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Supplemental Information

M1 Macrophage Polarization Is Dependent on TRPC1-Mediated Calcium Entry

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Supplemental figures and legends:

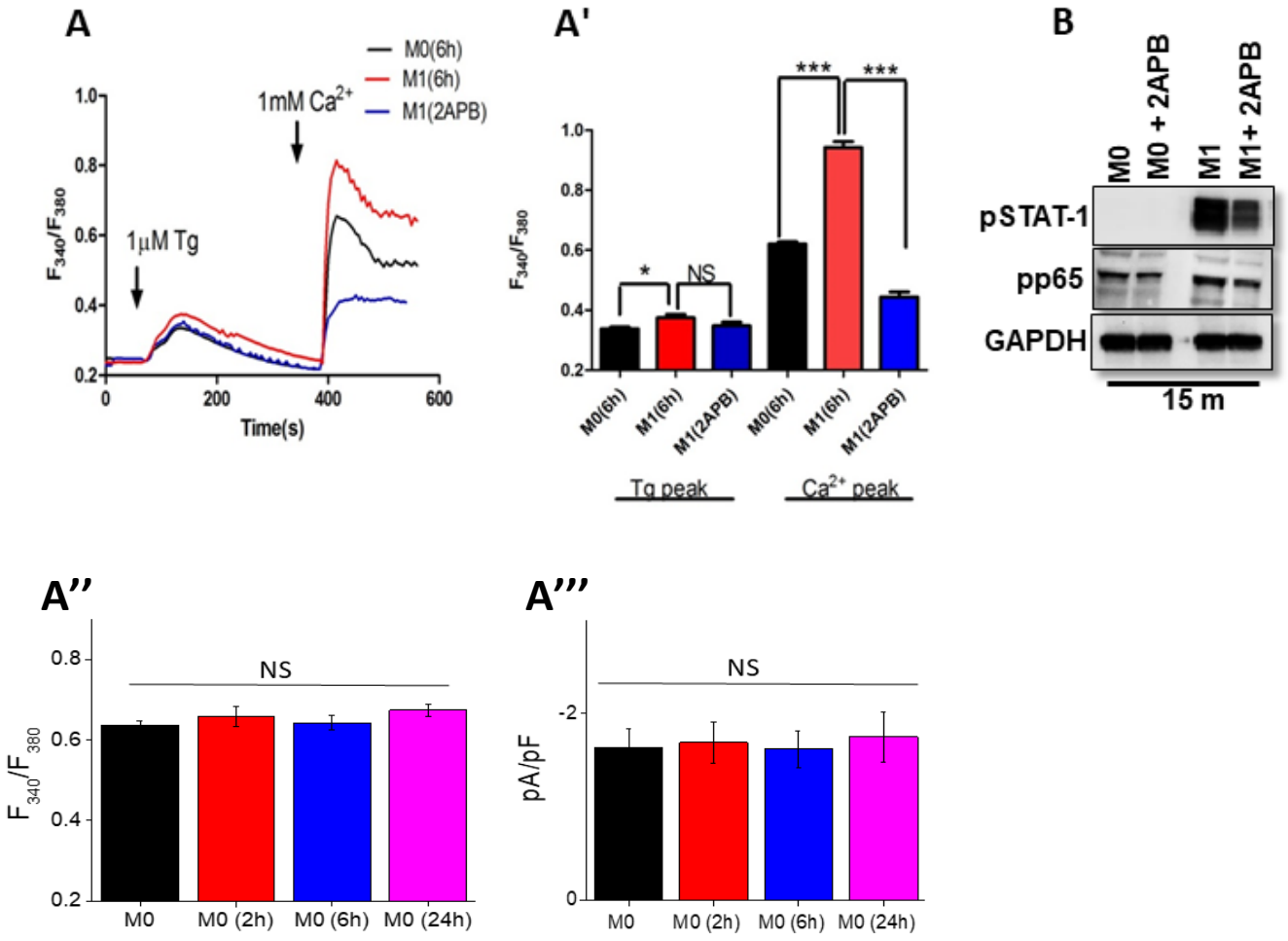

Figure S1. **IFNγ induced Ca^{2+} influx and shape M1-functional phenotype development in the presence or absence of 2APB *in vitro*, related to Figure 1.**

