iScience, Volume *23*

Supplemental Information

Interleukin-10 and Small Molecule SHIP1 Allosteric Regulators Trigger Anti-inflammatory Effects through SHIP1/STAT3 Complexes

Thomas C. Chamberlain, Sylvia T. Cheung, Jeff S.J. Yoon, Andrew Ming-Lum, Bernd R. Gardill, Soroush Shakibakho, Edis Dzananovic, Fuqiang Ban, Abrar Samiea, Kamaldeep Jawanda, John Priatel, Gerald Krystal, Christopher J. Ong, Artem Cherkasov, Raymond J. Andersen, Sean A. McKenna, Filip Van Petegem, and Alice L-F. Mui

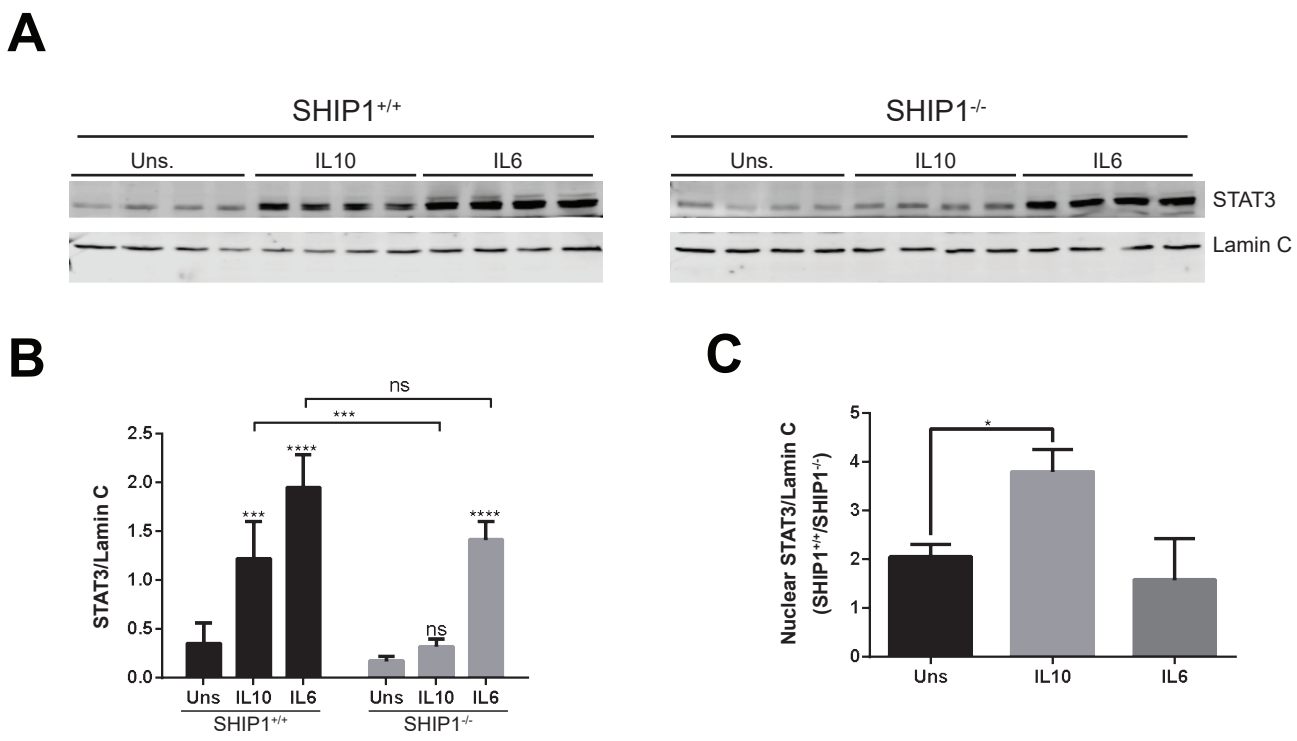


Figure S1,

IL10, but not IL6, induced nuclear translocation of STAT3 is diminished in SHIP1$^{-/-}$ cells, Related to Figure 4.
(A) J16 SHIP1$^{+/+}$ and J17 SHIP1$^{-/-}$ cells were stimulated with 1 ng/mL IL10 or IL6 for 15 minutes before lysing cells and the isolating nuclear fractions for immunoblot detection of STAT3 and Lamin C.
(B) STAT3 expression in the nuclear extracts, normalized to the detected level of nuclear envelop protein Lamin C. **** p<0.0001, *** p<0.001, * p<0.05, when comparing treatments (Two-way ANOVA with Tukey's correction). **(C)** The fold change in STAT3 nuclear expression in SHIP1$^{+/+}$/SHIP1$^{-/-}$ cells in response to IL10 or IL6. * p<0.05, comparisons calculated by One-way ANOVA with Tukey's correction.

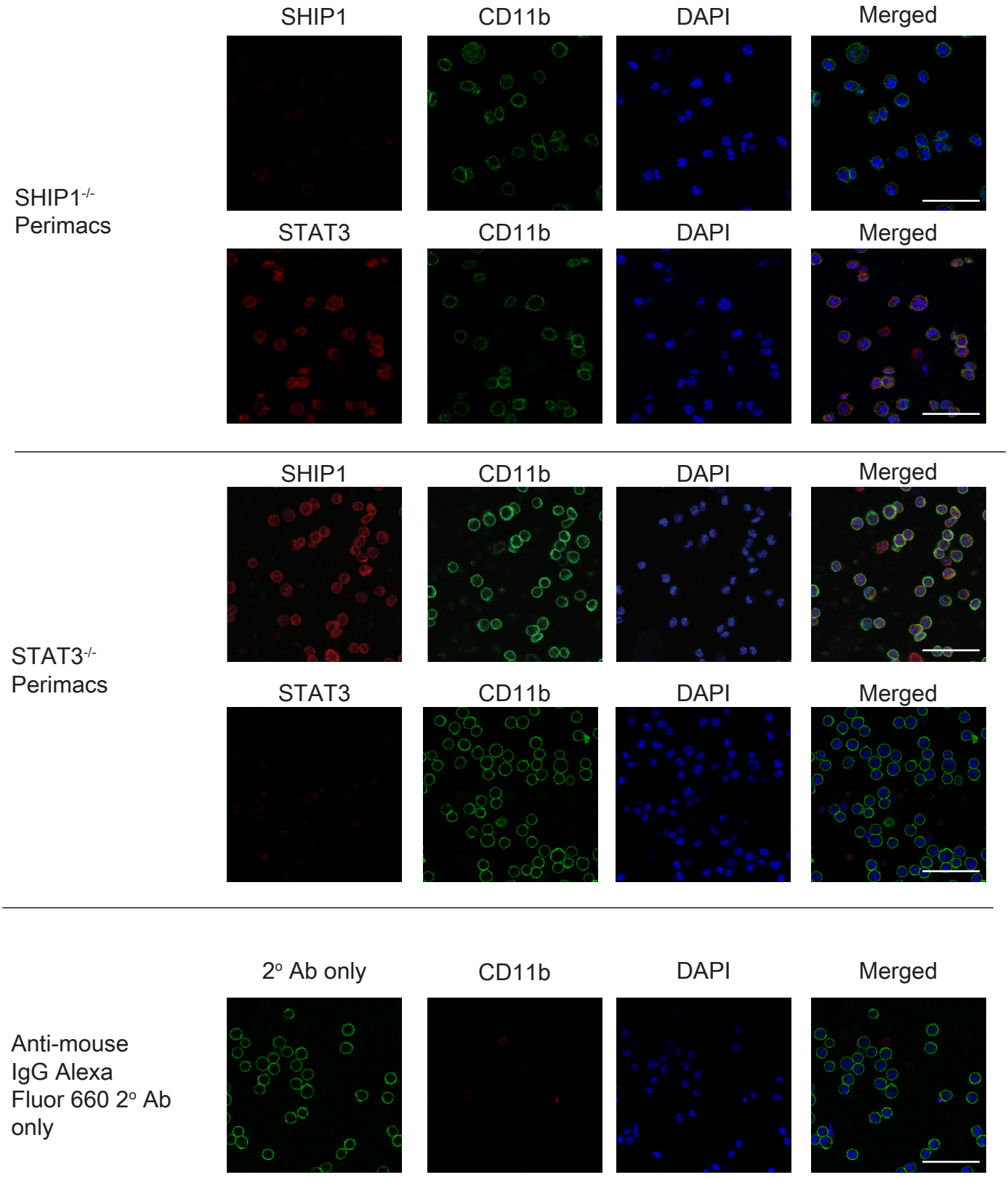
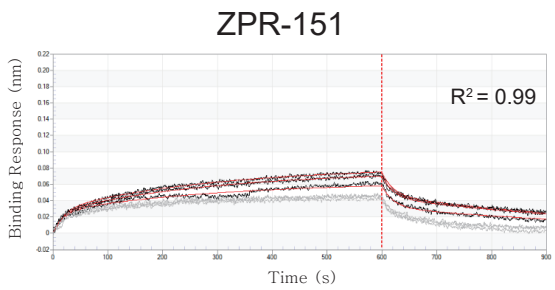


