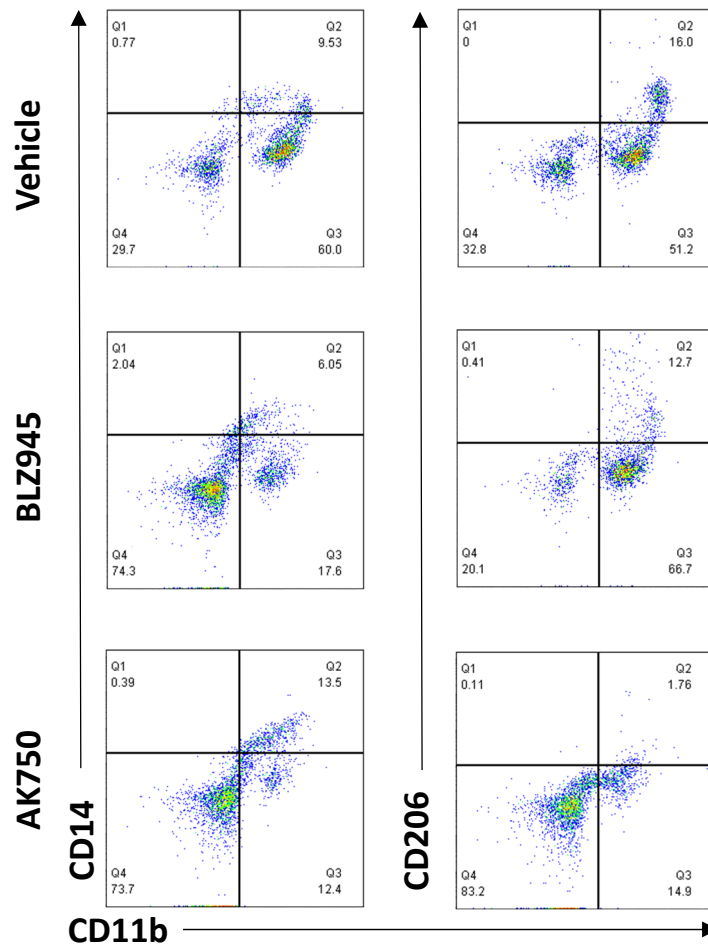
Suppl. Figure 5. Flow cytometry demonstrating expression of CD11b+CD206+, CD11b+MHC-II+, CD11b+CD86+ and CD11b+CD80+ on RAW264.7 cells stimulated with IL-4 for 24 hours followed by BLZ-945 or AK750 treatment across different time points, the figure is indicative of the (a) 12h, (b) 24h, (c) 48h and (d) 72h time points. Data are representative of 3 separate experiments.