(A) BMMΦ were pulsed with medium alone (M0), or 20 ng/ml IFNγ (M1) for 6h in the presence or absence of 50 μM 2APB and loaded with Fura-2AM. 1μM Tg was added (1st arrow) to the Fura-2AM- loaded cells bathed in Ca^{2+} -free medium to measure the internal Ca^{2+} release (1st peak), followed by addition of external Ca^{2+} (2nd arrow) to measure Ca^{2+} entry/ influx through PM (2nd peak). Average analog plots of the fluorescence ratio (340/380 nm) from an average of 40 to 50 cells are shown. (A') The corresponding bar graphs represent the mean ± SEM of Ca^{2+} release (1st peak) and store-operated Ca^{2+} entry (SOC) (2nd

peak) under these conditions. *** $p \leq 0.005$ (Student's t-test). (A'') The bar graphs represent the mean ± SEM of Ca^{2+} release (1^{st} peak) in BMMΦ were pulsed with medium alone for 0min (M0), 2h (M0(2h), 6h M0(6h), and 24h (M0(24h). (A''') IV curves were compared in BMMΦ pulsed with medium alone for 0min (M0), 2h M0(2h), 6h M0(6h), and 24h M0(24h) by whole-cell patch clamp recordings. Statistics from 8-10 recordings are shown in bar graph.

(B) The level of pNFκB p65 (pp65), or pSTAT1 in BMMΦ cultured with medium alone (M0) or IFNγ (M1) in the presence or absence of 2APB by immunoblot. GAPDH was used as loading control. Data shown are representative of three independent experiments with similar results.

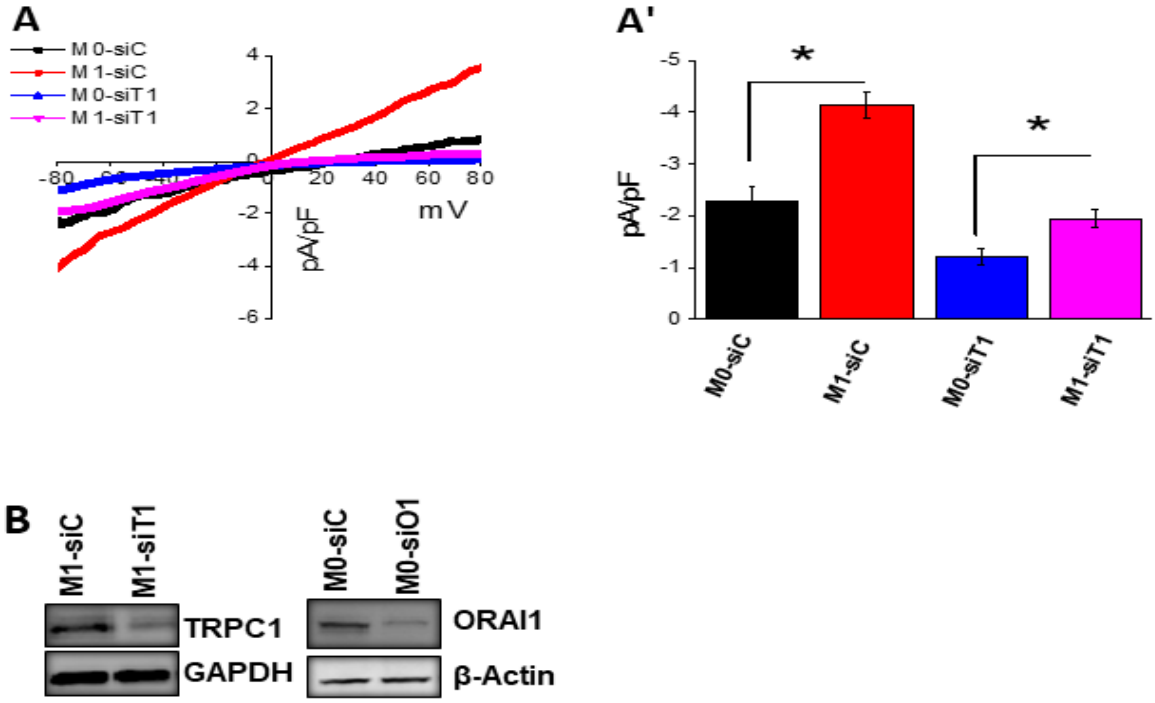

Figure S2. PM Ca^{2+} influx channels in M1-macrophages *in vitro*, related to Figure 2.

(A) BMMΦ transfected with control siRNA (siC), or siRNA specific for TRPC1 (siT1) were cultured under M0 and M1 conditions for 24h. IV curves were compared in control and TRPC1 knock-down cells by whole-cell patch clamp recordings. Statistics from 8-10 recordings are shown in bar graph (A').

(B) BMMΦ were pulsed with medium alone (M0), or IFNγ (M1) for 24h. M1 cells were transfected with siRNA specific for TRPC1 (siT1), or control siRNA (siC). Whereas, M0 cells were transfected with siRNA specific for ORAI1 (siO1), or control siRNA (siC).TRPC1 and ORAI1 protein expression was measured by western blot using anti-TRPC1 or anti-ORAI1 antibody respectively. GAPDH was used as loading control.

* $p \leq 0.05$ (Student's t-test).

