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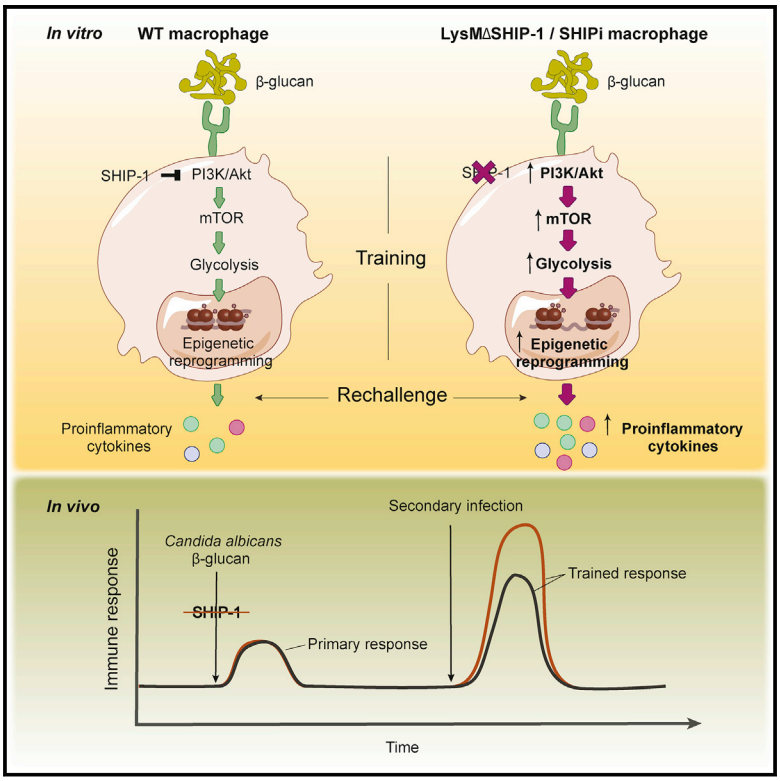
Targeting SHIP-1 in Myeloid Cells Enhances Trained Immunity and Boosts Response to Infection

Graphical Abstract



Highlights

- β-glucan-induced trained immunity is enhanced by SHIP-1 deletion in macrophages
- SHIP-1 regulates molecular, metabolic, and epigenetic hallmarks of trained immunity
- Myeloid SHIP-1 deficiency improves protection conferred by trained immunity
- SHIP-1 pharmacological inhibition enhances trained immunity in mice and human cells

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In Brief

Trained immunity leads to long-term protection, but strategies to boost it require further investigation. Saz-Leal et al. show that myeloid SHIP-1 deletion enhances trained immunity, improving the response to pathogen-specific or heterologous challenges. Pharmacological inhibition of SHIP-1 also potentiates this phenomenon, thereby revealing a potential tool to harness trained immunity.

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

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SUMMARY

β-Glucan-induced trained immunity in myeloid cells leads to long-term protection against secondary infections. Although previous studies have characterized this phenomenon, strategies to boost trained immunity remain undefined. We found that β-glucan-trained macrophages from mice with a myeloid-specific deletion of the phosphatase SHIP-1 (LysMΔSHIP-1) showed enhanced proinflammatory cytokine production in response to lipopolysaccharide. Following β-glucan training, SHIP-1-deficient macrophages exhibited increased phosphorylation of Akt and mTOR targets, correlating with augmented glycolytic metabolism. Enhanced training in the absence of SHIP-1 relied on histone methylation and acetylation. Trained LysMΔSHIP-1 mice produced increased amounts of proinflammatory cytokines upon rechallenge *in vivo* and were better protected against *Candida albicans* infection compared with control littermates. Pharmacological inhibition of SHIP-1 enhanced trained immunity against *Candida* infection in mouse macrophages and human peripheral blood mononuclear cells. Our data establish proof of concept for improvement of trained immunity and a strategy to achieve it by targeting SHIP-1.

INTRODUCTION

Innate immune cells challenged with certain stimuli undergo long-lasting changes that result in improved response to a second challenge by the same or different microbial insult, a process referred to as trained immunity (Quintin et al., 2012). Stimuli driving trained immunity lead to a shift to aerobic glycolysis (Arts et al., 2016b), accompanied by sustained changes in the epigenome, mainly via histone methylation and acetylation (Netea et al., 2016). Among trained immunity inducers, exposure to a low dose of *Candida albicans* or the fungal cell wall component β-glucan protects mice from secondary lethal systemic candidiasis (Bistoni et al., 1986) or heterologous *Staphylococcus aureus* septicemia (Di Luzio and Williams, 1978). This acquired resistance does not rely on T/B lymphocytes or natural killer (NK) cells but occurs in a myeloid-dependent manner (Cheng et al., 2014; Quintin et al., 2012).

The C-type lectin receptor Dectin-1 is critical for *Candida albicans* or β-glucan sensing, leading to immune training of monocytes (Quintin et al., 2012). These primed macrophages show heightened production of proinflammatory cytokines to a wide variety of insults (Ifrim et al., 2013; Quintin et al., 2012). Dectin-1-mediated training relies on activation of the PI3K (phosphoinositide 3-kinase)/mTOR (mammalian target of rapamycin) pathway (Cheng et al., 2014). PI3K-induced Akt signaling is tightly regulated by phosphoinositide phosphatases, which counterbalance PI3K activity (Eramo and Mitchell, 2016). Among those phosphatases, the hematopoietic-restricted SHIP-1 (SH2-containing inositol 5′-phosphatase 1) (Kerr, 2011) is of particular interest, as we showed that it binds to the intracellular tail of Dectin-1 receptor in granulocyte-macrophage colony-stimulating factor (GM-CSF) bone marrow-derived cells (Blanco-Menéndez et al., 2015).