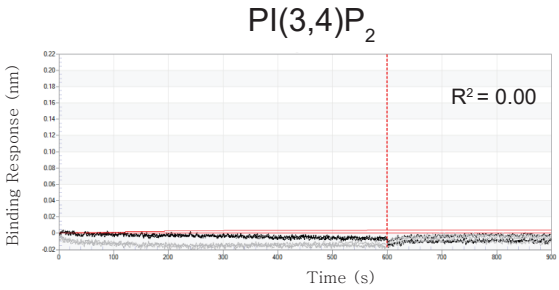
Figure S2,

SHIP1 and STAT3 immunofluorescence staining controls, Related to Figure 4.

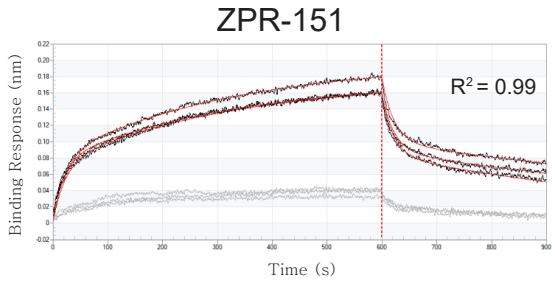
Representative confocal microscope images of unstimulated SHIP1$^{-/-}$ and STAT3$^{-/-}$ perimacs stained with anti-SHIP1 and anti-STAT3, CD11b antibodies and DAPI. Representative images of perimacs stained with anti-mouse IgG Alexa-Fluor 660 secondary antibody without anti-SHIP1/STAT3 primary antibody staining. Scale, 50 μM.

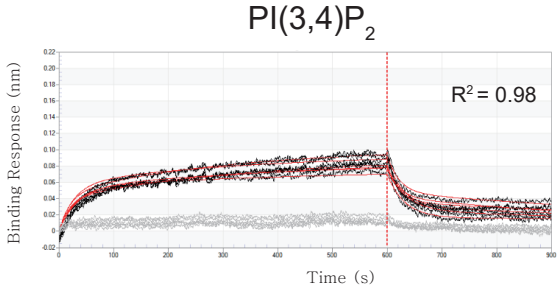
A K681A PAC2

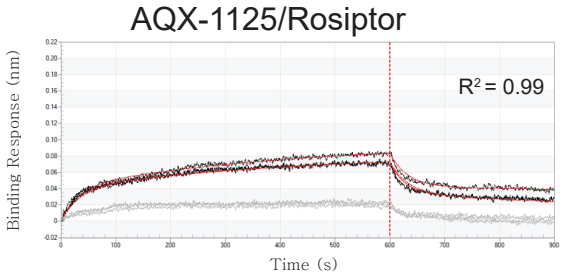


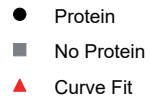


B WT PAC2









C

Protein	Compound	Replicates	k_{on} ($M^{-1}s^{-1}$)		k_{off} (s^{-1})	
			Mean	Std. Error	Mean	Std. Error
WT	ZPR-151	3	147	46.2	0.062	0.000749
WT	PI(3,4)P_2	3	42.6	21.9	0.0288	0.000435
WT	AQX-1125	3	65.5	53.4	0.0485	0.000943
K681A	ZPR-151	3	69.7	65.2	0.0477	0.00116
K681A	PI(3,4)P_2	3	250	1.163	<1.0E-07	

Figure S3

Bio-layer interferometry (BLI) curves from WT and K681A PAC2 loaded BLI sensors exposed to 20 mM of ZPR-151, PI(3,4)P_2 or AQX-1125/Rosiptor, **Related to Figures 6 and 7. (A)** Representative BLI curves for binding of 20 μM ZPR-151 and PI(3,4)P_2 to K681A PAC2. **(B)** Representative BLI curves for binding of 20 μM ZPR-151, AQX-1125 and PI(3,4)P_2 to wild-type (WT) PAC2. The black and gray lines (n=3) show the observed binding curves to PAC2 and no protein loaded biosensors respectively. The red lines show the curve fit of each black line. The curves are fitted to 2:1 heterogeneous ligand model indicating possibility the protein is heterogeneous (FortéBio. Technical Note 16: Small Molecule Binding Kinetics. https://www.fortebio.com/literature.html) **(C)** Representative of k_{on} and k_{off} values obtained from the fit curves.