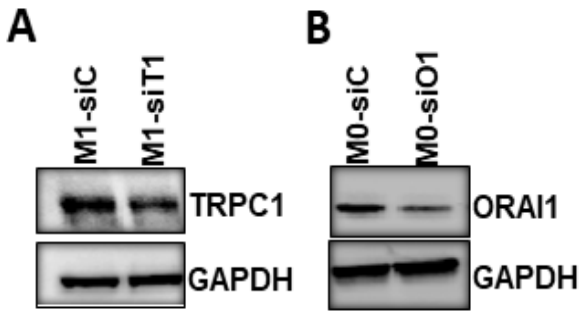

Figure S3. **TRPC1 and ORAI1 knock-down in peritoneal macrophages *in vivo*, related to Figure 3.**

(A) Peritoneal macrophages from mice i.p. injected with TRPC1 siRNA (siT1), or with control siRNA (siC) *in vivo* before the animals received IFNγ (M1). TRPC1 protein expression was measured by western blot using anti-TRPC1. GAPDH was used as loading control.

(A) Peritoneal macrophages (M0) from mice i.p. injected with ORAI1 siRNA (siO1), or with control siRNA (siC) *in vivo*. ORAI1 protein expression was measured by western blot using anti-ORAI1. GAPDH was used as loading control.

* $p \leq 0.05$ (Student's t-test).

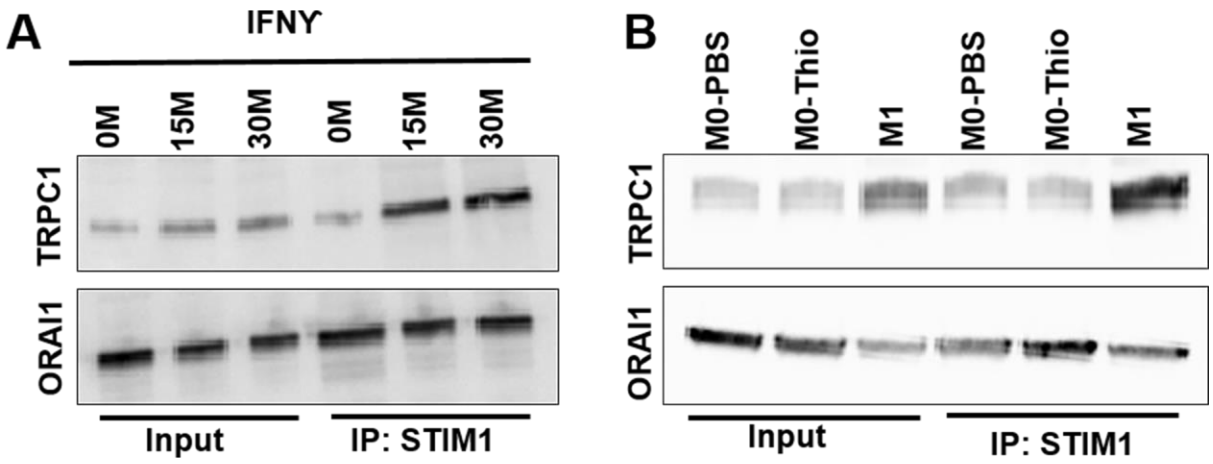

Figure S4. Level of STIM1-TRPC1 and STIM1-ORAI1 interaction after IFNγ stimulation of BMMΦ *in vitro* and **PMΦ *in vivo*, related to Figure 2 and Figure 4.**

A) BMMΦ from C57BL/6 mice were pulsed with IFNγ for indicates duration, and IP performed with anti-STIM1 antibody, followed by immunoblotting using anti-TRPC1 or anti-ORAI1 on 30 μg protein separated by SDS PAGE. Inputs shown here were 1/10th of the protein used for IP.

B) PMΦ from C57BL/6 mice injected with PBS only, Thio + PBS, or Thio + IFNγ as described in methods. IP was performed with anti-STIM1 antibody, followed by immunoblotting using anti-TRPC1 or anti-ORAI1 on 30 μg protein separated by SDS PAGE. Inputs shown here were 1/10th of the protein used for IP.

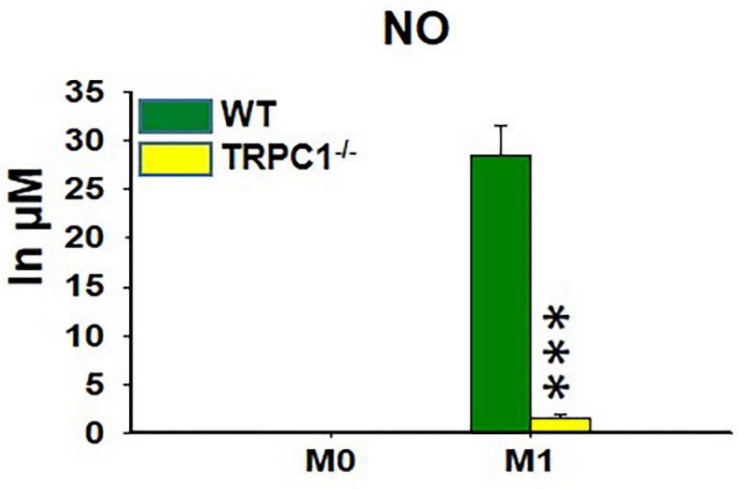

Figure S5. Effect of TRPC1 deficiency on the ability of IFNγ to induce NO *in vitro*, related to Figure 4.