Because *Candida albicans*-induced trained immunity relies on Dectin-1 and PI3K signaling, and SHIP-1 couples to Dectin-1 and counteracts PI3K function, we postulated that SHIP-1 targeting could modulate trained immunity triggered by Dectin-1. Our results indicate that SHIP-1 has a regulatory role in β-glucan-induced training, affecting all hallmarks involved in that process. Moreover, *in vivo* SHIP-1 deficiency in the myeloid compartment improves protection conferred by trained immunity. Notably, enhanced proinflammatory cytokine production and better protection was also achieved by pharmacological SHIP-1 inhibition in both mice and human peripheral blood mononuclear cells (PBMCs), providing a potential therapeutic approach to boost trained immunity.

RESULTS

SHIP-1 Deletion Boosts β-Glucan-Induced Trained Immunity in Macrophages

Dectin-1 sensing of β-glucan induces trained immunity in myeloid cells, including PBMCs (Ifrim et al., 2013) or bone

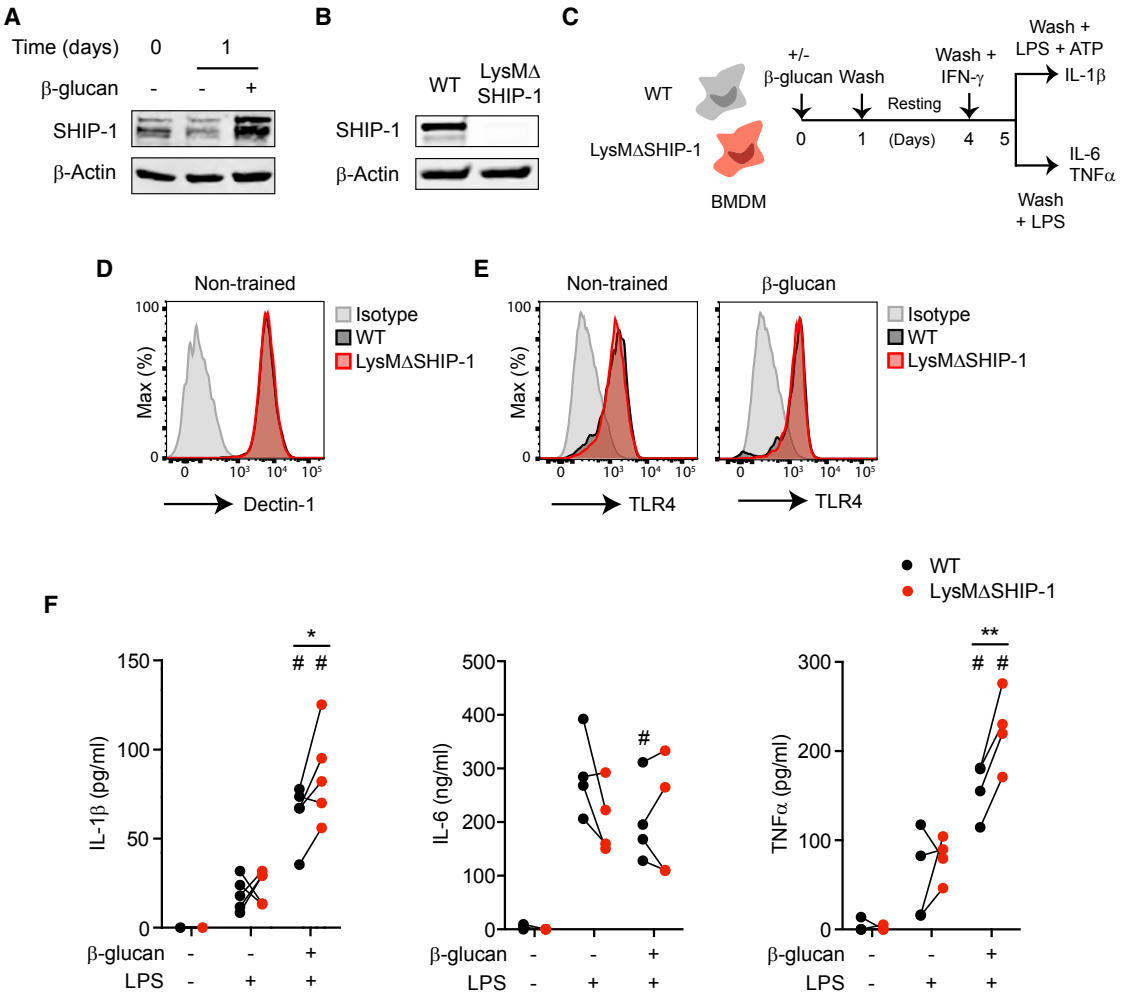

Figure 1. SHIP-1 Deletion Boosts β-Glucan-Induced Trained Immunity in Macrophages

(A) SHIP-1 expression by WB, normalized to β-actin, in bone marrow-derived macrophages (BMDMs) exposed (+) or not (−) to β-glucan (whole glucan particles) for the indicated time. Representative experiment of three performed.