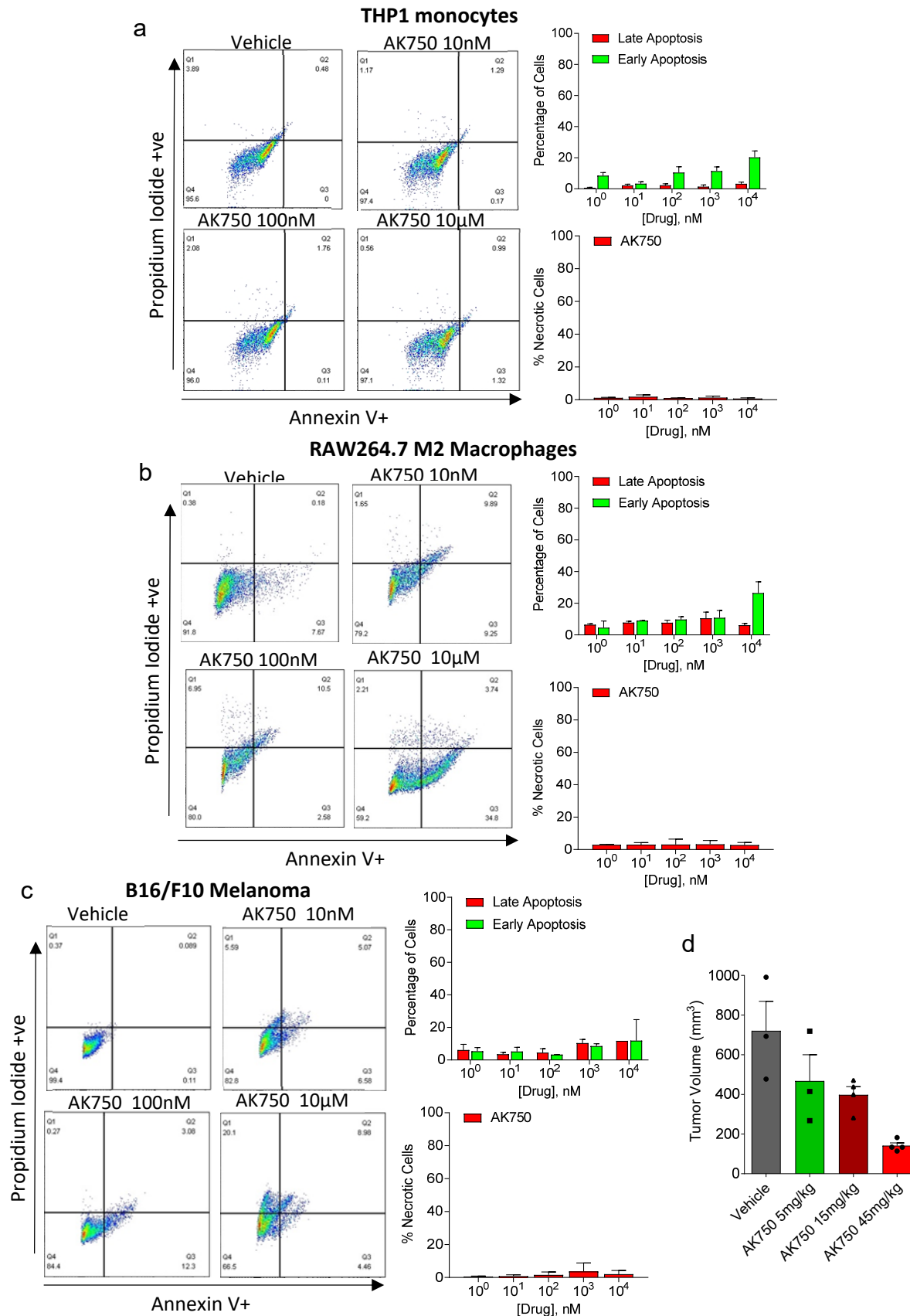
Suppl. Figure 5. continued



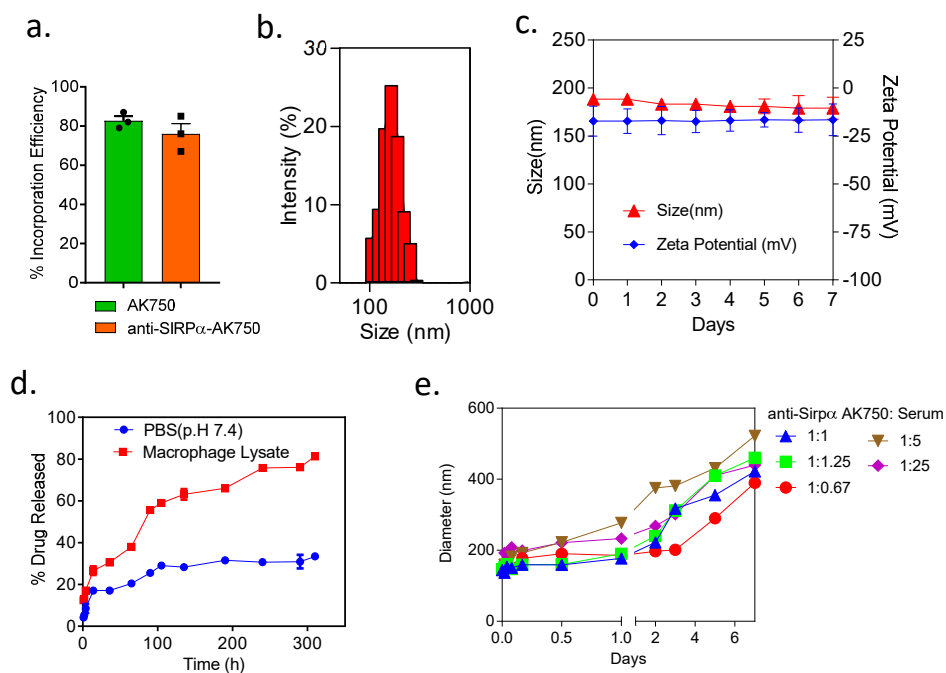
Suppl. Figure 6. (a) Graph showing internalization of BLZ945 and AK750 at 4h and 18h. The amount of drug internalized was quantified by UV-vis spectroscopy. Data shown are mean $\pm$ SEM from at least 3 replicates; * $p < 0.05$; * $p < 0.01$ (Student's t-test, one-sided); (b) Expression of phospho-CSF1R in RAW264.7 cells at 5 min, 1 hour, 6 hours, 24 hours, 48 hours and 72 hours after treatment with equimolar concentrations of either BLZ945 or AK750 (67nM) across different time points after treatment with B16F10 tumor conditioned media. Effect of BLZ-945 and AK750 on the inhibition of CSF1R phosphorylation when the drug or AK750 are already present in the cells prior to stimulation using B16F10 tumor conditioned media (TCM) was studied. The cropped blots are used in the figure, and full-length blots are presented in Supplementary Fig. 19; (c) Expression of iNOS in RAW264.7 macrophage cells at 5 min, 1 hour, 6 hours, 24 hours and 48 hours after treatment with B16F10 tumor conditioned media. Decrease in iNOS levels upon exposure to TCM across different time points was studied. The cells were treated with TCM for 24 hours, followed by which the TCM was replaced with DMEM basal media and cell lysates were collected at 5 min, 1 hour, 6 hours, 24 hours and 48 hours respectively; (d) Expression of iNOS and Arg-1 in RAW264.7 cells at 5 min, 1 hour, 6 hours and 24 hours after treatment with B16F10 tumor-conditioned media, followed by BLZ945 or AK750 treatment across different time points. The cropped blots are used in the figure, and full-length blots are presented in Supplementary Fig. 19; (e) Graph showing the quantification of iNOS/arginase levels.



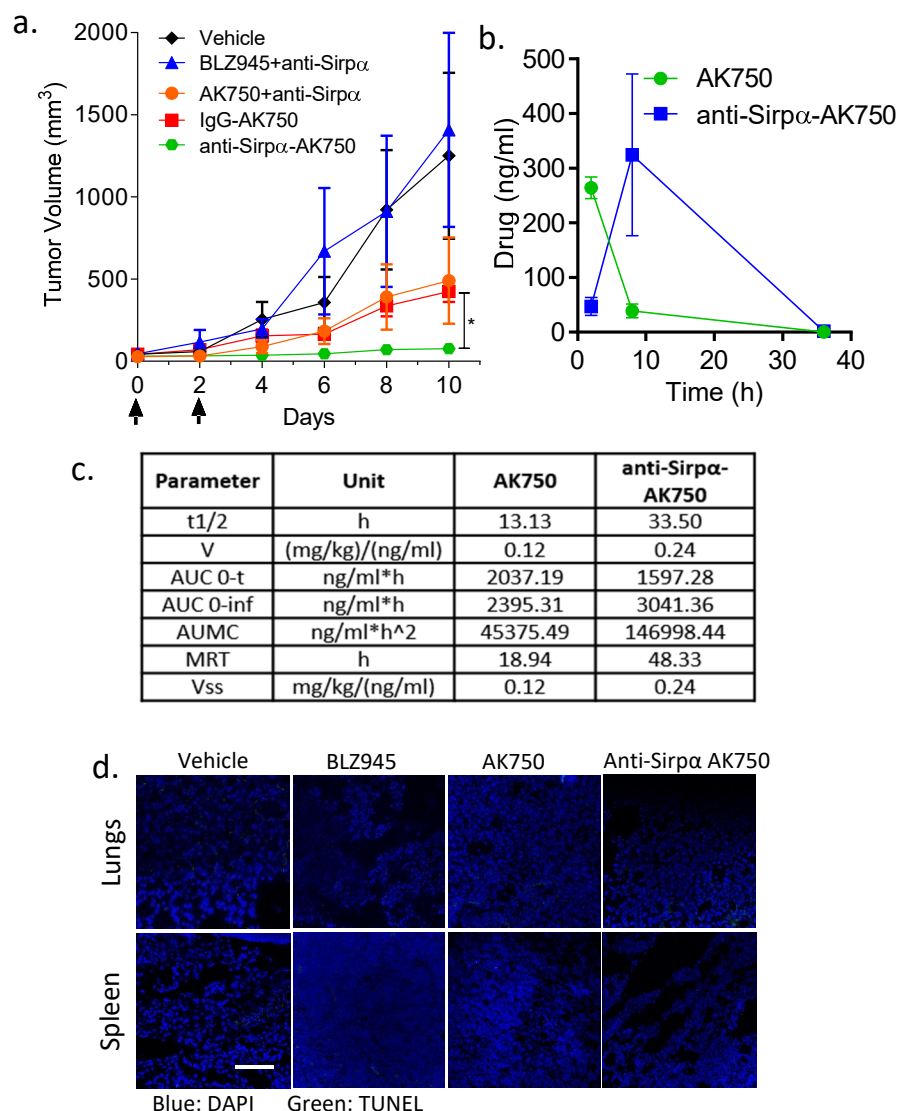
Suppl. Figure 7. AK750 inhibits differentiation of monocytes from bone marrow into a M2 lineage. (a) Flow cytometry demonstrating expression of CD11b+CD14+ and CD11b+CD206+ on monocytes. Effect of Vehicle, BLZ-945 or AK750 in inhibiting differentiation of monocytes to M2 macrophages upon CSF1- Stimulation was studied. Monocytes were treated with Vehicle, BLZ-945 or AK750 for 48 hours in the presence of CSF-1(10ng/ml) followed by which the cells were collected and the expression of monocyte and M2-macrophage phenotype markers were analyzed using FACS; (b) Graph represents the representative M2⁻/M2⁺ ratio by taking into account the individual monocyte and M2 markers that were analyzed using FACS (n = 2) .