BMMΦ from C57BL/6 and TRPC1-/- mice were cultured in the presence or absence of IFNγ. NO was assessed by colorimetric assay in supernatants collected at 24hrs from IFNγ treated (M1) or untreated (M0) cells.

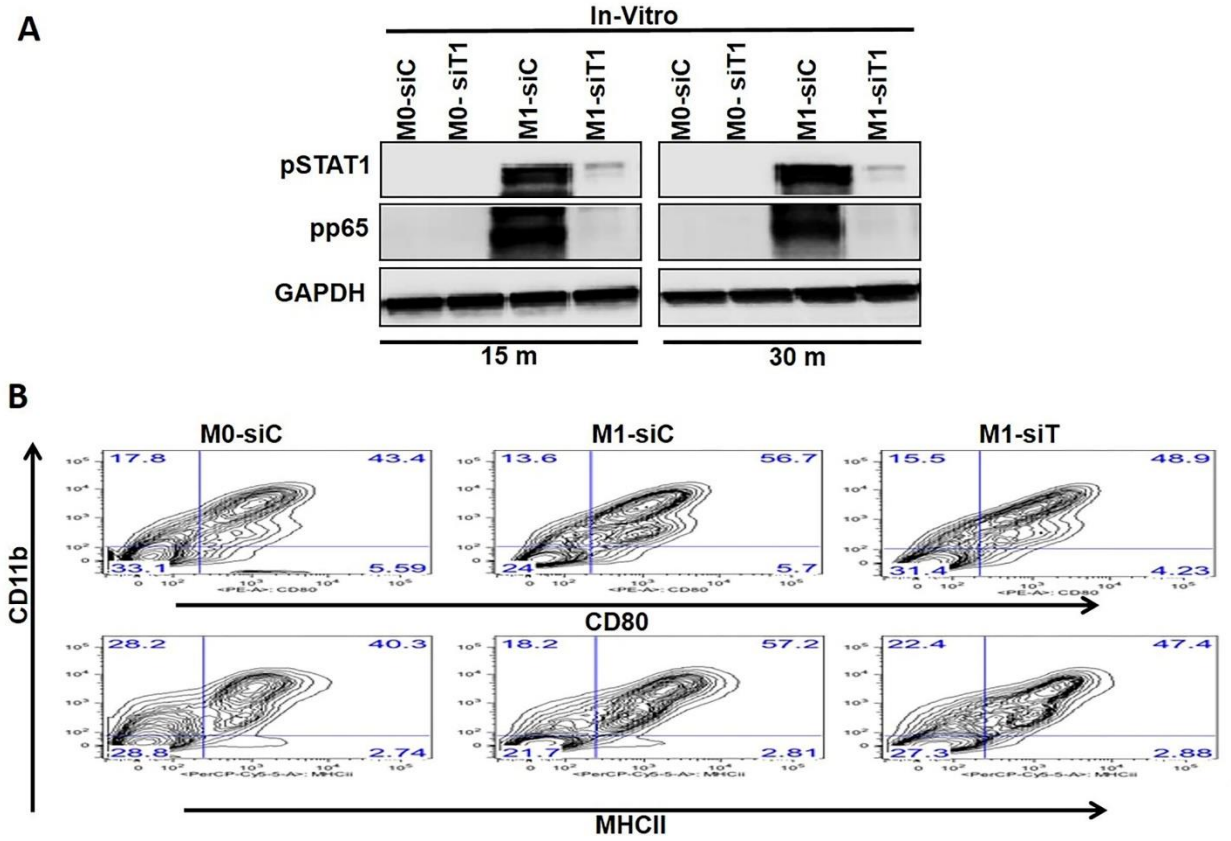

Figure S6. TRPC1 knock-down results in reduced IFNγ- induced phosphorylation of STAT1 and NFκB p65 as well as impaired maturation in BMMΦ *in vitro*, related to Figure 4.

(A) BMMΦ from C57BL/6 mice were transfected with non-targeting siRNA (siC) or TRPC1 siRNA to transiently knock down TRPC1. Cells were pulsed with medium alone (M0-siC, M0-siT1), or IFNγ (M1-siC, M1-siT1) for 15 min or 30min. Immunoblot using anti-pSTAT1 (Cell Signaling, 9167S) and anti-p65 (Cell Signaling, 3033S) is shown. GAPDH was used as loading control.