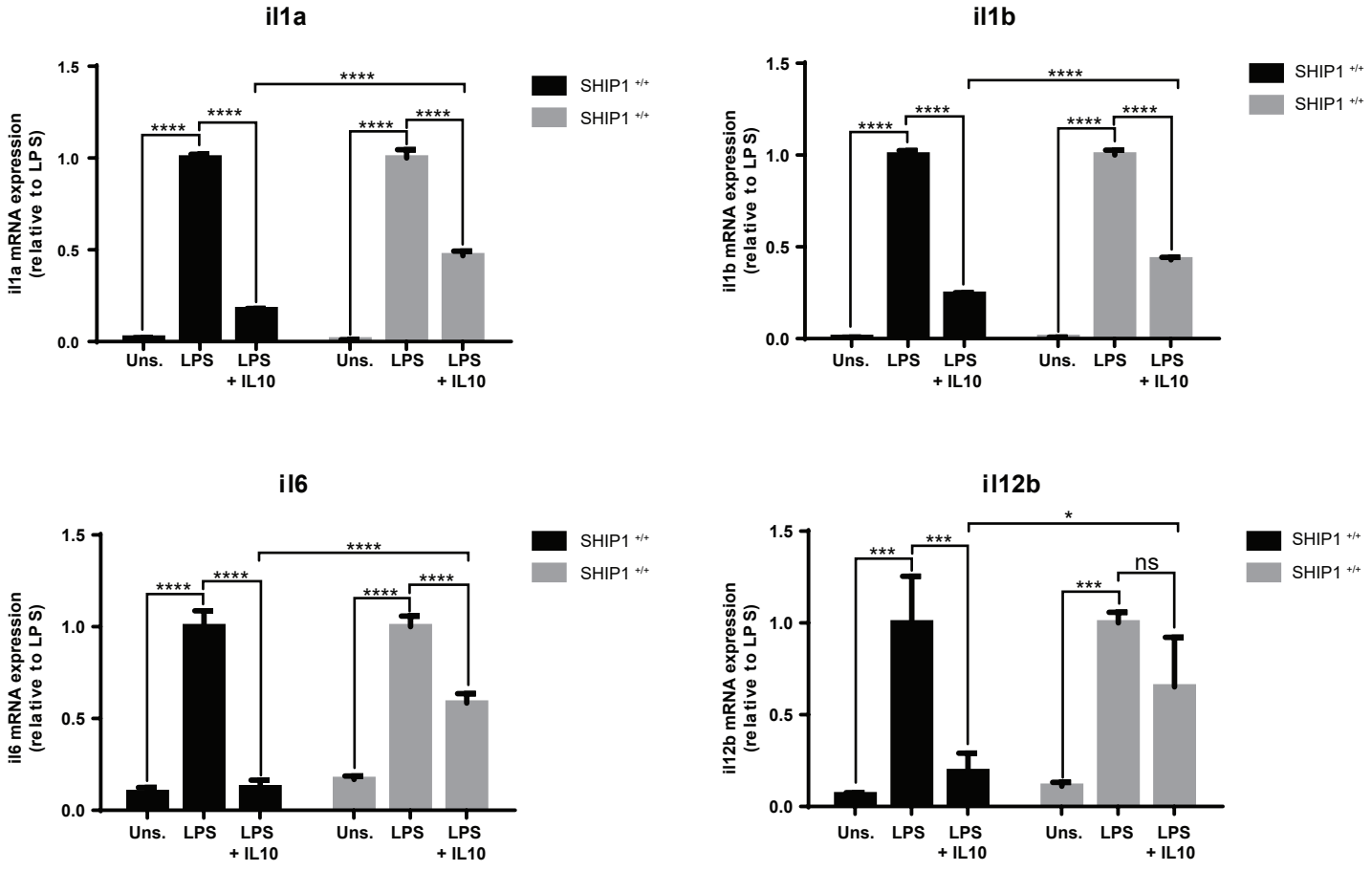


Figure S4

IL10 inhibition of IL1, IL6 and IL12β mRNA expression is diminished in SHIP1$^{-/-}$ cells. Related to Figure 1. SHIP1$^{+/+}$ and SHIP1$^{-/-}$perimacs were treated with LPS (1 ng/mL) ± IL10 (10 ng/mL) for 6 hours and RNA was extracted. The expression of the indicated cytokine mRNA was determined by qPCR. **** p<0.0001, *** p<0.001, * p<0.05, ns = not significant, comparisons between the treatments calculated by Two-way ANOVA with Tukey's correction.