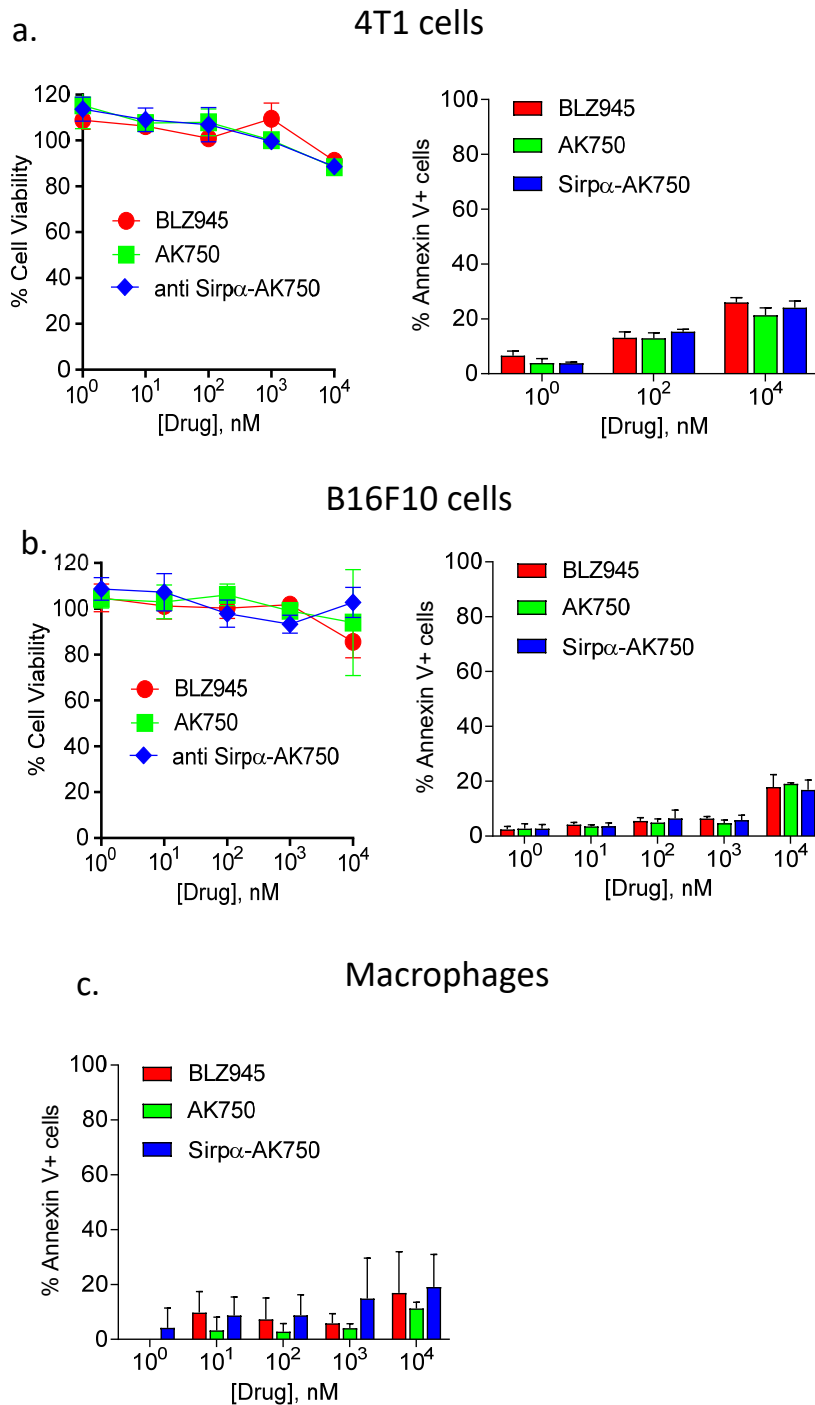
Suppl. Figure 8. FACS analysis of apoptosis and necrosis of (a-b) macrophages and (c) cancer cells following treatment with increasing concentration of AK750. At the concentrations used in the study, AK750 induced minimal apoptosis and no significant necrosis. Graphs show mean $\pm$ SEM (n=3). Representative FACS scatter plots are shown. **(d) AK750 exerts a dose-dependent anti-cancer effect, *in vivo*.** Graph shows the effect of different dose on tumor volume in 4T1 tumor bearing mice on day 12 after the first treatment. Each animal were injected with three doses of either Vehicle, 5 mg/kg, 15mg/kg and 45mg/kg of AK750, on day 0, day 4 and day 8. First day of treatment was considered as Day 0. End point for each animal was tumor size >1000mm³ or tumor ulceration or necrosis or animal death. Data shows mean $\pm$ SEM (n=5).