(B) BMMΦ transfected with control siRNA, or TRPC1 siRNA and pulsed for 24h with medium only (M0-siC, M0-siT1), or IFNγ (M1-siC, M1-siT1). The surface expression of maturation markers CD80 and MHC-II, in CD11b+ (myeloid cell marker) were measured by flow cytometry.

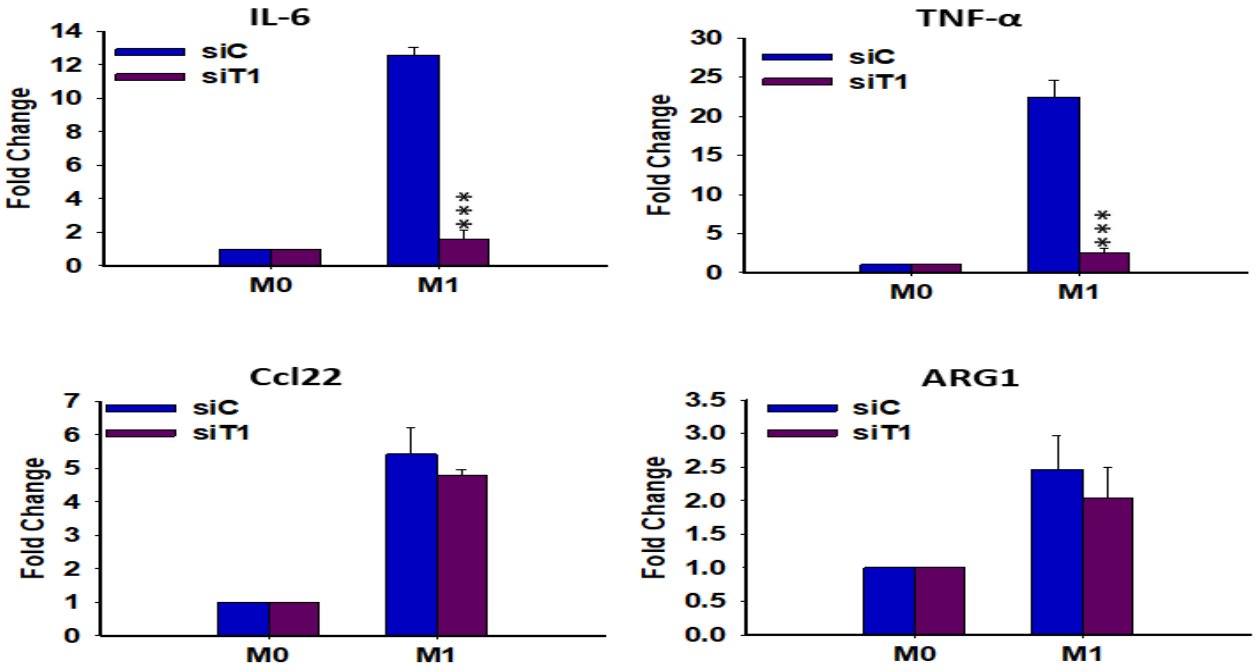

Figure S7. TRPC1 knock-down results in reduced IFNγ- induced production of M1 inflammatory mediators in BMMΦ *in vitro*, related to Figure 4.

BMMΦ from C57BL/6 mice were transfected with non-targeting siRNA or TRPC1 siRNA to transiently knock down TRPC1. Cells were cultured in the presence or absence of IFNγ and the level of M1-associated signature immune mediators, IL-6, TNF-α, and M2 anti-inflammatory mediators, CCL22, Arginase-1 (ARG-1) were analyzed in BMMΦ by qRT-PCR.