(B) SHIP-1 protein expression in BMDMs. Representative experiment of six performed.

(C) Trained immunity *in vitro* model in mouse BMDMs. See also Figure S1A.

(D and E) Dectin-1 expression in BMDMs before β-glucan stimulation (D) or TLR4 expression both under non-trained (left) or β-glucan-primed (right) conditions, just before LPS stimulation (E), according to Figure 1C. FACS histograms representative of four independent experiments. See also Figures S1B and S1C.

(F) BMDMs were stimulated (+) or not (−) with β-glucan or LPS, and IL-1β (left), IL-6 (middle), and TNFα production (right) was analyzed in supernatants according to Figure 1C.

See also Figure S2. Independent experiments (N = 4 or 5) are shown. *p < 0.05 and **p < 0.01, paired Student's t test comparing wild-type (WT) and LysMΔSHIP-1. #p < 0.05, paired Student's t test comparing stimulated or not with β-glucan within the same genotype.

marrow-derived macrophages (BMDMs) (Walachowski et al., 2017). We initially stimulated BMDMs with purified particulate β-glucan from *S. cerevisiae*, a well-known ligand for Dectin-1 (Rosas et al., 2008). SHIP-1 basal expression in BMDMs was further induced after 1 day of β-glucan stimulation (Figure 1A). To study the potential involvement of SHIP-1 in Dectin-1-triggered trained immunity, we generated BMDMs from wild-type (WT) mice or mice bearing a myeloid-specific deletion of SHIP-1 in the myeloid compartment (LysMΔSHIP-1) (Collazo et al., 2012) (Figure 1B). Next, we adapted the proposed *in vitro* long-term scheme of trained immunity (Quintin et al., 2012) to IFN-γ-primed BMDMs, evaluating whether training with β-glucan boosts cytokine production in response to lipopolysaccharide (LPS) (Figure 1C). Of note, as previously described (Mosser and Zhang, 2008), IFN-γ priming was required to detect LPS-induced cytokines in BMDMs (Figure S1A), regardless of the induction of training. Surface expression of the receptors involved in β-glucan (Dectin-1; Figures 1D and S1B) and LPS (TLR4; Figures 1E and S1C) recognition were comparable between WT and LysMΔSHIP-1 BMDMs. We found that β-glucan-induced training resulted in increased cell viability in WT BMDMs (Figure S2), concurring with previous results (Bekkering et al., 2016; Garcia-Valtanen et al., 2017). Non-trained SHIP-1-deficient BMDMs showed higher viability than

their WT counterparts, but the relative cell number after β-glucan training was similar between genotypes (Figure S2). To ensure the analysis of cell-intrinsic responses as described (Bekkering et al., 2016), cytokine production was normalized to the relative cell number present in each treatment.

Pre-incubation of WT BMDMs with β-glucan prompted a greater production of IL-1β and TNFα in response to LPS (Figures 1F and S1A), reproducing trained immunity (Quintin et al., 2012). Notably, β-glucan-trained LysMΔSHIP-1 BMDMs showed an increased production of these trained immunity-associated cytokines compared with trained WT BMDMs (Figure 1F). Conversely, IL-6 was not induced following training or in the absence of SHIP-1 in this setting (Figure 1F). Of note, SHIP-1 deletion did not affect any of these inflammatory responses under non-trained conditions. These data indicate that SHIP-1 modulates the extent of LPS-induced proinflammatory cytokine production specifically during β-glucan training.

SHIP-1 Regulates Molecular and Metabolic Hallmarks of Trained Immunity

We tested whether the boost in β-glucan training in the absence of SHIP-1 was accompanied by regulation of key hallmarks involved in the process. Regarding the molecular pathway, Akt was phosphorylated in response to β-glucan in a time-dependent manner in WT BMDMs (Figure 2A, left, and Figure S3A), concurring with previous results (Cheng et al., 2014). Notably, LysMΔSHIP-1 BMDMs showed significantly increased and sustained Akt phosphorylation upon β-glucan stimulation (Figure 2A, left, and Figure S3A). The analysis of mTOR targets, S6 and 4EBP1, also revealed a maintained and significantly increased phosphorylation during the treatment with β-glucan in SHIP-1-deficient BMDMs (Figure 2A, right, and Figure S3B). Of note, a basal activation of the Akt pathway occurs in LysMΔSHIP-1 BMDMs. This is consistent with previous results in which the absence of SHIP-1 was associated with Akt overactivation and survival advantage (Antignano et al., 2010; Wang et al., 2002), which would concur with our results in non-trained BMDMs (Figure S2).

Next, we measured the extracellular acidification rate (ECAR) in β-glucan-trained BMDMs in a glycolysis stress test prior to LPS stimulation (Figure 2B). Training with β-glucan for 5 days increased ECAR in WT BMDMs, a metabolic shift that was significantly boosted in trained SHIP-1-deficient BMDMs (Figure 2B), as reflected by enhanced basal (Figure 2C) and maximal (Figure 2D) glycolysis, together with a higher glycolytic reserve (Figure 2E). Increase in glycolytic reserve is the first metabolic signature associated with SHIP-1-deficient BMDMs upon β-glucan training (Figure S4). Consistent with data on signaling pathway activation (Figures 2A and S3), basal enhanced glycolysis was found in LysMΔSHIP-1 BMDMs (Figures 2C–2E and S4), although it did not result in higher cytokine production unless β-glucan-induced training was established (Figure 1F). These results suggest that SHIP-1 controls the extent of the glycolytic switch. Therefore, SHIP-1 deficiency may promote a pro-glycolytic state that could boost inflammatory response upon β-glucan-trained conditions.