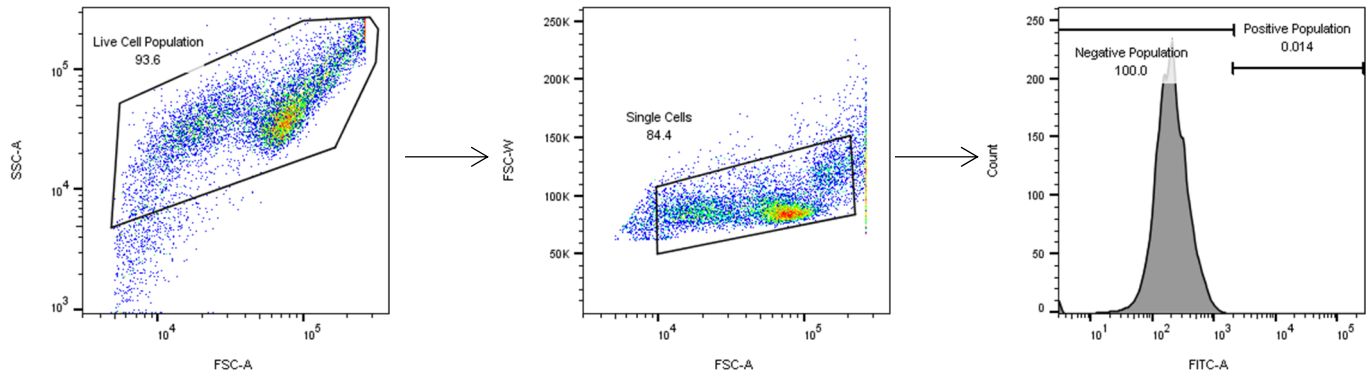
Suppl. Figure 9. Physicochemical characterization of anti-SIRPα-AK750. (a) Graph shows the total CSF1R-inhibiting amphiphile incorporated in AK750 and anti-SIRPα-AK750 supramolecules. Data is expressed as a % of the starting concentration, hence expressed as incorporation efficiency (mean ± SEM; n=3). (b) The size distribution of anti-SIRPα-AK750 as measured by Dynamic Light Scattering; (c) Graph shows the stability of the anti-SIRPα-AK750 as a function of the change in size distribution and zeta potential, measured by Dynamic Light Scattering. Data shown are mean ± SD (n=3). (d) Graph shows the release of free drug from the anti-Sirpα AK750 in PBS (pH 7.4) or when incubated with macrophage cell lysate. Data shown are mean ± SD (n=3). (e) Graph shows the stability of anti-SIRPα AK750 as a function of the change in size distribution, measured by Dynamic Light Scattering when incubated in different concentrations of human serum.



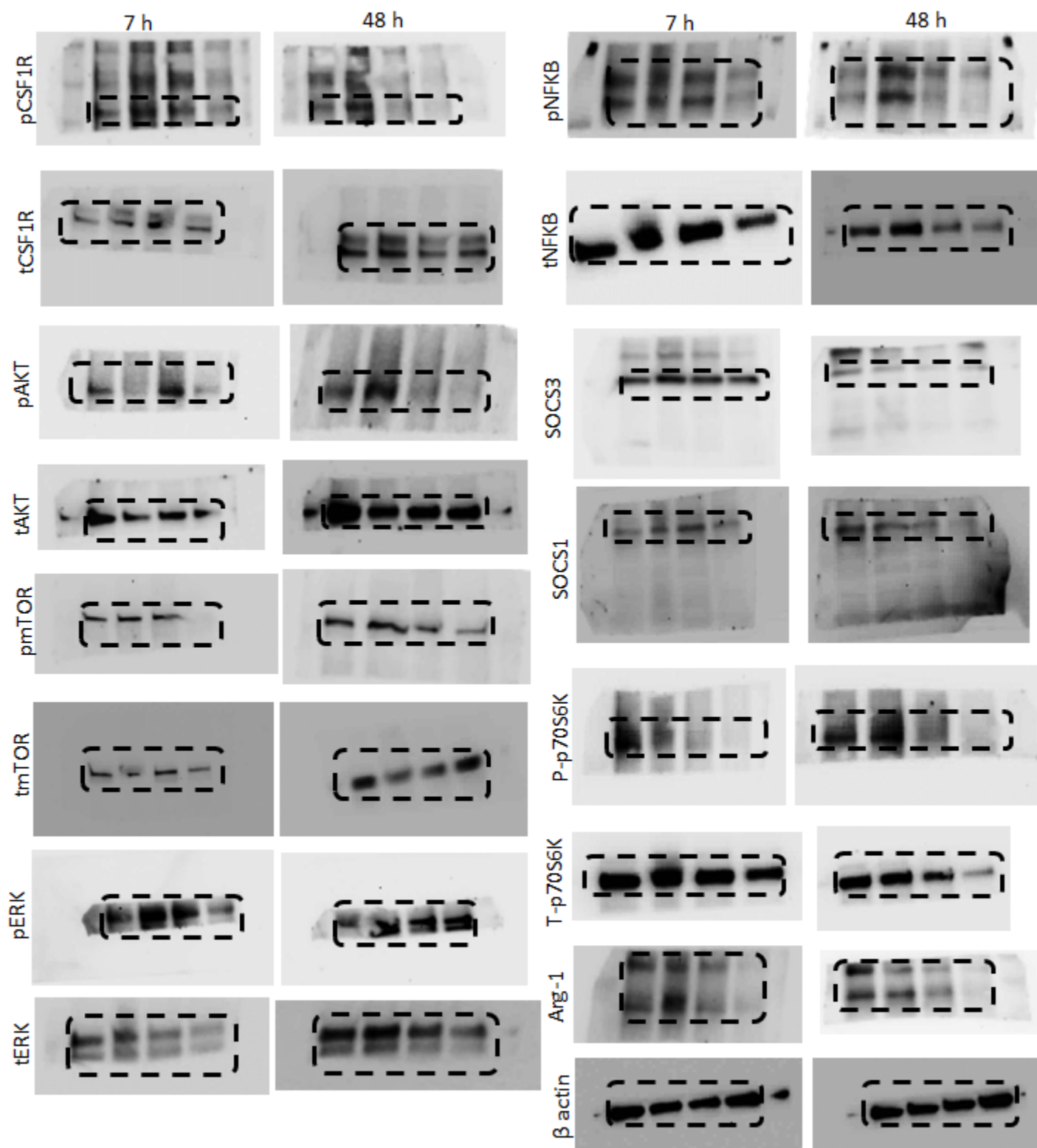
Suppl. Fig. 10. Preclinical characterization of anti-Sirpα-AK750. (a) Growth curves show effect of different multi-dose treatments on tumor volume in B16/F10 tumor bearing mice. Each animal was injected with two doses of either vehicle (for control group) or BLZ945 (45mg/kg) + anti-Sirpα antibody (5mg/kg), AK750 (molar equivalent to 45mg/kg dose of BLZ945) + anti-Sirpα IgG (5mg/kg), IgG-AK750 (molar equivalent to 45mg/kg dose of BLZ945) and anti Sirpα-AK750 (molar equivalent to 45mg/kg dose of BLZ945) on day 0 and day 2. First day of treatment was considered as Day 0. Data shown are mean ± SEM (n = 3, *P<0.05, ANOVA). (b) Graph shows plasma pharmacokinetic profile of AK750 and anti-Sirpα-AK750 in C57/BL6 mice. The graph shows plasma concentration of drug at different time points post injection. The mice were sacrificed and blood was collected through cardiac puncture at different time points after tail vein injection of AK750, anti Sirpα-Ak750 (mole equivalent to 15 mg/kg dose of BLZ945). The drug concentrations were quantified using LC/MS/MS. Data shown are mean ± SEM (n=3). (c) Table shows different pharmacokinetic parameters. (d) Representative cross sections of lungs and spleen (major RES organs), isolated from animals treated with different treatments, were stained for apoptosis (TUNEL) as a marker for systemic toxicity.