*** $p \leq 0.001$ (Student's t-test).

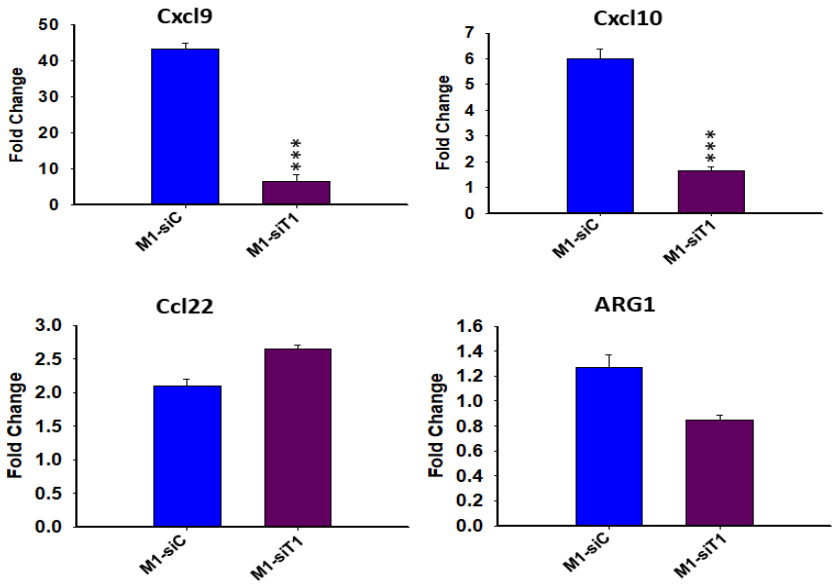

Figure S8. **TRPC1 knock-down results in reduced IFNγ- induced production of M1 inflammatory mediators in peritoneal macrophages *in vivo*, related to Figure 5.**

PMΦ transiently deficient in TRPC1 or control cells from mice that received siRNA specific for TRPC1 or non-targeting siRNA were harvested 24h after i.p. injection with vehicle (M0-siC, M0-siT1), or IFNγ (M1-siC, M1-siT1). The expression of M1 associated inflammatory mediators, CXCL9, CXCL10, and M2 anti-inflammatory mediators, CCL22, Arginase-1 (ARG-1) were measured by qRT-PCR. *** $p \leq 0.001$ (Student's t-test).

TRANSPARENT METHODS

Mice and primary cell culture conditions

Female mice 6-8 wk old were used in this study. Both TRPC1$^{-/-}$ and WT (C57BL/6) mice were bred in the UND animal facility. All animal experiments were conducted under the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of UND. All primary cells isolated were cultured in RPMI medium with 10% (vol/vol) FCS, 2mM glutamine, 100IU ml^{-1} of penicillin, 0.1 mg ml^{-1} of streptomycin and 20mM HEPES buffer, pH 7.2–7.5 (all from Invitrogen) and 2mM β–mercaptoethanol (Sigma-Aldrich).

Human subjects and circulating macrophage/ monocytes isolation and Analysis

For this study, patients (18-60 years) were enrolled from 2015 to 2017 in Altru Clinic Intensive Care Units (Grand Forks, ND, USA). The collection of human samples has been approved by institutional review board (UND IRB protocol 201503-298 and Altru IRB ST151). Written informed consent from the patient or a next-of-kin was required for enrolment. The inclusion criteria were two signs or more of at least two of the following: temperature <36 or > 38 degrees C; heart rate >90/min; respiration rate >20/min or arterial PCO2<32 mm Hg; and white blood cell (WBC) count >12,000/mm^3 or <4000/mm^3 or shift to the left of the differential WBC count with band forms >10%. Exclusion criteria included: pregnancy, cancer, altered mental state, chronic renal failure/ Insufficiency with a baseline creatinine > 2, steroids or any immunosuppressant within 30 days, chronic liver failure, HIV/AIDS, or Hepatitis B or C, drug or alcohol use. All patients were clinically followed up for 10 days or till discharged. Control samples were collected from matched healthy blood donors (age ± 5 years, sex, race). Twenty ml of blood were collected from healthy control, or patient starting day 1 of ICU admission in EDTA blood collection tubes (BD Biosciences, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using histopaque gradient (Sigma-Aldrich, St Louis, MO,

USA) as per the manufactures' suggestions. Monocytes/ macrophages in PBMCs were separated using CD11b microbeads (Miltenyi Biotec, Gladbach, Germany) by following the magnetic cell sorting protocol provided by the manufacturer. Western Blotting and RT-PCR analysis were performed as described in detail below. Specific primers used for RT-PCR analysis are listed in Table S1.

Polarization to M1 macrophage inflammatory phenotype, and bacterial burden

For *in vitro* studies, bone marrow cells were isolated from mice and differentiated to macrophages (Chauhan et al., 2014). Bone marrow-derived macrophage (BMMΦ) on day 6 of differentiation were used for experiments. Naïve macrophages cells were exposed to IFNγ (20ng/ml, Peprotech) to generate the M1- inflammatory phenotype. For *in vivo* studies, mice were injected intraperitoneally (i.p.) with 4% thioglycollate on day-0. On day-3 mice were injected i.p. IFNγ (50µg/kg) to drive peritoneal macrophage (PMΦ) polarization to M1-phenotype, or vehicle (PBS) (mock control). Whereas, in studies involving peritonitis due to *Klebsiella pneumoniae* (KPn) infection, mice were injected i.p. with with 30000 CFU of KPn (American Type Culture Collection strain 43816) for 24 h to drive peritoneal macrophage (PMΦ) polarization to M1-phenotype. Mock control mice instead received vehicle (PBS). From mice twenty-four hours after receiving IFNγ, bacterial infection, or PBS, peritoneal exudate cells (PECs) were harvested and PMΦ were analyzed for expression of immune mediators by flow cytometry, RT-PCR, and western blot.