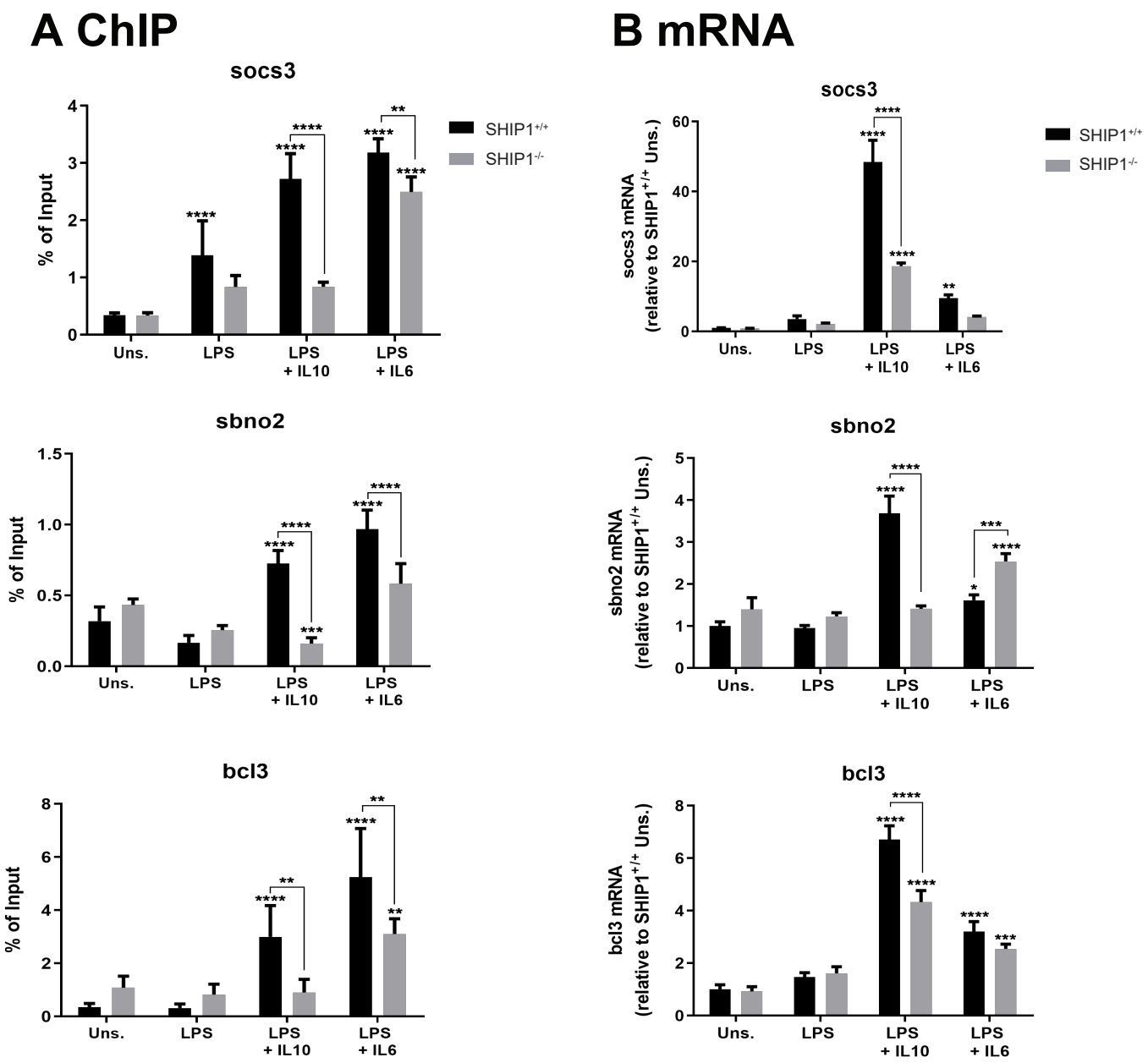


Figure S5

STAT3 recruitment to promoters of IL10 and IL6 regulated genes. Related to Figure 4.
(A) Evaluation, by chromatin immunoprecipitation (ChIP) of STAT3 recruitment to the socs3, sbno2 and bcl3 promoters of J16 SHIP1$^{+/+}$ and J17 SHIP1$^{-/-}$ cells stimulated with LPS (1 ng/mL) ± IL10 or IL6 (1 ng/mL) for 20 mins. Co-immunoprecipitated DNA was expressed as precent of the total input.

(B) mRNA expression, of the corresponding genes, in SHIP1$^{+/+}$ and $^{-/-}$ perimacs stimulated with LPS (1 ng/mL) ± IL6 (1 ng/mL) for 1 hour. Data is normalized to GAPDH and expressed relative to the unstimulated (Uns.) SHIP1$^{+/+}$cells.

**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05 comparison between treatments were calculated by Two-way ANOVA with Tukey's correction.

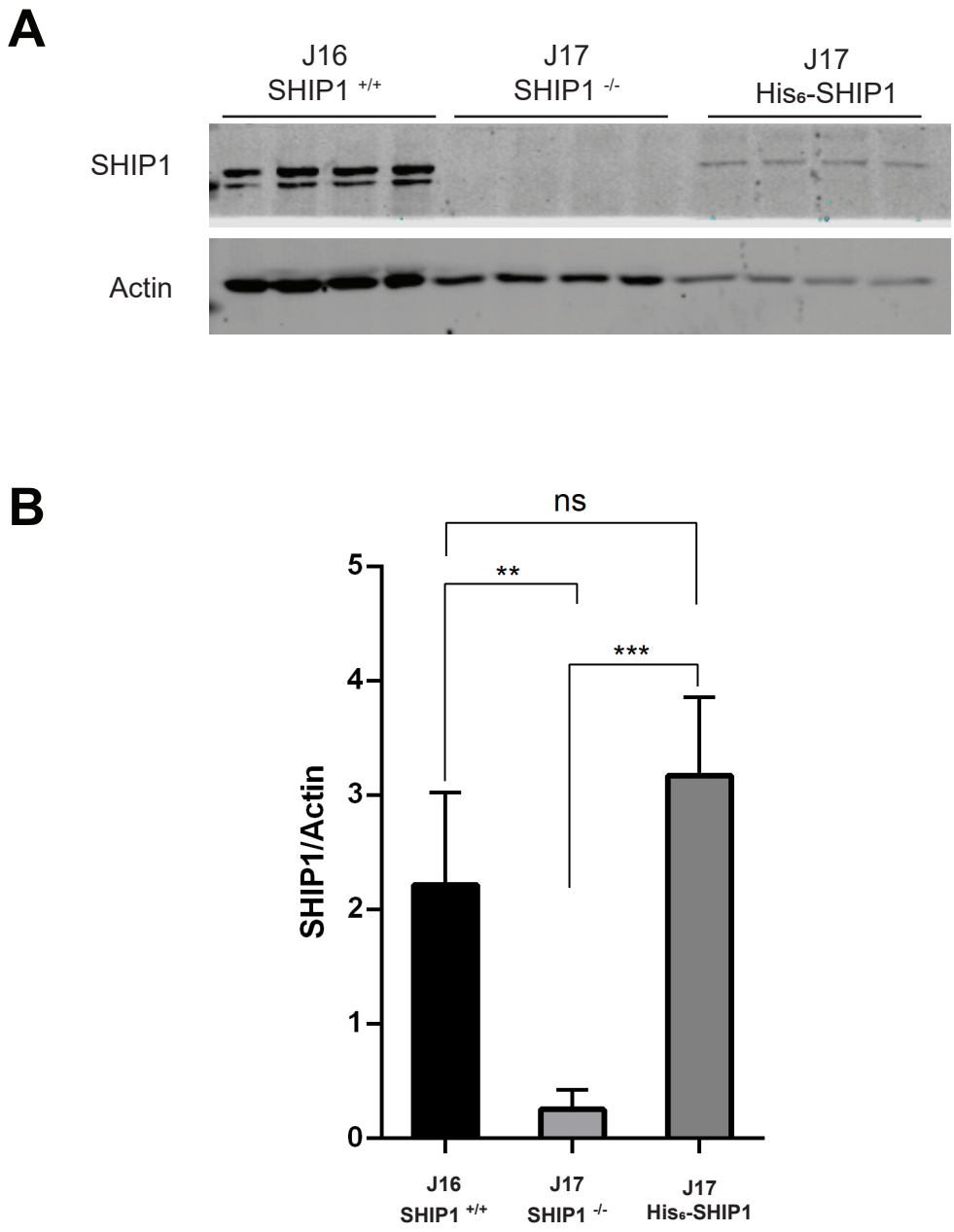


Figure S6

Relative Expression of endogenous wild-type SHIP1 and the transduced His_6-SHIP1 proteins. Related to Figures 2 and 3. Lysates from J16 $SHIP1^{+/+}$, J17 $SHIP1^{-/-}$ and J17 $SHIP1^{-/-}$ cells reconstituted with His_6-SHIP1 were collected. **(A)** Expression of endogenous SHIP1 and transduced His-SHIP1 level was detected by immunoblotting. **(B)** Quantification of SHIP1 protein level normalized to actin. (One Way ANOVA, *** $p < 0.001$,** $p < 0.01$, ns = not significant).