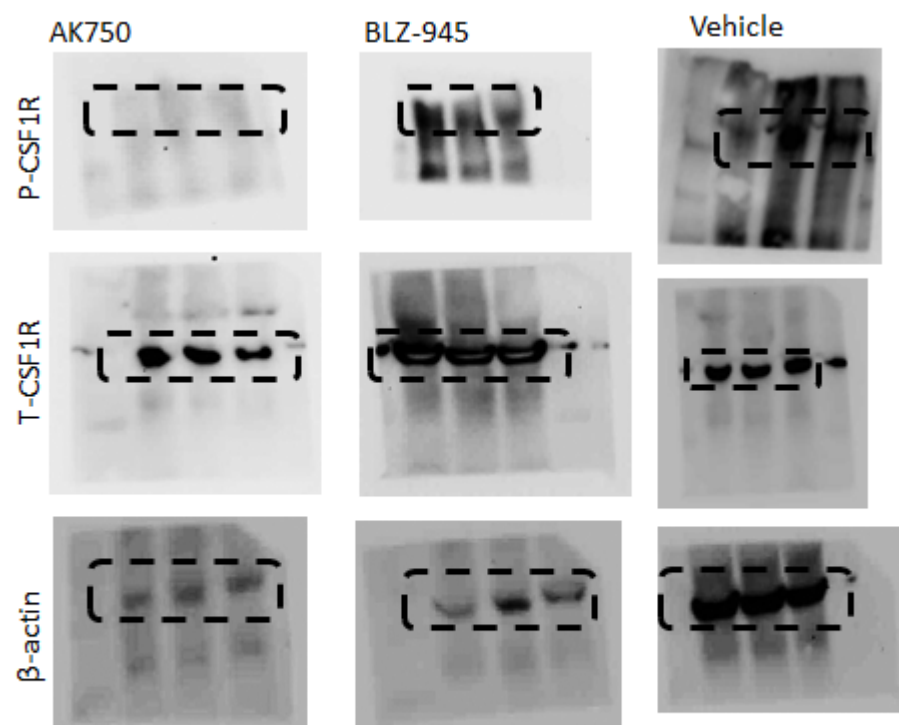
Suppl. Figure 11. (a) Graphs shows the concentration effect of AK750, anti-SIRP α AK750 and BLZ 945 on cell viability (left) and apoptosis (right) of T1 cells at 72h of treatment. Data shown are mean $\pm$ SEM. **(b)** Graphs shows the concentration effect of AK750, anti-SIRP α AK750 and BLZ 945 on cell viability (left) and apoptosis (right) of B16F10 at 72h of treatment. Data shown are mean $\pm$ SEM. **(c)** Graph shows quantification of apoptosis staining in M2-polarized Macrophages at 72h after treatment with different concentrations of AK750, anti-SIRP α AK750 and BLZ 945. Apoptotic cells were shown as % Annexin-V+ve cells quantified using FACS. The concentrations in different groups were all normalized to the molar equivalent of BLZ945. Data shown are mean $\pm$ SEM (n=3).



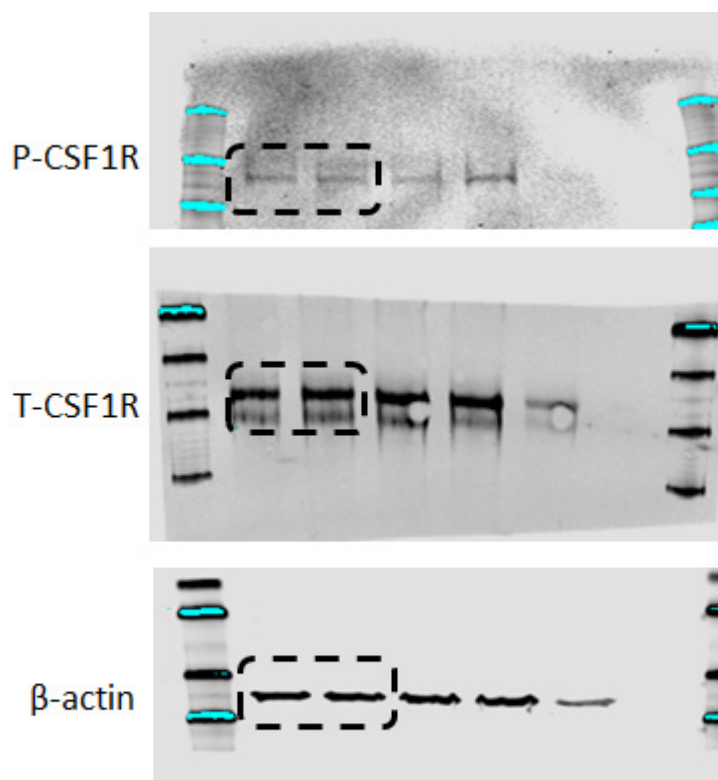
Suppl. Figure 12. The flow cytometry gating strategy.



