Cell Reports, Volume *25*

Supplemental Information

Targeting SHIP-1 in Myeloid Cells Enhances Trained Immunity and Boosts Response to Infection

Paula Saz-Leal, Carlos del Fresno, Paola Brandi, Sarai Martínez-Cano, Otto M. Dungan, John D. Chisholm, William G. Kerr, and David Sancho

SUPPLEMENTAL INFORMATION

Figures S1-S4

Supplemental Experimental Procedures

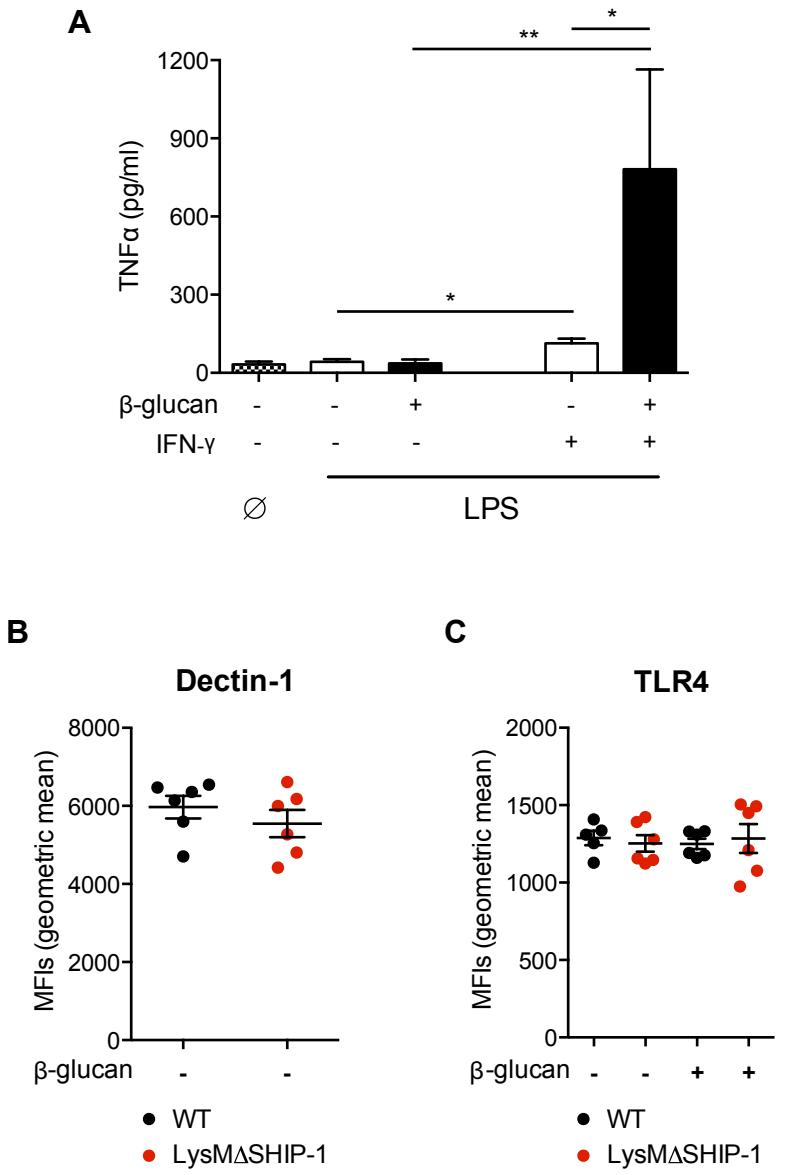

Figure S1 Experimental set-up of trained immunity *in vitro* model applied to BMDMs, related to Figure 1.

(A) WT and LysMΔSHIP-1 BMDMs were stimulated (+) or not (-) with β-glucan and primed (+) or not (-) with IFN-γ prior to LPS stimulation, according to the model in Figure 1C. (Ø) represent BMDMs without any stimuli. TNFα in supernatants was analyzed. Mean ± SEM from five independent experiments is shown. (B) Dectin-1 surface expression was analyzed by FACS in WT and LysMΔSHIP-1 BMDMs before β-glucan stimulation. (C) TLR4 surface expression was analyzed by FACS in WT and LysMΔSHIP-1 BMDMs both under non-trained (-) or β-glucan primed (+) conditions, before LPS stimulation. (B, C) Individual data and mean ± SEM from a pool of two experiments is shown including three BMDMs cultures per experiment. Each dot represents an independent cell culture.