To assess whether SHIP-1 could affect epigenetic reprogramming induced by β-glucan, we tested the presence of the activating histone 3 Lys 4 trimethylation (H3K4me3) (Cheng et al., 2014; Quintin et al., 2012). Chromatin immunoprecipitation (ChIP) analysis showed that H3K4me3 was specifically enriched by β-glucan training at TNFα promoter in WT BMDMs and further augmented in trained SHIP-1-deficient macrophages (Figure 2F), concurring with final enhanced TNFα production (Figure 1F). Moreover, inhibition of histone methyltransferases using 5′-deoxy-5′-(methylthio)adenosine (MTA) (Quintin et al., 2012) abolished TNFα overproduction in the absence of SHIP-1 upon training, whereas the histone demethylase inhibitor pargyline (Quintin et al., 2012) did not have any effect (Figure 2G). Considering that β-glucan-induced training relies also on histone acetylation, training in the presence of the histone deacetylase activator resveratrol (Cheng et al., 2014) or the histone acetyltransferase inhibitor EGCG (Ifrim et al., 2014) inhibited the enhanced TNFα produced by trained SHIP-1-deficient macrophages (Figure 2H). These results highlight SHIP-1 as a regulator of trained immunity by dampening the Akt/mTOR molecular pathway and the glycolytic switch and relying on the epigenetic reprogramming induced by β-glucan.

Myeloid-Specific Deletion of SHIP-1 Improves Trained Immunity *In Vivo*

The generation of trained immunity *in vivo* leads to cross-protection against diverse secondary infections (Netea et al., 2016). Signaling through PI3K is the canonical molecular pathway implicated in the development of these trained responses (Arts et al., 2016a; Cheng et al., 2014). To test the role of myeloid SHIP-1 in cytokine production under β-glucan training *in vivo*, WT and LysMΔSHIP-1 mice were challenged with LPS after training with β-glucan (Cheng et al., 2014), and serum cytokines were measured (Figure 3A). LPS-induced levels of IL-6 and TNFα were increased in sera from WT mice upon β-glucan pre-treatment (Figure 3B), indicative of the generation of a trained response (Quintin et al., 2012). Notably, serum levels of IL-1β, IL-6, and TNFα were further increased in LysMΔSHIP-1 trained mice compared with trained WT mice (Figure 3B), supporting the regulatory role of SHIP-1 upon β-glucan training also *in vivo*.

Protective response against lethal systemic *Candida albicans* infection by trained immunity relies on monocytes and macrophages (Quintin et al., 2012). After training with β-glucan, WT and LysMΔSHIP-1 mice were intravenously infected with a lethal dose of the clinical isolate *C. albicans* SC5314 (Figure 3C). Both WT and LysMΔSHIP-1 non-trained mice rapidly succumbed upon these infectious conditions (Figure 3D, dashed lines), indicating that SHIP-1 expression in the myeloid compartment is redundant for the primary response to lethal candidiasis. The protective effect of β-glucan administration against a lethal *C. albicans* infection was significantly improved in LysMΔSHIP-1 mice compared with WT littermates (Figure 3D, solid lines).

As trained immunity can be defined as a protection mechanism from secondary lethal *C. albicans* infection induced by a nonlethal encounter with the same pathogen (Quintin et al., 2012), we trained mice with a low dose of *C. albicans* followed by a lethal dose of the fungus (Figure 3E). Again, the training stimulus enlarged the survival time of WT mice and LysMΔSHIP-1 trained mice were more resistant than WT to lethal systemic candidiasis (Figure 3F, solid lines). Notably, we observed enhanced production of IL-1β and TNFα in *Candida*