Protein sample	SHIP1 PAC2-cc
PDB ID	6DLG
Data collection	APS 23ID-D
Wavelength (Å)	1.03319
Space group	P 21 21 21
Unit cell parameters (Å)	a=45.10, b=73.20, c=124.21
Unit cell angles (°)	α=90.0, β= 90.0 γ= 90.0
Resolution (Å)*	47.36-1.50 (1.59-1.50)
R_{meas} (%)	5.9 (78.7)
$CC_{1/2}$ (%)	99.9 (68.2)
I / σ(I)	13.79 (1.85)
Completeness (%)	98.9 (98.6)
No. unique reflections	66070 (10528)
Redundancy	3.28 (3.29)
Wilson B factor (Å^2)	25.38
Refinement	Phenix 1.13-2998
Resolution (Å)	38.4-1.50
Solvent content (%)	38
R_{work} / R_{free} (%)	17.85/20.04
Ramachandran plot	
favoured / outlier (%)	99.3 / 0.2
No. of atoms	
Protein	3628
Isopropanol	4
Water	246
B-factors (Å^2)	
Protein	24
Isopropanol	26.6
Water	30.9
RMSD bond lengths (Å)	0.008
RMSD bond angles (°)	0.966

*Values of the highest resolution shell are listed within parentheses

Table S1

Related to Figure 6. Protein crystallization data collection and refinement statistics

TRANSPARENT METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse colonies. BALB/c mice wild type ($^{+/+}$) or SHIP1 knockout ($^{-/-}$) mice were provided by Dr. Gerald Krystal (BC Cancer Research Centre, Vancouver, BC). The generation of STAT3$^{-/-}$ mice started with crossing C57BL/6 STAT3 flox/flox mice (Dr. Shizuo Akira, Hyogo College of Medicine, Nishinomiya, Japan) with C57BL/6 LysMcre mice (Jackson Laboratory). Their offspring were then crossed with homozygous STAT3 flox/flox mice to produce to generate both STAT3 flox/flox /LysMCre$^{+/-}$ mice (referred to be STAT3$^{-/-}$ mice) and STAT3 flox/flox mice (STAT3$^{+/+}$ mice) in the same litters. All mice were maintained in accordance with the ethic protocols approved by the University of British Columbia Animal Care Committee.

Constructs. The mammalian (lentiviral) expression plasmids of SHIP1 in FUGWBW were generated using Gateway LR reactions from pENTR1A (Invitrogen, Burlington, ON) constructs. A pENTR1A-His$_6$-SHIP1 WT (SHIP1 Uniprot ID Q9ES52) plasmid was used as the template for standard primer based, site-directed mutagenesis to generate the K681A, Y190F, Y799F, Y659F and Y657F mutants. The phosphatase disrupted SHIP1 construct (P671A, D675A, and R676G in the phosphatase domain) was kindly provided by Dr. KS Ravichandran (University of Virginia). The constructs were confirmed by DNA sequencing. Subsequently, a Gateway LR reaction was performed between pENTR1A construct and FUGWBW (FUGW in which the green fluorescent protein was replaced by the Gateway cassette, and a blasticidin S resistance gene expression cassette was inserted downstream of the Gateway cassette (Peacock et al., 2009). Success of the LR reaction was confirmed by restriction enzyme digest. For Clover/mRuby2 based FRET experiments (Lam et al., 2012), pENTR1A Clover-SHIP1 was constructed by inserting a Clover fragment from pcDNA3 Clover (Addgene) to the N-terminal of SHIP1 in pENTR1A-His$_6$-SHIP1 WT, replacing the His$_6$.pENTR1A STAT3-mRuby2 was constructed by cloning murine STAT3 (Uniprot ID P42227) into pENTR1A followed by insertion of a mRuby2 fragment from pcDNA3 mRuby2 (Addgene) to the N-terminus of STAT3. Constructs were confirmed by sequencing and transferred to FUGWBW as above.

Bacteria expression vectors to produce recombinant proteins for crystallography and biolayer interferometry were generated by ligase-independent cloning (LIC) methodology in the LIC-HMT vector (Van Petegem et al., 2004). This plasmid contains an N-terminal tag composed of His$_6$ and maltose binding protein (MBP), followed by a TEV protease cleavage site (abbreviated as the HMT-tag). The PCR product was purified and treated with T4 DNA polymerase (LIC-quality) (Novagen, Madison, WI) in the presence of dCTP only. The LIC-HMT vector was digested with *Ssp*I and the linearized plasmid was treated with T4 DNA polymerase in the presence of dGTP only. Equal volumes of insert and vector were mixed and incubated at room temperature for 10 minutes, followed by transformation into chemical competent *E. coli DH5*α cells using the standard heat shock protocol, and selection on kanamycin-containing LB agar plates. To generate different PAC2 mutants, standard site-directed mutagenesis was employed. Identities of all plasmids were confirmed by DNA sequencing.

Cell lines. J16 and J17 cell lines derived from SHIP1$^{+/+}$ and $^{-/-}$ BMDM respectively were previously described (Ming-Lum et al., 2012) and cultured in Mac media (IMDM supplemented with 10% (v/v) FCS, 10 µM β-mercaptoethanol, 150 µM monothioglycolate and 1 mM L-glutamine). J17 cells expressing wild type and

mutant His_6-SHIP1, Clover-SHIP1 or mRuby2-STAT3 constructs were generated by lentivirus mediated gene transfer as described (Cheung et al., 2013). Transduced cells were selected with 5 µg/ml blasticidin. Clover-SHIP1 and mRuby2-STAT3 cells were further subjected to fluorescent activated cell sorting to select the brightest cells on a FACS Aria II cytometer.

Isolation of mouse peritoneal macrophages. Primary peritoneal macrophages (perimacs) were isolated from mice by peritoneal lavage with 3 ml of sterile Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, Nepean, ON). Perimacs were collected and transferred to Mac media.

Production of bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were generated by first collecting femurs and tibias from mice, and then flushing out the bone marrow through a 26-G needle. Extracted cells were plated, in Mac media supplemented with 5 ng/ml each of CSF-1 and GM-CSF (Stem Cell Technologies, Vancouver, BC), on a 10-cm tissue culture plate for 2 hours at 37°C. Non-adherent cells were collected and replated at 9×10^6 cells per 10-cm tissue culture plate. Cells were then cultured in the presence of CSF-1 and GM-CSF. Differentiated BMDMs were used after 7 to 8 days. All cells were maintained in a 37°C, 5% CO2, 95% humidity incubator.

METHOD DETAILS

Continuous Flow Cultures. The continuous flow apparatus facilitates constant stimulation and removal of cell supernatants to determine kinetic profiles of cytokine production over time. BMDMs were seeded at $3x10^5$ cells per well in a 24-well tissue culture plate that had been coated with poly-L-lysine (Thermo Fisher Scientific, Nepean, ON) and rinsed with PBS. After overnight incubation, culture media was removed and Leibovitz's L-15 (L-15) media (Invitrogen, Burlington, ON) supplemented with 3% FCS, 10 µM β-mercaptoethanol and 150 µM monothioglycolate was added. Cells were allowed to equilibrate in L-15 media for 1 hour before being placed in the continuous flow apparatus. Stimulation solution was made in the same media equilibrated at 37°C, and was passed through a modified inlet fitted to the well by a syringe pump (New Era Syringe Pumps Inc., Farmingdale, NY). A flow rate of 150 µl per minute was used. At the same time, cell supernatants were removed from the well at the same flow rate, and fractions were collected at 5-minute intervals over the course of 3 hours. These fractions were analyzed for secreted TNFα levels by ELISA.

Real-time quantitative PCR. . Total RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON) according to manufacturer's instructions. About 2-5 µg of RNA were treated with DNAseI (Roche Diagnostics, Laval, QC) according to the product manual. For mRNA expression analysis, 120 ng of RNA were used in the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Laval, QC), and 0.1 µl to 0.2 µl of cDNA generated were analyzed by SYBR Green-based real time PCR (real time-PCR) (Roche Diagnostics, Laval, QC) using 300 nM of gene-specific primers . Expression levels of mRNA were measured with the StepOne Plus RT-PCR system (Applied Biosystems, Burlington, ON), and the comparative Ct method was used to quantify mRNA levels using GAPDH as the normalization control.