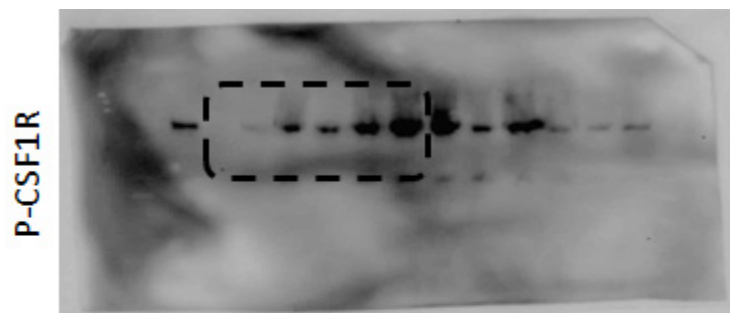
Suppl. Figure 13. Full length blots for Fig. 2c. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



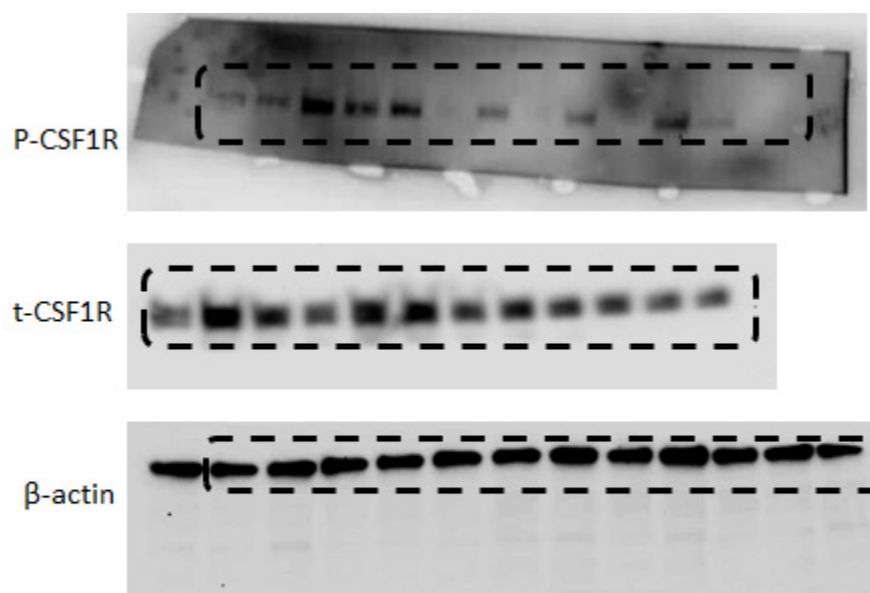
Suppl. Figure 14. Full length blots for Fig. 3e. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



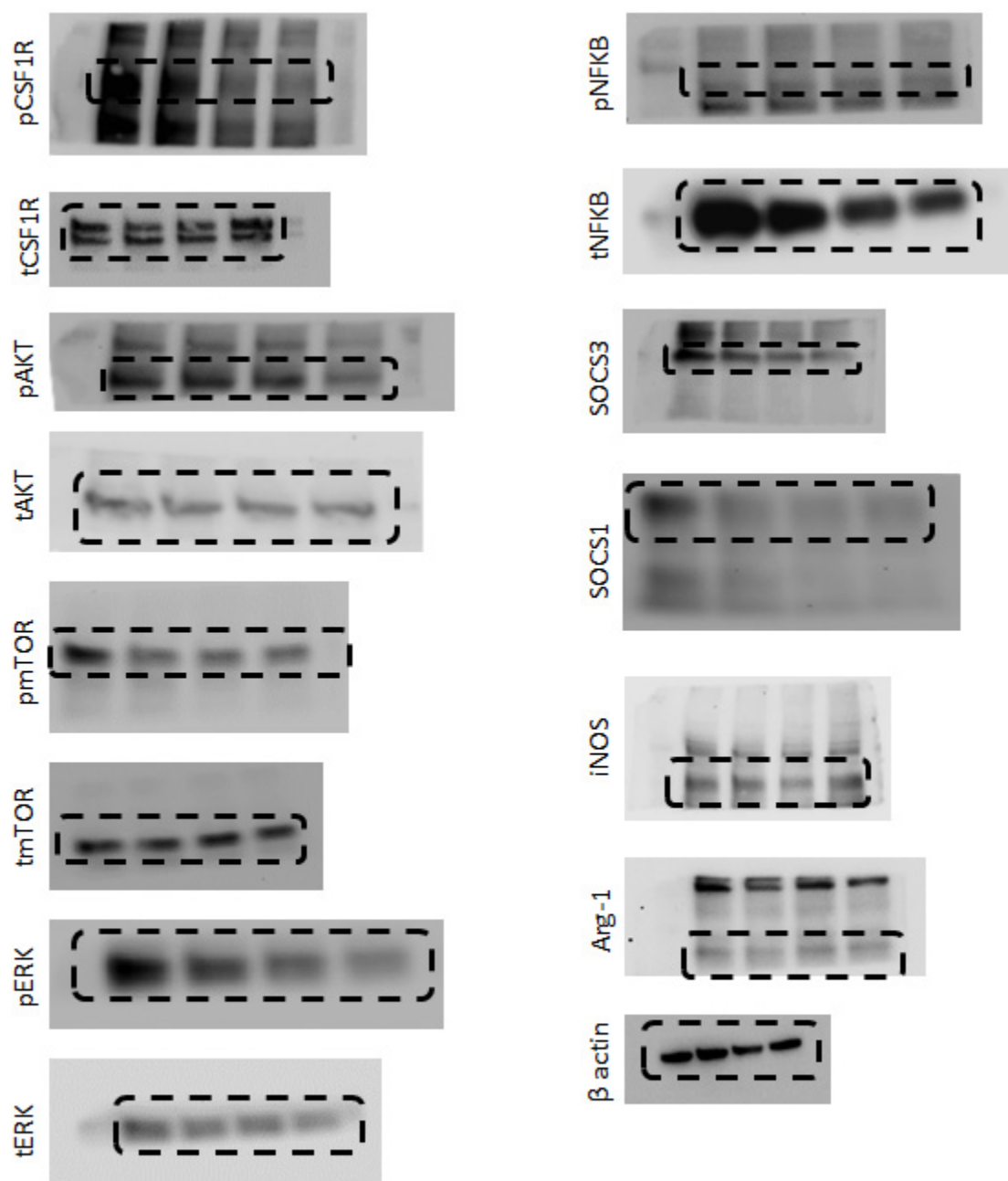
Suppl. Figure 15. Full length blots for Fig. 5j. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