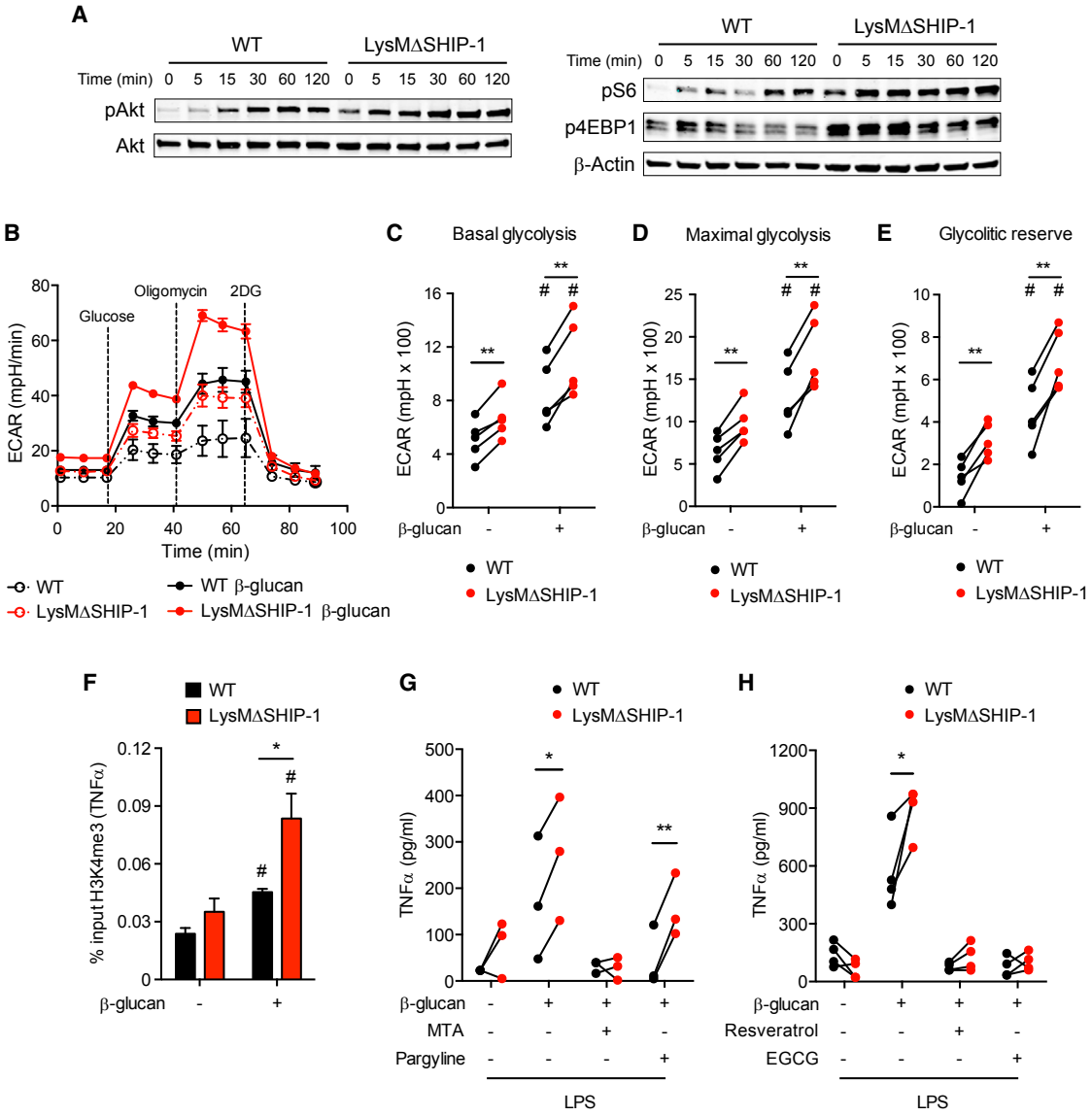

Figure 2. SHIP-1 Regulates Molecular and Metabolic Hallmarks of Trained Immunity

(A) BMDMs were exposed to β-glucan for the indicated time, and phospho-Akt, Akt, phospho-S6, phospho-4EBP1, and β-actin were analyzed by WB. Representative experiment of five performed. See also Figure S3.

(B–E) BMDMs were left untreated (dashed lines) or treated for 1 day with β-glucan (solid lines), washed, rested for 3 days, and re-plated in equal numbers for determination of extracellular acidification rate (ECAR) in a glycolysis stress test upon sequential addition of glucose, oligomycin, and 2-deoxyglucose (2DG) as indicated (B). Analysis of basal glycolysis (C), maximal glycolysis (D), and glycolytic reserve (E). See also Figure S4. Mean ± SEM (B) or individual data (C–E) of five independent cultures are shown.

(F) BMDMs were trained (+) or not (−) with β-glucan for 1 day, washed, and rested for 4 days and chromatin immunoprecipitation (ChIP) against H3K4me3 was performed in which enrichment on the TNFα promoter was analyzed using qPCR. Mean ± SEM of five independent experiments is shown.

(G and H) BMDMs were incubated (+) or not (−) with the methyltransferase inhibitor 5′-deoxy-5′-(methylthio)adenosine (MTA), the histone demethylase inhibitor pargyline (G), the histone deacetylase activator resveratrol, or the histone acetyltransferase inhibitor EGCG (H) for 30 min before β-glucan training and after wash-out. TNFα production was analyzed in supernatants after LPS stimulation according to Figure 1C. Individual data corresponding to three (G) or four (H) independent experiments are shown.

In (C)–(H), *p < 0.05 and **p < 0.01, paired Student's t test comparing WT and LysMΔSHIP-1. In (C)–(F), #p < 0.05, paired Student's t test comparing stimulated or not with β-glucan within the same genotype.

lethally infected kidneys from LysMΔSHIP-1 trained mice (Figure 3G), together with a decreased renal fungal burden (Figure 3H). These data indicate that SHIP-1 deficiency in myeloid cells enhances β-glucan- and *Candida*-induced trained immunity *in vivo*, improving the response to pathogen-specific or heterologous challenges.