In some experiments, the mice were euthanized at 24h p.i. and peritoneal lavage, blood, and liver were aseptically homogenized in cold PBS with CompleteTM protease inhibitor cocktail (Roche Diagnostics, Germany) (Tripathi et al., 2018, Jondle et al., 2016, Mishra et al., 2013). For the bacterial burden analyses, serially diluted liver homogenates, peritoneal lavage, and blood were plated on LB agar and incubated at 37^{o}C overnight (Tripathi et al., 2018, Jondle et al., 2016,

Mishra et al., 2013). Electrophysiological and biochemical analysis of these cells were performed to assess Ca^{2+} influx and properties of the channels involved in this process.

RNAi Transfections

Lipofectamine 2000 (Invitrogen) was used for siRNA transfection as per supplier's instructions. siRNA duplexes targeting the coding sequence of mouse TRPC1 (TRPC1-siRNA, Cat. # Sc-42665), ORAI1 (ORAI1-siRNA, Cat. # Sc-76002), or scrambled control siRNA (siRNA-sc, Cat. # Sc-36869) were purchased from Santa Cruz Biotechnologies. For *in vitro* studies BMMΦ in 6 well culture plates were typically used 24h posttransfection with 60 pmol of appropriate siRNAs that had been added 0.5 ml of transfection mix. For *in vivo* studies, mice were injected i.p. with 3 ml of 4% thioglycollate on day 0. Thioglycollate injected mice on day 2 received 250 pmol of TRPC1-siRNA, ORAI1-siRNA, or siRNA-sc i.p. in 1 ml of OptiMem media (Cat # 31985070, Gibco) before receiving 50μg/kg IFNγ or vehicle on day 3. On day 4 mice were euthanized to harvest PECs and analyze macrophages to measure Ca^{2+} influx, or expression of various cytokines, chemokines, transcription factors, and surface maturation markers.

Calcium measurements

Measurements were performed by imaging Fura-2 loaded cells using the Olympus IX50 microscope and Polychrome 4 (TILL Photonics) system (Chauhan et al., 2014; Pani et al., 2009). Images were acquired using a Photometrics CoolSNAP HQ camera (Photometrics) and the MetaFluor software (Molecular Devices).

Electrophysiological Measurements

All electrophysiological experiments were performed on cells (Pani et al., 2009; Selvaraj et al., 2012; Sun et al., 2017). Whole cell-attached patch clamp measurements were performed at

room temperature (22°C to 25°C) using an Axopatch 200B amplifier (Molecular Devices). Cells in the recording chambers were perfused continuously through a custom-designed, gravity-driven, speed-controlled system with an external Ringer's solution containing:145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4 adjusted with NaOH. Patch pipette resistance was 3 to 6 mΩ filled with standard intracellular solution containing: 145 mM cesium methanesulfonate, 8 mM NaCl, 10 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH 7.2 (CsOH). Cells were activated by including thapsigargin (Tg) in the pipette solution (as indicated in the figures). Voltage ramps were applied from −90 to +90 mV (over a period of 1 s, imposed every 4 s) from a holding potential of 0 mV. Currents were digitized at a rate of 1 kHz. A liquid junction potential of <8 mV was not corrected, and capacitive currents and series resistance were determined and minimized. For analysis, the first ramp was used for leak subtraction for the subsequent current records. The current was normalized to the initial size of the cell to obtain current densities (pA/pF).

Western blotting

Cells were solubilized in SDS-PAGE sample buffer (Chauhan et al., 2014; Sun et al., 2014). Proteins in the extracts were resolved on 10% SDS-PAGE followed by Western blot analysis using the desired antibodies as described earlier. The following antibodies were used for western blot analysis: anti- NFκB p65 (p65) (Cell Signaling, 8242S), anti- pNFκB p65 (pp65) (Cell Signaling, 3033S), anti-STAT1 (Cell Signaling, 9172S) and anti-pSTAT1 (Cell Signaling, 9167S), anti-TRPC1 (Abcam, ab192031), anti-ORAI1 (Alamo Lab, ACC-060), β-Actin (Cell Signaling, 4970S) and anti-GAPDH (Gen Script, A00191). Immunoreactivity of p65, pp65, STAT1, pSTAT1, TRPC1, ORAI1, or GAPDH were detected using super signal west Pico Chemiluminisecnt detection reagent (Thermo Fisher Scientific) and analyzed on BioRad Reader (Bio-Rad Laboratories, Hercules, CA, USA) using Chembio software (Medford, NY, USA). Densitometry of

individual bands was done using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation and Western blot analyses