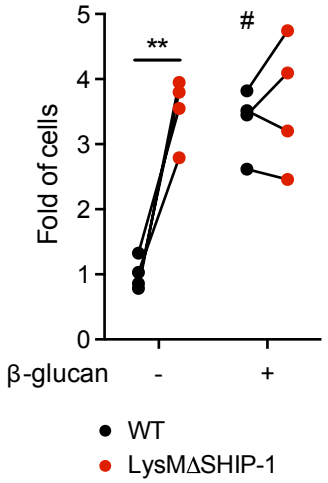

Figure S2. Relative amount of BMDMs recovered before LPS stimulation, related to Figure 1. WT and LysMΔSHIP-1 BMDMs were exposed (+) or not (-) to β-glucan according to model in Figure 1C. At day 5 and before LPS stimulation, the number of viable BMDMs was determined. Fold cell number was calculated by dividing live cell number in each experimental condition by the average number of WT non-trained cells in all the experiments. Individual data from four independent experiments are shown. $^{**}p < 0.01$, paired Student's *t*-test comparing WT and LysMΔSHIP-1. $\#p < 0.05$, paired Student's *t*-test comparing stimulated or not with β-glucan within the same genotype.

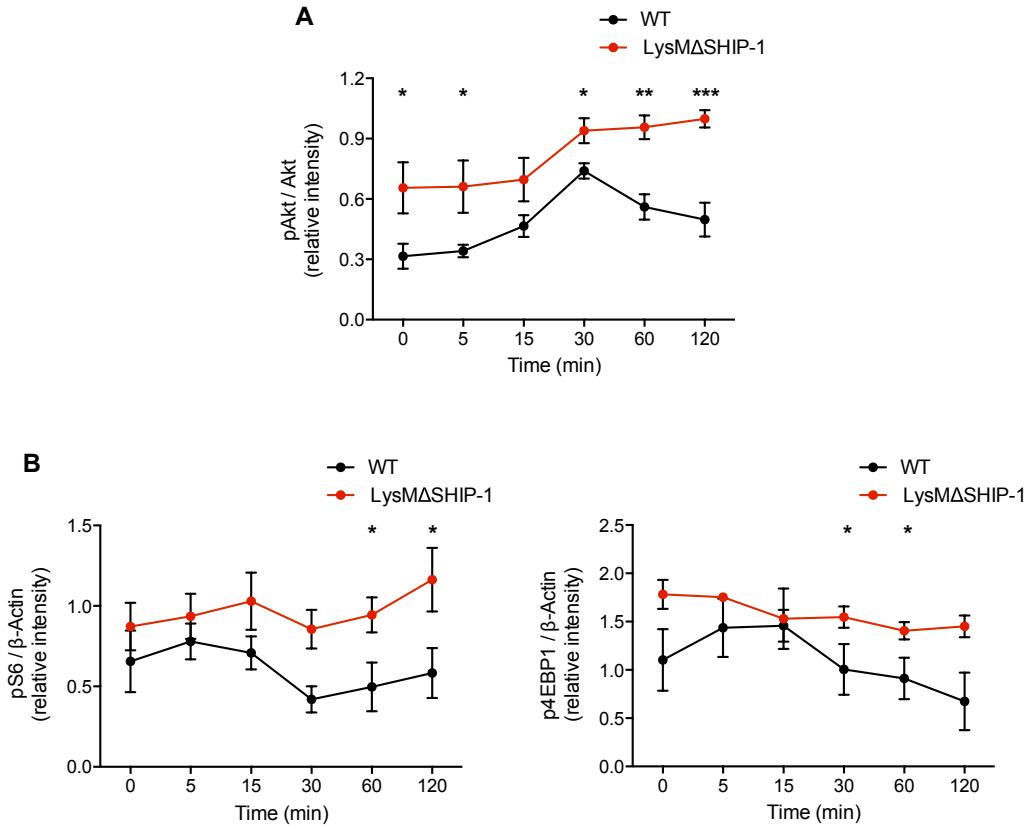

Figure S3. Quantification of WB kinetics, related to Figure 2. WT and LysMΔSHIP-1 BMDMs were exposed to β-glucan for the indicated time and phospho-Akt, Akt (A), phospho-S6, phospho-4EBP1 and β-Actin (B) analyzed by WB and quantified by ImageJ software. Relative band intensity is shown. Mean ± SEM from a pool of five experiments performed. *$p < 0.05$, **$p < 0.01$, ***p>0.001, paired Student's *t*-test comparing WT and LysMΔSHIP-1 at any time point.