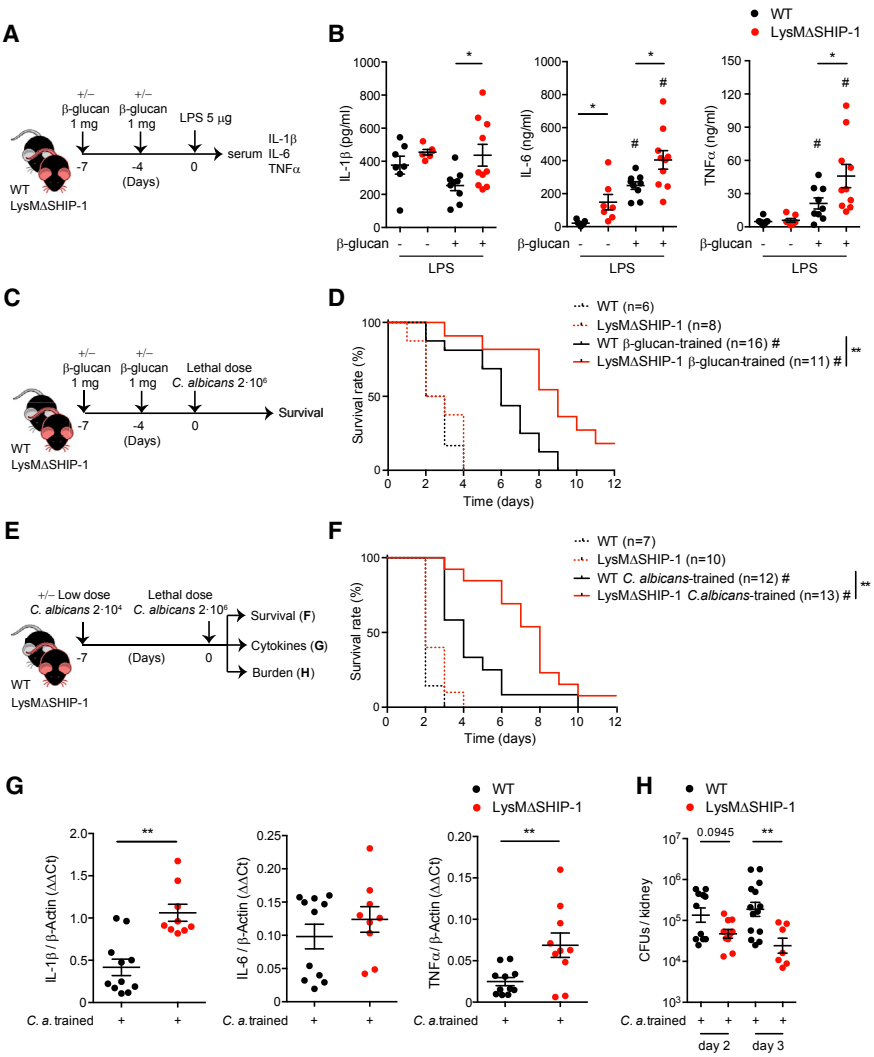

Figure 3. Myeloid-Specific Deletion of SHIP-1 Improves Trained Immunity *In Vivo*

(A) *In vivo* model of training by two intraperitoneal (i.p.) β-glucan injections and secondary i.p. LPS challenge for measuring serum cytokines.

(B) Mice were treated according to Figure 3A. Serum was collected after 60 min (TNFα) or 90 min (IL-1β and IL-6) of LPS challenge, and cytokines were analyzed.

(C) *In vivo* model of training as in (A) but with secondary *Candida albicans* lethal infection.

(D) Survival curve according to Figure 3C.

(E) *In vivo* model of training by a systemic infection with a low dose of *C. albicans* followed by a secondary lethal challenge with the same pathogen.

(F) Survival curve according to Figure 3E.

(G and H) Renal cytokines on day 2 post-infection (p.i.) (G) and kidney fungal burden (H) at indicated time points p.i. were evaluated in trained mice, following model in Figure 3E.

In (B), (G), and (H), single dots correspond to individual mice. Mean ± SEM of two (B and H) or three (G) pooled experiments is shown, including at least 5 mice per condition. *$p < 0.05$ and **$p < 0.01$, unpaired Student's t test comparing WT and LysMΔSHIP-1. #$p < 0.05$, unpaired Student's t test comparing the same genotype stimulated or not with β-glucan. In (D) and (F), a pool of two experiments is shown, including between 6 and 16 mice per group as indicated. **$p < 0.01$, log rank test between WT and LysMΔSHIP-1 mice. #$p < 0.05$, log rank test comparing trained or not with β-glucan (D) or *C. albicans* (F) within the same genotype.

Pharmacological Inhibition of SHIP-1 Enhances Trained Immunity

The relevance of the PI3K pathway in distinct pathologies has promoted the development of chemical SHIP-1 phosphatase inhibitors such as 3α-aminocholestane (3AC; SHIPi) (Brooks et al., 2010). We thus tested 3AC as a potential tool to boost trained immunity. BMDMs were trained with β-glucan in presence of different doses of 3AC (half maximal inhibitory concentration [IC_{50}] = 13.5 μM; Brooks et al., 2015), and LPS-induced TNFα was measured (Figure 4A). Upon β-glucan training, SHIP-1 inhibition boosted TNFα production in a dose-dependent manner (Figure 4B).

To analyze the effect of 3AC SHIPi under *in vivo* infectious conditions, mice were administered SHIPi twice on consecutive days following the published regimen (Gumbleton et al., 2017), and coincident with the second day of 3AC administration, mice were trained with a low dose of *C. albicans*. Seven days later, mice were lethally infected with the same fungus (Figure 4C). Inhibition of SHIP-1 did not affect the survival of non-trained mice (Figure 4D, dashed lines) but improved the survival of *Candida*-trained mice (Figure 4D, solid lines).