Measurement of TNFα production. Cells were seeded at 50 x 10^4 cells per well in a 96-well tissue culture plate and allowed to adhere overnight. Media was changed the next day 1 hour prior to stimulation. Cells were stimulated with 1 or 10 ng/ml LPS +/- various concentrations of IL10 for 1 hour. Supernatant was collected and secreted TNFα protein levels were measured using a BD OptEIA Mouse TNFα Enzyme-Linked Immunosorbent Assay (ELISA) kit (BD Biosciences, Mississauga, ON). Triplicates wells were used for each stimulation condition. IC50 values were calculated from three independent experiments and differences in IL10 IC50 values from cells expressing SHIP1 mutants vs SHIP1 WT protein were analyzed by one-way ANOVA.

***In vitro* phosphatase assay.** The phosphatase assay was performed in 96-well microtiter plates with 10 ng of enzyme/well in a total volume of 25 μL in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20, 10 mM $MgCl_2$ as described (Ong et al., 2007). Enzyme was incubated with or ZPR-MN100 (dissolved in ethanol) for 10 minutes at 23°C, before the addition of 50 μM of inositol-1,3,4,5-tetrakisphosphate (IP_4) (Echelon Bioscience Inc., Salt Lake City, Utah). The reaction was allowed to proceed for 10 minutes at 37°C and the amount of inorganic phosphate released was assessed by the addition of Malachite Green reagent and absorbance measurement at 650 nm. For enzyme kinetic determination, different concentrations of IP_4 (0 – 300 μM) were used and the reactions were stopped at different time points. Initial velocities were calculated, and K_{cat} and K_M were determined using GraphPad 6 software.

***In vitro* pull down assay.** J17 His_6-SHIP1 and Y190F cells were seeded at 2 x 10^6 cells per well in a 6-well plate. After overnight incubation, fresh cell media was added for 30 minutes before stimulation with 100 ng/ml IL10, IL6 or 20 μM ZPR-151 for 5 minutes. Cells were lysed with Protein Solubilization Buffer (PSB, 50 mM Hepes, pH 7.5, 100 nM NaF, 10 mM Na Pyrophosphate, 2 mM NaVO4, 2 mM Na Molybdate, 2 mM EDTA) containing 1% octylglucoside, 0.01 M imidazole, and protease inhibitor cocktail (Roche Diagnostics, Laval, QC) for 30 minutes at 4°C and centrifuged at 10000 rpm for 15 minutes. EDTA resistant Ni beads (Roche Diagnostics, Laval, QC) were added to supernatants and the mixture incubated at 4°C for 1 hour before spinning down and washing of beads three times with 0.1% octylglucoside wash buffer in PSB. Bead samples and starting material cell lysates were separated on a 7.5 % SDS-PAGE gel.

Immunoblotting. Protein lysates were separated on SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Etobicoke, ON). Membranes were blocked and probed, where appropriate, with the following primary antibodies overnight: SHIP1 (P1C1) (Santa Cruz Biotechnology), pSHIP1 (Y190) (Kinexus), STAT3 (9D8) (ThermoFisher Scientific), pSTAT3 (Y705) (Thermo Fisher Scientific), STAT1 (BD Transduction Laboratories), pSTAT1 (Y701) (Upstate Biotechnology), GAPDH and actin (Sigma-Aldrich). Membranes were developed with either Alexa Fluor® 660 anti-mouse IgG or Alexa Fluor® 680 anti-rabbit IgG antibodies (Thermo Fisher Scientific) and imaged using a LI-COR Odyssey Imager.

Acceptor Photobleaching FRET analysis. J17 $SHIP1^{-/-}$cells expressing Clover-SHIP1 and/or mRuby2-STAT3 were seeded at 50 x 10^4 cells per well in 8-well Ibidi μ-Slides (Ibidi GmbH, Martinsried, Germany). After overnight incubation, cells were serum-starved with Mac media containing 1% serum for 3 hours before media replacement with Leibovitz's (L-15) media (Invitrogen, Burlington, ON) supplemented with 1%

serum, 10 μM β-mercaptoethanol, 150 μM monothioglycolate and 1 mM L-glutamine for confocal microscopy imaging. Cells were imaged on a Leica SP8X on DMi8 confocal microscope system with a 63X/1.3 Gly HC PL APO CS2 objective using a white light laser line with 488 nm for donor excitement and 555 nm for acceptor excitement. Photobleaching FRET analysis was performed by measuring Clover-SHIP1 donor fluorescence intensity before and after bleaching the acceptor, mRuby2-STAT3, within a field of cells 'region of interest' (ROI), at 100% laser intensity for 60 frames. Acceptor photobleaching was performed first in resting cells then at 1 minute following 'mock' stimulation with L-15 media, or L-15 media containing 100 ng/ml IL10, IL6 or 20 μM ZPR-151. Donor and acceptor fluorescence intensity of individual cells within the bleached ROI was quantified before and after bleaching. Percentage FRET efficiency was calculated as %FRETeff = 100 x ($D_{post} - D_{pre}$)/D_{post} where D_{pre} and D_{post} represent Clover-SHIP1 donor fluorescence intensity before and after bleaching, respectively.

Immunofluorescence. Perimacs were seeded at 3 x 10^5 cells per well in 18-well Ibidi μ-Slides (Ibidi GmbH, Martinsried, Germany) and allowed to adhere for 3 hours before washing with PBS to remove non-adherent cells. CD8+ T cells were seeded at 2 x 10^6 cells in 12-well tissue culture plates. Cells were stimulated with either 100 ng/ml IL10 or 20 μM ZPR-151 for 2 or 20 minutes followed by 3 x PBS washes and fixing of cells in 4% paraformaldehyde for 15 minutes at room temperature. Cells were incubated with anti-mouse CD16/CD32 Fc Block (BD Pharmingen) for 1 hour followed by an overnight incubation at 4°C with either anti-SHIP1 antibody (P1C1) (Santa Cruz Biotechnology) or anti-STAT3 antibody (9D8) (ThermoFisher Scientific). Cells were then incubated with anti-mouse IgG (H+L)-Alexa Fluor 660 secondary antibody (ThermoFisher Scientific) for 1 hour, followed by, for perimacs, anti-CD11b-FITC antibody (BD Pharmingen) for 30 minutes. CD8+ T cells were mounted onto 18-well Ibidi μ-Slides prior to confocal microscopy and cells were stored in Ibidi Mounting Media supplemented with ProLong Gold antifade reagent with DAPI (Molecular Probes, Life Technologies). Cells were imaged on a Leica SP5II on DM6000 confocal microscope with a 63x/1.4-0.6 Oil PL APO objective using 405, 488 and 633 nm laser lines for excitation. Final images were scanned sequentially acquiring eight Z-stacks with a frame-average of four. Co-localization analysis was performed using ImageJ software by first combining individual z-stack confocal images then performing deconvolution and co-localization using CUDA deconvolution and JACoP plugins respectively. Pearson's coefficient values were produced as a measurement of the degree of overlap between SHIP1 or STAT3 with CD11b (for Perimacs) or DAPI.