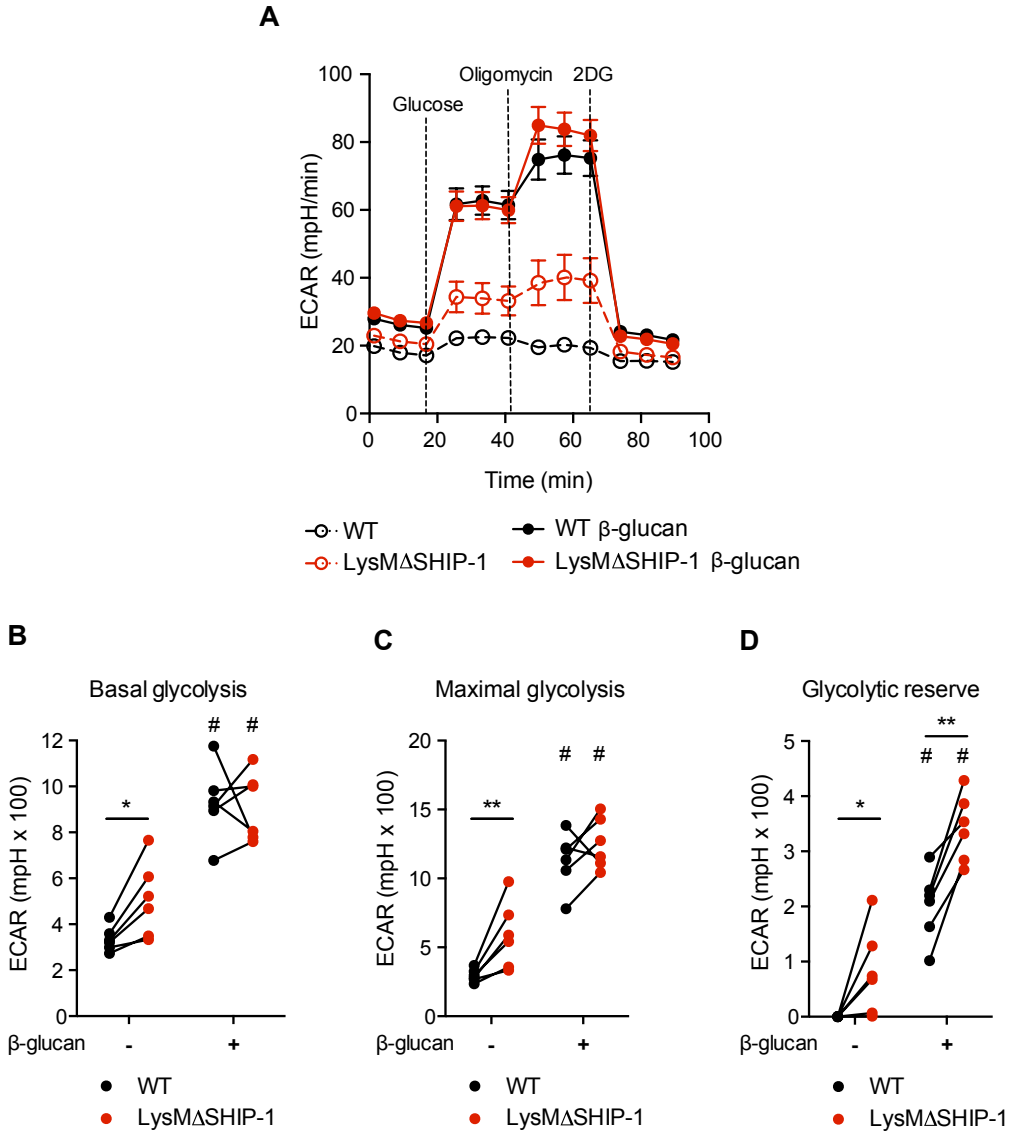

Figure S4. SHIP-1 controls the extent of the early glycolytic metabolism, related to Figure 2. (A-D) WT and LysMΔSHIP-1 BMDMs were left untreated (dashed lines, A) or treated overnight with β-glucan (solid lines, A) and extracellular acidification rate (ECAR) was determined. ECAR in a glycolysis stress test was analyzed upon sequential addition of glucose, oligomycin and 2-deoxyglucose (2DG) as indicated (A). Analysis of basal glycolysis (B), maximal glycolysis (C) and glycolytic reserve (D). (A-D) Mean ± SEM (A) or individual data (B-D) of six independent cultures are shown**.** (B-D) *$p < 0.05$, **$p <$ 0.01, paired Student's *t*-test comparing WT and LysMΔSHIP-1. #$p< 0.05$, paired Student's *t*-test comparing stimulated or not with β-glucan within the same genotype.

Supplemental Experimental Procedures

Candida albicans
Candida albicans (strain SC5314, kindly provided by Prof. C. Gil, Complutense University, Madrid, Spain) was grown on YPD-agar plates (Sigma) at 30ºC for 48h.

In vitro cell differentiation and culture

Mouse bone marrow-derived macrophage differentiation. To obtain mouse bone marrow-derived macrophages (BMDMs) from WT and LysMΔSHIP-1 mice, femurs were collected and flushed, and red blood cells were lysed using RBC Lysis Buffer (Sigma) for 3 minutes at room temperature (RT). Cell suspensions were plated in non-treated cell culture plates (Corning) in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 1 mM pyruvate (Lonza), 100 µM non-essential aminoacids (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all three from Lonza) and 50 µM 2-mercaptoethanol (Merck), herein called R10, plus M-CSF (30% mycoplasma-free L929 cell supernatant) at 37ºC for 5 days. At day 5, BMDMs were detached in phosphate buffered saline (PBS, Gibco) supplemented with 5 mM EDTA (PBS/EDTA, Life Technologies), counted, plated in R10 at the required concentration and rested overnight before any stimulation.

Peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by differential centrifugation using Biocoll Separating Solution (Cultek). Cells were washed twice in PBS, resuspended in DMEM (Sigma) supplemented with 10% heat-inactivated FBS, 100 µM non-essential aminoacids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol, herein called D10; counted and plated for stimulation.

ELISA

Mouse cytokines were analyzed in supernatants of BMDMs using the following reagents: for IL-1β, Mouse IL-1β/IL-1F2 DuoSet, R&D Systems; for IL-6, Purified rat anti-mouse IL-6, Biotin rat anti-mouse IL-6-both from BD Biosciences- and Streptavidin Horseradish Peroxidase (HRP) Conjugate from Invitrogen; for TNFα, OptEIA ELISA kit, BD Biosciences.
Human cytokines were analyzed in supernatants of PBMCs by using the Human IL-1β/IL-1F2 Duoset, Human IL-6 Duoset and Human TNFα DuoSet kits, all from R&D Systems.

Western Blot

Cell lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors (Roche). Samples were run on Mini-PROTEAN TGX PRECAST Gels and transferred onto a nitrocellulose membrane (both from Bio-Rad Laboratories) for blotting with the following antibodies: β-Actin (C4) and SHIP-1 (P1C1) from Santa Cruz; pAkt (Ser473, #4058S), Akt (#2920S), pS6 (Ser235/236, #4858T) and p4EBP1 (Thr37/46, #9459S), all from Cell Signaling. Alexa Fluor-680 (Life Technologies) or Qdot-800 (Rockland) conjugated secondary antibodies were used. Gels were visualized in an Odyssey instrument (LI-COR) and band intensity was quantified by using ImageJ software (Bitplane).