To further explore the potential relevance of the use of 3AC SHIPi, we trained human PBMCs in the presence of SHIPi, rested and stimulated with LPS, and cytokine production was measured (Figure 4E). This scheme mirrors the stimulation pattern proposed for human PBMCs elsewhere (Quintin et al., 2012). Importantly, SHIP-1 inhibition boosted IL-1β, IL-6, and TNFα production in these β-glucan-trained human PBMCs (Figure 4F). Thus, our data indicate that SHIP-1 can be targeted with pharmacological inhibitors in both mice and human cells to boost trained immunity.

DISCUSSION

Herein, we define SHIP-1 in myeloid cells as a target to improve trained immunity. Additionally, we provide a pharmacological approach, the SHIP-1 inhibitor 3AC, improving training-induced resistance to *Candida* infection and trained immunity in human PBMCs. Because modulation of myeloid progenitors in the bone marrow is an integral component of trained immunity (Mitroulis et al., 2018), and 3AC administration expands the hematopoietic stem cell compartment (Brooks et al., 2015), SHIP-1

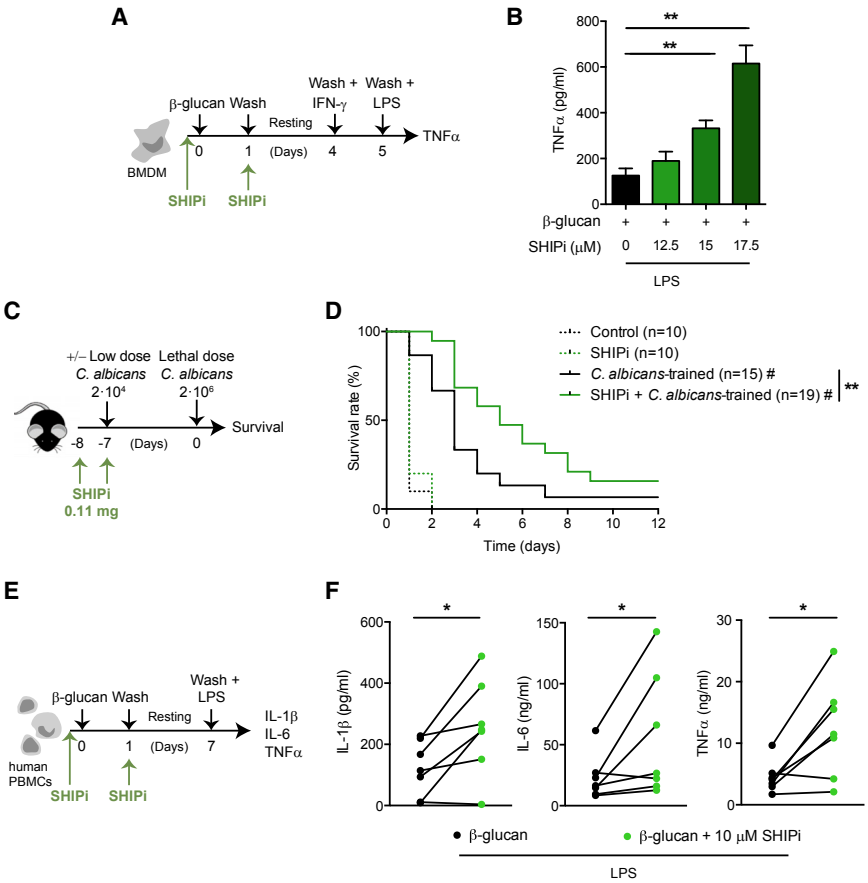

Figure 4. Pharmacological Inhibition of SHIP-1 Enhances Trained Immunity

(A) *In vitro* experimental model applied to mouse BMDMs, indicating when the SHIP-1 inhibitor (SHIPi) 3α-aminocholestane (3AC) was added.

(B) Mouse BMDMs were incubated with the SHIPi at the indicated concentrations. TNFα production was analyzed in supernatants of β-glucan-trained cells after LPS stimulation according to Figure 4A. Mean + SEM of four independent experiments is shown. **$p < 0.01$, paired Student's t test between SHIPi-treated and non-treated cells.

(C) *In vivo* model of training by a systemic infection with a low dose of *Candida albicans* in the presence of SHIPi followed by a second lethal challenge with the same pathogen. When indicated, the inhibitor was administered intraperitoneally.

(D) Survival curve of 0.3% hydroxypropylcellulose (control) or SHIPi-treated mice according to Figure 4C. A pool of two experiments is shown, including between 10 and 19 mice per group as indicated. **$p < 0.01$, log rank test between trained control and SHIPi-treated. #$p < 0.05$, log rank test comparing trained or not with *C. albicans* within the same treatment.

(E) *In vitro* experimental model applied to human peripheral blood mononuclear cells (PBMCs) indicating when SHIPi was added.

(F) IL-1β, IL-6, and TNFα production was analyzed in supernatants of β-glucan-trained human PBMCs after LPS stimulation according to Figure 4E. Samples from seven independent donors are shown. *$p < 0.05$, paired Student's t test.

inhibition could also influence this compartment. Although *Candida*-induced training and the primary response to the fungus are T/B cell independent (Bär et al., 2014; Quintin et al., 2012), systemic inhibition of SHIP-1 can also influence NK and T cells (Gumbleton et al., 2017), and we cannot rule out indirect effects on non-myeloid cells.