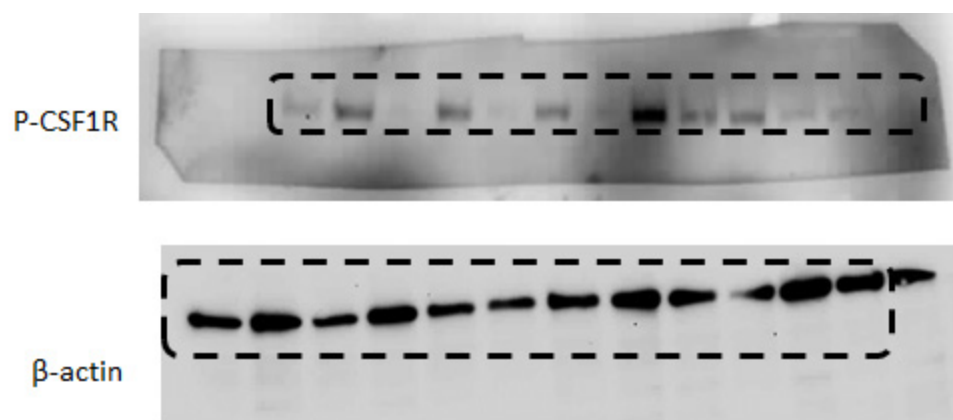
Suppl. Figure 16. Full length blots for Supp. Fig. 3a. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



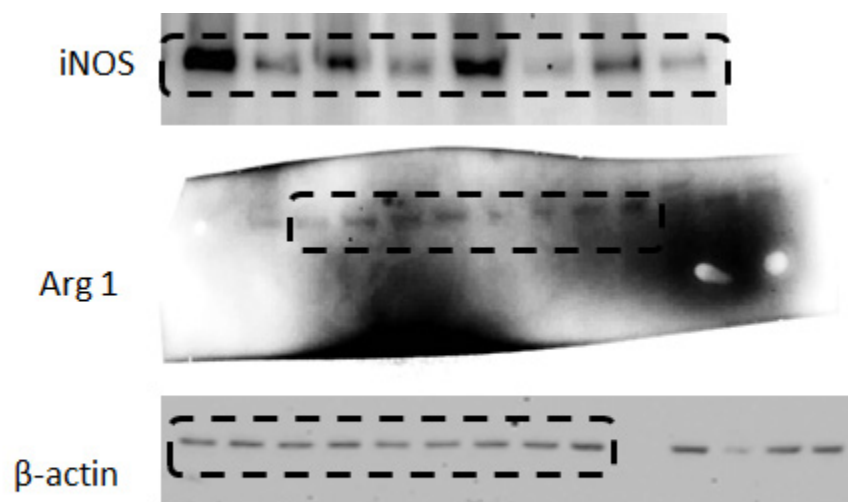
Suppl. Figure 17. Full length blots for Supp. Fig. 3c. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



Suppl. Figure 18. Full length blots for Supp. Fig. 4c. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



Suppl. Figure 19. Full length blots for Supp. Fig. 6b. Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.



Suppl. Figure 19. Full length blots for Supp. Fig. 6c Dotted lines show the cropped sections. Brightness was adjusted during processing of the blots.

Table 1. Antibodies Used for Flow Cytometry:

(Dilutions were used as per manufacturer's recommendations)

Antibody	Supplier name	Catalogue number	Clone name	Lot number	Selected Citations from the Supplier's Website
CD45	BioLegend	103225	RA3-6B2	B200232	1. George A, <i>et al.</i> 1994. <i>J. Immunol.</i> 152:1014. 2. Asensi V, <i>et al.</i> 1989. <i>Immunology</i> 68:204. 3. Domiati-Saad R, <i>et al.</i> 1993. <i>J. Immunol.</i> 151:5936.
F4-80	BioLegend	123120	BM8	B193622	1. Kobayashi M, <i>et al.</i> 2008. <i>J. Leukoc. Biol.</i> 83:1354. 2. Poeckel D, <i>et al.</i> 2009. <i>J. Biol Chem.</i> 284:21077. 3. Glass AM, <i>et al.</i> 2013. <i>J. Immunol.</i> 190:4830.
CD206	BioLegend	141707	C068C2	B190965	1. Keller J, <i>et al.</i> 2012. <i>Biochem Biophys Res Commun.</i> 417:217. 2. Ito H, <i>et al.</i> 2012. <i>J Am Soc Nephrol.</i> 23:1797. 3. Taguchi K, <i>et al.</i> 2014. <i>J Am Soc Nephrol.</i> 25:1680. 4. Canan CH, <i>et al.</i> 2014. <i>J Leukoc Biol.</i> 96:473. Giordano C, <i>et al.</i> 2015. <i>Hum Mol Genet.</i>
CD11b	BioLegend	101212	M1/70	B243489	1. Springer T, <i>et al.</i> 1978. <i>Eur. J. Immunol.</i> 8:539. 2. Springer TA, <i>et al.</i> 1982. <i>Immunol. Rev.</i> 68:171. (Block) 3. Flotte TJ, <i>et al.</i> 1983. <i>Am. J. Pathol.</i> 111:112.
Ly6c	BioLegend	128005	HK1.4	B195147	1. Jutila MA, <i>et al.</i> 1988. <i>Eur. J. Immunol.</i> 18:1819. 2. Havran WL, <i>et al.</i> 1988. <i>J. Immunol.</i> 140:1034 3. Makaroff LE, <i>et al.</i> 2009. <i>P. Natl. Acad. Sci. USA</i> 106:4799.
CD86	BioLegend	105110	PO3	B179311	1. Nakajima A, <i>et al.</i> 1995. <i>Eur. J. Immunol.</i> 25:3060. 2. Nuriya S, <i>et al.</i> 1996. <i>Int. Immunol.</i> 8:917. 3. Kasprovicz DJ, <i>et al.</i> 2000. <i>J. Immunol.</i> 165:680. 4. Saito K, <i>et al.</i> 1998. <i>J. Immunol.</i> 160:4225.
CD80	BioLegend	104706	16-10A1	B207221	1. Harlan DM, <i>et al.</i> 1994. <i>P. Natl. Acad. Sci. USA</i> 91:3137. 2. Hathcock KS, <i>et al.</i> 1994. <i>J. Exp. Med.</i> 180:631. 3. Herold KC, <i>et al.</i> 1997. <i>J. Immunol.</i> 158:984. 4. Ma XT, <i>et al.</i> 2006. <i>Cancer Res.</i> 66:1169.
MHC2	BioLegend	107605	M5/114.15.2	B199709	1. Bhattacharya A, <i>et al.</i> 1981. <i>J. Immunol.</i> 127:2488. (IP) 2. Viville S, <i>et al.</i> 1993. <i>Cell</i> 72:635. (IHC) 3. Nelson AJ, <i>et al.</i> 1993. <i>J. Immunol.</i> 151:2453. (IHC) 4. Shi Y, <i>et al.</i> 1998. <i>J. Exp. Med.</i> 187:367. (Block) 5. Yamashita I, <i>et al.</i> 1993. <i>Int. Immunol.</i> 5:1139.