Antibodies and flow cytometry

Samples were stained with the appropriate antibody cocktails in ice-cold FACS Buffer at 4ºC for 15 minutes. Antibodies included mouse PE-anti-TLR4 (BioLegend) and APC-anti-Dectin-1 (Bio-Rad). Dead cells were excluded by Hoechst 33258 (Invitrogen) incorporation. Purified anti-FcγRIII/II (2.4G2, TONBO Bioscience) was used to block murine Fc-receptors at 4ºC for 10 minutes in all the stainings. Events were acquired using FACSCanto 3L (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

Glycolytic flux evaluation

The assay was performed in DMEM supplemented with 1mM glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin. The pH was adjusted to 7.4 with KOH (herein called Seahorse medium). Cells were washed with PBS and 175µl of Seahorse medium was added. Plates were incubated at 37°C without CO_2 for 1h prior to the assay. Extracellular acidification rate (ECAR) was determined by using the glycolysis stress test in an XF-96 Extracellular Flux Analyzer (Agilent Technologies). Three consecutive measurements were performed under basal conditions and after sequential addition of 80 mM glucose (Merck), 9 µM oligomycin A (Sigma) and 500 mM 2-deoxy-glucose (2DG, Sigma). Basal and maximal glycolysis were defined as ECAR after addition of glucose and oligomycin, respectively. Glycolytic reserve was defined as the difference maximal and basal glycolysis.

Chromatin immunoprecipitation (ChIP) analysis

ChIP was performed using the Magna ChIP A – Chromatin Immunoprecipitation kit together with the ChIPAb+ Trimethyl-Histone3 (Lys4) (H3K4me3) – ChIP validated antibody, both from Millipore-Merck, following the provider's instructions. In brief, cells were fixed with 1% formaldehyde for 10 minutes at RT, exposed to glycine to quench unreacted formaldehyde and washed twice with ice-cold PBS supplemented with the provided protease inhibitor cocktail. After scraping the cells in ice-cold PBS, they were pelleted, lysed and sonicated for 15 minutes (30 seconds on/30 seconds off) at high intensity by using a Bioruptor UCD-200TM-TX water bath sonicator (Diagenode). Sonicates were diluted and incubated with antibodies plus protein A magnetic beads for 1 hour with rotation at 4 ºC. Beads were magnetically collected and washed extensively. Protein-DNA complexes were disrupted from the beads upon proteinase-K treatment and recovered DNA was purified. Immunoprecipitated DNA and input DNA were amplified by means of quantitative PCR with specific primers for the promoter region of TNFα (Fw: 5'-CAACTTTCCAAACCCTCTGC-3'; Rv: 5'-CTGGCTAGTCCCTTGCTGTC-3') with input DNA to generate a standard curve. ChIPdata are represented as a percentage of input.

RNA extraction and quantitative-PCR

RNeasy Plus Mini Kit, from Qiagen, was used for RNA extraction. cDNA was prepared using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in a 7900-FAST-384 instrument (Applied Biosystems) by using the GoTaq qPCR master mix from Promega. Primers used in this work (synthetized by Sigma) were as follows: β-actin Fw: 5′-GGCTGTATTCCCCTCCATCG-3′; β-actin Rv: 5′-CCAGTTGGTAACAATGCCATGT-3′; IL-1β Fw: 5'-CTGAACTCAACTGTGAAATGCCA-3'; IL-1β Rv: 5'-AAAGGTTTGGAAGCAGCCCT-3'; IL-6 Fw: CCGTGTGGTTACATCTACCCT-3'; IL-6 Rv: 5'-CGTGGTTCTGTTGATGACAGT-3' TNFα Fw: 5'-CCCTCACACTCAGATCATCTTCT-3'; TNFα Rv: 5'-GCTACGACGTGGGCTACAG-3'; mRNA levels were normalized to β-Actin expression. Data are shown as relative expression to β-Actin (ΔΔCt).