Mouse Endotoxemia Model. Groups of 6-8 week old BALB/c SHIP1$^{+/+}$ and SHIP1$^{-/-}$ mice were intraperitoneally injected with either 1 or 5 mg/kg of LPS with or without co-administration of 1 mg/kg of IL10 or 5 mg/kg ZPR-MN100. Blood was drawn 1 hour later by cardiac puncture for determination of plasma cytokine levels by ELISA.

Mouse Colitis Model. Colitis was induced in 6-8 week old BALB/c IL10$^{-/-}$ mice by administering the colonic contents of conventional C57BL/6 mice diluted 1:10 in PBS by oral gavage. Mouse weights and fecal consistencies were monitored and colitis allowed to develop for 6 weeks. Ethanol (Vehicle) and ZPR-MN100 (3 mg/kg) was diluted in cage drinking water and dexamethasone (0.4 mg/kg) was administered every 2 days by oral gavage for 3 weeks. At the end of the dosing period, proximal, medial and distal colon

sections were collected for paraffin embedding or stored in RNALater (Invitrogen, Mississauga, ON) for RNA extraction. Slides were prepared, stained with hematoxylin and eosin, and mounted by the UBC Department of Pathology and Laboratory Medicine Histochemistry Facility. Specimens were assigned pathological scores by two, independent, blinded investigators according to a method described by Madsen *et al* (Madsen et al., 2001). In brief, colonic inflammation was graded using a 4-point scale, scoring 0-3 for each of submucosal edema, immune cell infiltration, goblet cell ablation, and integrity of the epithelial layer. For analysis of mRNA expression, colon sections were homogenized and total RNA extracted as described above and analyzed by real time PCR using gene specific primers for IL17, CCL2, and GAPDH (normalization control).

Expression of PAC2 for crystallography. LIC-HMT-PAC2 expression vector was transformed into *E. coli Rosetta(DE3) pLacI* cells. Overnight culture was inoculated with a 250-fold dilution to start the actual culture. The cells were grown at 37°C in LB medium (supplemented with 50 µg/ml of kanamycin and 34 µg/ml of chloramphenicol) with shaking at 225 rpm. When OD_{600} reached about 0.6, the culture was cooled down to room temperature before the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the expression of recombinant protein. Cultures were left in the shaker overnight (usually 16-18 hours) at 22°C, and then collected by centrifugation (5000 g for 10 minutes at 4°C). The cell pellet was subsequently resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 350 mM NaCl, 10 mM TCEP, 5 mM imidazole, supplemented with 1X EDTA-free Protease Inhibitor Cocktail (PIC) (Roche Diagnostics, Laval, QC) and 25 µg/ml lysozyme), and lysed via sonication (2 cycles of 2 minutes pulse) on ice. Cell debris was removed by two rounds of centrifugation, first at 5000 g for 15 minutes at 4°C followed by 18000 rpm for 30 minutes at 4°C. Supernatant was filtered with a 0.45 µm filter and loaded onto a Talon Co^{2+}-affinity column, previously equilibrated with Buffer A (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 1 mM TCEP), and washed with 10 column volume (CV) of Buffer B (Buffer A + 5 mM imidazole). Bound proteins were eluted with 6 CV of Buffer C (Buffer A + 50 mM imidazole).

To remove the HMT tag, TEV protease (purified in house as a His_6-tagged protein) was added to the eluted protein, which was then dialyzed against Buffer D (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 1 mM TCEP) overnight at 4°C with gentle stirring. The dialyzed sample was loaded onto the Amylose column (New England Biolabs, Whitby, ON), and the flow through, which contained the untagged protein, was loaded onto the Talon column to remove the His_6-TEV protease. The flow through from the Talon column was dialyzed against Buffer E (20 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM TCEP) overnight at 4°C with gentle stirring, and then loaded onto the ResourceQ column (6 ml column volume) (GE Healthcare, Mississauga, ON), followed by washes with 3 CV of Buffer E. To elute the protein, Buffer F (20 mM Tris-HCl pH 7.4, 1000 mM NaCl, 1 mM TCEP) was used. A gradient from 25 mM NaCl (0% buffer G) to 200 mM NaCl (20% Buffer G) was used across 20 CV to separate the components in the protein sample. The fractions were analyzed by SDS-PAGE. PAC2 usually eluted from the ResourceQ column at ~130 mM NaCl. The purified protein was concentrated to about 5-10 mg/ml using Amicon concentrators with 30K MWCO (Millipore, Etobicoke, ON), and exchanged into the desired buffer. For protein crystallization, the desired buffer contained 50 mM Tris-HCl pH7.4, 25 mM NaCl and 0.5 mM TCEP. For Biolayer Interferometry, HMT-PAC2

proteins eluted from the first Talon column were directly purified on the ResourceQ column without cleavage of the HMT tag.

Expression of PAC2-Avi tag for Biolayer interferometry. A sequence corresponding to Avi-tag (GLNDIFEAQKIEWHE) was added to the c-terminal end of the PAC2 in LIC-HMT-PAC2 expressing vector via standard restriction digestion and ligation. The LIC-HMT-PAC2-Avi expressing vector was then co-transformed into *E.coli BL21* cells with pBirAcm expression vector in 1:1 molar ratio. Overnight culture was inoculated with a 250-fold dilution to start the actual culture. The cells were grown at 37^{o}C in LB medium (supplemented with 50 μg/mL of kanamycin and 10 μg/mL of chloramphenicol) with shaking speed of 225 rpm. When OD_{600} reached about 0.6, 5 mM of biotin in bicine buffer pH 8.3 was added to the culture to have final concentration of 125 μM of biotin. The culture was then cooled down to room temperature before the addition of 0.4 mM isopropyl-B-D-1-thiogalactopyranoside (IPTG) to induce the expression of the recombinant protein with Avi-tag. The rest of the method is identical as written in "Expression of PAC2 for crystallography".

Protein crystallization, data collection, phasing and refinement. Initial crystallization hits were obtained via sparse matrix screening in 96-well plates using commercially available crystallographic solutions (Qiagen, Toronto, ON). Optimization of crystallization conditions was performed in 24-well plate format using the hanging drop vapor diffusion method. Diffraction-quality protein crystals were obtained at 4-7 mg/ml protein at room temperature with 0.1 M HEPES-NaOH pH 6.7, 20% PEG1500 and 5 mM $MgCl_2$. The PAC2-cc protein contained surface entropy reduction mutations (E770A, E772A, E773A) and aided in improving crystal quality. Unique fragments of crystal clusters of protein were soaked for 5 to 10 seconds in the crystallization solution containing 25% isopropanol, and flash-frozen in liquid nitrogen.