Table 2. Antibodies Used for Western Blot Studies:

Antibody	Supplier name	Catalogue number	Clone name	Lot number	Selected Citations from the Supplier's Website
SOCS3 (1:1000 dilution)	Cell Signalling Technology (CST)	2923S	NA	2	1. Alexander, W.S. et al. (1999) J Leukoc Biol 66, 588–92. 2. Chen, X.P. et al. (2000) Immunity 13, 287–90.
SOCS1 (A156) (1:1000 dilution)	CST	3950S	NA	1	1. Alexander, W.S. et al. (1999) J Leukoc Biol 66, 588–92. 2. Starr, R. et al. (1997) Nature 387, 917–21. 3. Endo, T.A. et al. (1997) Nature 387, 921–4
Arginase 1 (1:1000 dilution)	CST	9819S	NA	3	1. Rodriguez, P.C. et al. (2004) Cancer Res 64, 5839–49. 2. Wu, G. and Morris, S.M. (1998) Biochem J 336 (Pt 1), 1–17.
P-Akt (S473) (D9E) (1:1000 dilution)	CST	2965S	NA	14	1. Franke, T.F. et al. (1997) Cell 88, 435–7. 2. Burgering, B.M. and Coffey, P.J. (1995) Nature 376, 599–602. 3. Sarbassov, D.D. et al. (2005) Science 307, 1098–101.
Akt (pan) (11E7) (1:1000 dilution)	CST	4685S	NA	3	1. Franke, T.F. et al. (1997) Cell 88, 435–7. 2. Burgering, B.M. and Coffey, P.J. (1995) Nature 376, 599–602. 3. Sarbassov, D.D. et al. (2005) Science 307, 1098–101
β- Actin (1:2000 dilution)	CST	4970S	NA	14	1. Lim, Y. P. et al. (2004) Clin. Cancer Res. 10, 3980–3987. 2. Kayalar, C. et al. (1996) Proc. Natl. Acad. Sci. USA. 93, 2234–2238.
iNOS (D6B6S) (1:1000 dilution)	CST	13120S	NA	1	1. Son, H. et al. (1996) Cell 87, 1015–10123. 2. Hawkins, R.D. (1996) Neuron 16, 465–467. 3. Bogdan, C. (2001) Nat Immunol 2, 907–16
P-mTOR (Ser2448) (D9C2) (1:1000 dilution)	CST	5536S	NA	2	1. Brown, E.J. et al. (1994) Nature 369, 756–8 2. Sabatini, D.M. et al. (1994) Cell 78, 35–43. 3. Dennis, P.B. et al. (2001) Science 294, 1102–5.
mTOR 7C10 (7C10) (1:1000 dilution)	CST	2983S	NA	14	1. Sabers, C.J. et al. (1995) J. Biol. Chem. 270, 815–822. 2. Brown, E.J. et al. (1994) Nature 369, 756–758. 3. Sabatini, D.M. et al. (1994) Cell 78, 35–43. 4. Dennis, P.B. et al. (2001) Science 294, 1102–1105.
P-NF-kappaB p65 (S536) (1:1000 dilution)	CST	3031S	NA	10	1. Baeuerle, P.A. and Baltimore, D. (1996) Cell 87, 13–20. 2. Thompson, J.E. et al. (1995) Cell 80, 573–582.

NF-kappaB p65 (D14E12) (1:1000 dilution)	CST	8242S	NA	4	1. Baeuerle, P.A. and Baltimore, D. (1996) Cell 87, 13-20. 2. Haskill, S. et al. (1991) Cell 65, 1281-9. 3. Thompson, J.E. et al. (1995) Cell 80, 573-82.
P-Erk 1/2 p44/42 MAPK (3A7) (1:1000 dilution)	CST	9102S	NA	23	1. Meloche, S. and Pouyssegur, J. (2007) Oncogene 26, 3227-39. 2. Roberts, P.J. and Der, C.J. (2007) Oncogene 26, 3291-310.
Erk 1/2 p44/42 MAPK (3A7) (1:1000 dilution)	CST	9107S	NA	7	1. Meloche, S. and Pouyssegur, J. (2007) Oncogene 26, 3227-39. 2. Roberts, P.J. and Der, C.J. (2007) Oncogene 26, 3291-310.
P-M-CSFR (Y723) (49C10) (1:1000 dilution)	CST	3155S	NA	9	1. Byrne, P.V. et al. (1981) J. Cell. Biol. 91, 848-853. 2. Bourette, R.P. et al. (2000) Growth Factors 17, 155-166. 3. Bourette, R.P. et al. (1997) EMBO J. 16, 5880-5893
M-CSFR (Y723) (49C10) (1:1000 dilution)	CST	3152S	NA	4	1. Byrne, P.V. et al. (1981) J. Cell. Biol. 91, 848-853. 2. Bourette, R.P. et al. (2000) Growth Factors 17, 155-166. 3. Bourette, R.P. et al. (1997) EMBO J. 16, 5880-5893.
P-p70 S6 K (S371) (1:1000 dilution)	CST	9208	NA	3	1. Dufner, A. and Thomas, G. (1999) Exp. Cell Res. 253, 100-109. 2. Weng, Q.P. et al. (1998) J. Biol. Chem. 273, 16621-16629. 3. Pullen, N. et al. (1998) Science 279, 707-710.
p70 S6 K (S371) (1:1000 dilution)	CST	2708S	NA	7	1. Dufner, A. and Thomas, G. (1999) Exp. Cell Res. 253, 100-109. 2. Weng, Q.P. et al. (1998) J. Biol. Chem. 273, 16621-16629. 3. Pullen, N. et al. (1998) Science 279, 707-710.