Co-immunoprecipitation and Western blot analyses were performed on cell lysate (Chauhan et al., 2014; Pani et al., 2013; Selvaraj et al., 2012). Following stimulation between 5-10 million cells from *in vitro* or *in vivo* experiments were lysed in 0.5 ml of 1× RIPA buffer (Sigma-Aldrich, 20-188) supplemented with 0.05% SDS, 1% Triton X-100, 20% glycerol, 1mM phenylmethylsulfonyl fluoride, and 1× protease and phosphatase inhibitors (Thermo Scientific) for immunoprecipitation using anti-STIM1 (Cell Signaling, 4916S, 1:50) Immune complexes were separated using Protein A Agarose Plus beads (Pierce, Rockford, IL, USA), proteins were resolved on 10% SDS-PAGE followed by Western blotting using the desired antibodies. The following antibodies were used for western blot analysis: anti-STIM1 (Cell Signaling, 4916S), anti-TRPC1 (Abcam, ab192031), and anti-ORAI1 (Alamo Lab, ACC-060).

Determination of Nitric oxide production

Cells (1 million/well in 2ml) were pulsed with medium alone for (M0) or with IFNγ for (M1) activation phenotype. At 24h of culture, nitric oxide (NO) levels in the culture supernatant was measured using Griess reagent (Promega [Fitchburg, Wisconsin, United States]) as per manufacturer's instructions.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) analysis

The qRT-PCR analysis was performed on cDNA (Chauhan et al., 2015). Total RNA from cells was isolated using Trizol reagent following manufacturers' instructions. One microgram of total RNA from each sample was reverse transcribed into cDNA by using a high capacity cDNA

reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, CA, USA). Transcript levels of M1-inflammatory mediators or the housekeeping ribosomal 18 S RNA were analyzed by RT-PCR using specific primers (Table S1). Expression levels for all the genes were normalized to the mRNA level of the housekeeping 18 S RNA gene in the same sample. The fold change was calculated by dividing the normalized value of the gene of interest in stimulated samples with the corresponding normalized value in unstimulated samples.

Table S1. Sequences of the specific primers used, related to Figure 1, and Figures 4, 5, 6, 7.

Mouse Genes	Sense	Anti-Sense
18s	5′- CAT GTG GTG TTG AGG AAA GCA-3′	5′- GTC GTG GGT TCT GCA TGA TG-3′
Nos-2	5′ -AGGAGGAGAGAGATCCGATTTAG-3′	5′- TCAGAGTTCCCTGTCTCAGTAG-3′
Cxcl9	5′ - CATCATCTTCCTGGAGCAGTG -3′	5′- GAGGGATTTGTAGTGGATCGTG-3′
Cxcl10	5′ -TCAGGCTCGTCAGTTCTAAGT-3′	5′ -CCTTGGGAAGATGGTGGTTAAG-3′
IL-6	5′- TTC ATC CAG TTG CCT TCT TG-3′	5′- GGG AGT GGT ATC CTC TGT GAA GTC-3′
TNF-α	5′- GGGTGTTCATCCATTCTCTACC -3′	5′- TTGGACCCTGAGCCATAATC-3′
IL-23	5′-CTGAGAAGCAGGGAACAAGAT-3′	5′-CATGCAGAGATTCCGAGAGAG-3′
Ccl22	5′-CAACGACGCCACCTTTACT-3′	5′-GGGATAAGCTGGAAGGGATAGA-3′
Arg-1	5′-GTGGCAGAGGTCCAGAAGAATG5′-	5′-GGGAGTGTTGATGTCAGTGTGAGC-3′
Human genes	Sense	Anti-Sense
RPLP0	5′-TGCTGATGGGCAAGAACA-3′	5′-GAACACAAAGCCCACATTCC-3′
Cxcl9	5′-GACTACATAAGAGACCACTTCACC-3′	5′-GCCATCCTCCTTTGGAATGATA-3′
Cxcl10	5′-CCCATCTTCCAAGGGTACTAAG-3′	5′-GCAGTGGAAGTCCATGAAGTA-3′
Ccl22	5′-CGCGTCGTGAAACACTTCTA-3′	5′-GATCGGCACAGATCTCCTTAT-3′

Flow cytometry

Expression of surface maturation markers on macrophages was analyzed by flow cytometry (Chauhan et al., 2014; Jondle et al., 2016). Single cell suspensions were prepared at 2 X 10^7 cells/ ml in staining buffer (10% FCS in PBS) and pre-incubated with 1µg of the 2.4G2 antibodies for 5-10 minutes on ice prior to staining. 50µl of cell suspension (equal to 10^6 cells) were dispensed into each tube or well along with a previously determined optimal concentration of cell surface specific antibody against CD11b, MHCII, CD80, and CD86 in 50µl of staining buffer. Cell surface expression of these maturation markers was measured on a BD LSR II flow cytometer (BD Biosciences). The collected events were analyzed with FlowJo v7.6 (Treestar).

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