Diffraction data set were collected at the Advance Proton Source (APS) beamline 23-ID-D-GM/CA and processed with XDS through XDSGUI[45]. The phase problem was solved with an unpublished structure as search model in Phaser MR [47]. The initial model was refined with COOT [48] and Refmac5 [49]. Towards the final model occupancy refinement of sidechains was used in Phenix(Adams et al., 2010) and three TLS groups were defined. Data collection and refinement statistics are shown in **Table S1**. The model and data were deposited under protein database ID 6DLG.

Small angle X-ray scattering. PAC1 samples in 190 μM in 50 mM TrisHCl (pH7.4) 150 mM NaCl, 1 mM TCEP and 2% EtOH with and without 570 μM ZPR-MN100 (6 fold molar excess). Dynamic Light Scattering (DLS) data for PAC1 and PAC1-ZPR-MN100 complexes were collected prior to SAXS data collection to confirm that all the samples are highly pure and suitable for data collection. The data collection was performed on a 3-pinhole camera (S-MAX3000; Rigaku Americas, The Woodlands, TX) equipped with a Rigaku MicroMaxþ002 microfocus sealed tube (Cu Kα radiation at 1.54 Å) and a Confocal Max-Flux (CMF) optics system operating at 40 W (Rigaku). Scattering data were recorded using a 200 mm multiwire two-dimensional detector. The data for both samples and buffer were collected for 3 h for each sample within the range of $0.008 \leq s \leq 0.26$ Å−1 and processed according to the method previously described, where s

= 4πsin θ/λ (Patel et al., 2011, Patel et al., 2010, Patel et al., 2012). The Normalized Spatial Discrepancy (NSD) of the non-liganded and liganded PAC1 models were 0.6 and 1.0 respectively.

Biolayer interferometry. The binding affinity between the PAC2 protein and small molecule allosteric regulators was examined via bio-layer interferometry (BLI) experiments using super-streptavidin (SSA) biosensor tips and an Octet Red 96 instrument (ForteBio, Fremont, CA). SSA biosensor tips were hydrated in assay buffer 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM TCEP, 0.2% Tween-20 prior to protein immobilization. 0.5 ug/mL of protein was immobilized to the SSA biosensor overnight at 4^{o}C. After immobilization of protein to the biosensor, the tips were blocked with 0.1% BSA for 90 minutes followed by 20 minutes of wash with assay buffer supplemented with 1% EtOH. The kinetic measurement was done at 30^{o}C with orbital flow of 1,000 RPM. The baseline was achieved with the assay buffer supplemented with 1% EtOH for 60 s. The association was measured for 600 s at an analyte concentration of 20 µM followed by dissociation for 300 s in the same buffer as the baseline. The raw data was analyzed using the Octet Red Data Analysis software (ver. 8.2). The raw data were aligned to the baseline and subtracted using both single and double reference subtraction.

QUANTIFICATION AND STATISTICAL ANALYSIS

The band quantification of all immunoblots were performed using LI-COR Odyssey imaging system and Image Studio™ Lite software (LI-COR Biosciences, Lincoln, NE). GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA) was used to perform all statistical analyses. Statistical details can be found in figure legends. Values are presented as means ± standard deviations. Unpaired *t* tests were used where appropriate to generate two-tailed P values. One-way or Two-way ANOVA were performed where required with appropriate multiple comparisons tests. Differences were considered significant when $p \leq 0.05$.

SUPPLEMENTAL REFERENCES

ADAMS, P. D., AFONINE, P. V., BUNKOCZI, G., CHEN, V. B., DAVIS, I. W., ECHOLS, N., HEADD, J. J., HUNG, L.-W., KAPRAL, G. J., GROSSE-KUNSTLEVE, R. W., MCCOY, A. J., MORIARTY, N. W., OEFFNER, R., READ, R. J., RICHARDSON, D. C., RICHARDSON, J. S., TERWILLIGER, T. C. & ZWART, P. H. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica Section D,* 66**,** 213-221.

CHEUNG, S. T., SO, E. Y., CHANG, D., MING-LUM, A. & MUI, A. L. 2013. Interleukin-10 inhibits lipopolysaccharide induced miR-155 precursor stability and maturation. *PLoS One,* 8**,** e71336.

LAM, A. J., ST-PIERRE, F., GONG, Y., MARSHALL, J. D., CRANFILL, P. J., BAIRD, M. A., MCKEOWN, M. R., WIEDENMANN, J., DAVIDSON, M. W., SCHNITZER, M. J., TSIEN, R. Y. & LIN, M. Z. 2012. Improving FRET dynamic range with bright green and red fluorescent proteins. *Nat Methods,* 9**,** 1005-12.

MADSEN, K., CORNISH, A., SOPER, P., MCKAIGNEY, C., JIJON, H., YACHIMEC, C., DOYLE, J., JEWELL, L. & DE SIMONE, C. 2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology,* 121**,** 580-91.

MING-LUM, A., SHOJANIA, S., SO, E., MCCARRELL, E., SHAW, E., VU, D., WANG, I., MCINTOSH, L. P. & MUI, A. L. 2012. A pleckstrin homology-related domain in SHIP1 mediates membrane localization during Fcgamma receptor-induced phagocytosis. *FASEB J,* 26**,** 3163-77.

ONG, C. J., MING-LUM, A., NODWELL, M., GHANIPOUR, A., YANG, L., WILLIAMS, D. E., KIM, J., DEMIRJIAN, L., QASIMI, P., RUSCHMANN, J., CAO, L.-P., MA, K., CHUNG, S. W., DURONIO, V., ANDERSEN, R. J., KRYSTAL, G. & MUI, A. L.-F. 2007. Small-molecule agonists of SHIP1 inhibit the phosphoinositide 3-kinase pathway in hematopoietic cells. *Blood,* 110**,** 1942-1949.

PATEL, T. R., MEIER, M., LI, J., MORRIS, G., ROWE, A. J. & STETEFELD, J. 2011. T-shaped arrangement of the recombinant agrin G3-IgG Fc protein. *Protein Sci,* 20**,** 931-40.

PATEL, T. R., MORRIS, G. A., ZWOLANEK, D., KEENE, D. R., LI, J., HARDING, S. E., KOCH, M. & STETEFELD, J. 2010. Nano-structure of the laminin gamma-1 short arm reveals an extended and curved multidomain assembly. *Matrix Biol,* 29**,** 565-72.

PATEL, T. R., REUTEN, R., XIONG, S., MEIER, M., WINZOR, D. J., KOCH, M. & STETEFELD, J. 2012. Determination of a molecular shape for netrin-4 from hydrodynamic and small angle X-ray scattering measurements. *Matrix Biol,* 31**,** 135-40.

PEACOCK, J. W., PALMER, J., FINK, D., IP, S., PIETRAS, E. M., MUI, A. L., CHUNG, S. W., GLEAVE, M. E., COX, M. E., PARSONS, R., PETER, M. E. & ONG, C. J. 2009. PTEN loss promotes mitochondrially dependent type II Fas-induced apoptosis via PEA-15. *Mol Cell Biol,* 29**,** 1222-34.

VAN PETEGEM, F., CLARK, K. A., CHATELAIN, F. C. & MINOR, D. L., JR. 2004. Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. *Nature,* 429**,** 671-5.