We show that SHIP-1 inhibition potentiates the canonical PI3K/Akt activation pathway, which also mediates trained immunity in response to other stimuli such as the bacillus Calmette-Guérin (BCG) vaccine (Arts et al., 2016a). SHIP-1 inhibition could represent a broad strategy to boost trained immunity. Indeed, SHIP-1 displays an inhibitory function in NOD2 signaling (Condé et al., 2012), the BCG-mediated trained immunity pathway (Kleinnijenhuis et al., 2012). Considering that BCG vaccination confers cross-protection to human viral infections (Arts et al., 2018b), SHIP-1 inhibitor could improve the protective effect of BCG.

Enhanced trained immunity could raise as an important host defense mechanism against infections or sepsis (Netea et al., 2016). However, because diverse endogenous danger signals from injured tissues can trigger innate immune memory hallmarks (Crișan et al., 2016b), caution is needed regarding the potential deleterious function of boosting trained immunity in diseases characterized by excessive inflammation. This notion could apply to atherosclerosis (Leentjens et al., 2018), cardiovascular events (Hoogeveen et al., 2017), gout (Crișan et al., 2016a), and a variety of autoimmune diseases and autoinflammatory disorders such as rheumatoid arthritis, systemic lupus erythematosus, and hyper-IgD syndrome (Arts et al., 2018a), in which monocytes and macrophages share a detrimental trained immunity-like phenotype. Similarly, boosting microglia-dependent training (Wendeln et al., 2018) through SHIP-1 inhibition could be detrimental for neurological disorders and stroke. Under these settings, SHIP-1 activators such as AQX-1125 (Stenton et al., 2013) could be potentially used to ameliorate an excessive and detrimental activation of trained immunity.

In conclusion, although studies on trained immunity have focused on the characterization of this phenomenon, strategies to enhance trained immunity deserve further investigation. Our data indicate that the trained immunity process can be boosted by targeting SHIP-1 in myeloid cells. Moreover, SHIP-1 inhibitors could be proposed as potential pharmacological tools to improve trained immunity in clinical settings in which enhancement of inflammatory responses is beneficial.

EXPERIMENTAL PROCEDURES

Mice and Human Samples

Mice were bred at Centro Nacional de Investigaciones Cardiovasculares (CNIC) under specific pathogen-free conditions. WT C57BL/6J mice were

used for SHIP-1 inhibition experiments. LysM$^{+/+}$SHIP-1$^{flox/flox}$ (WT) and LysM$^{Cre/+}$SHIP-1$^{flox/flox}$ (LysMΔSHIP-1) (Collazo et al., 2012) were kept as littermates. Experiments were conducted with 8- to 12-week-old age-matched mice (regardless of gender). Experiments were approved by the animal ethics committee at CNIC and conformed to Spanish law under Real Decreto 1201/2005. Animal procedures were also performed in accordance to European Union (EU) Directive 2010/63EU and Recommendation 2007/526/EC.

Buffy coats from healthy volunteers were obtained from the Andalusian Biobank after ethical approval was obtained from the local Instituto de Salud Carlos III (ISCIII) Research Ethics Committee (PI 36_2017).

Trained Immunity *In Vitro* Models

BMDMs

BMDMs (10^5) were plated in 96-well plates (200 μL final volume; Corning) and stimulated with R10 or 100 μg/mL β-glucan (whole glucan particles, Biothera) for 24 hr. Then, cells were washed and rested 3 days in culture medium. On day 4, unless indicated, BMDMs were washed again and primed with 25 ng/mL IFN-γ (BD Biosciences) for 24 hr. On day 5, a final wash was performed, and cells were stimulated with R10 or 1 μg/mL standard *Escherichia coli* LPS (EK; Invivogen). To measure IL-1β production, following 4 hr of LPS challenge, cells were further stimulated for 2 hr with 5 mM ATP (Sigma-Aldrich), needed for inflammasome activation and pro-IL-1β processing (Schroder and Tschopp, 2010), and supernatants were harvested for ELISA assay. For TNFα and IL-6, after 24 hr of LPS stimulation, supernatants were collected for ELISA.

When required, BMDMs were pre-incubated for 30 min prior to β-glucan stimulation with 500 μM 5′-deoxy-5′-(methylthio)adenosine (MTA), 6 μM pargyline, 50 μM resveratrol, and 50 μM (-)-epigallocatechin-3-gallate (EGCG) (all from Sigma-Aldrich). SHIP-1 inhibitor (SHIPi; 3AC; Calbiochem) was also used at the indicated doses on β-glucan-trained BMDMs (toxic for non-trained cells). Inhibitors were also added in the first wash-out, before the resting period.

To assess receptor expression and cell viability, 6 × 10^5 BMDMs were plated in non-treated 24-well plates (1,200 μL final volume; Corning) and followed the training scheme described above. Dectin-1 expression was evaluated on day 0 prior to β-glucan addition. Cell viability and TLR4 expression were assessed on day 5 before LPS stimulation. At indicated times, cells were collected in PBS/EDTA and stained on ice-cold fluorescence-activated cell sorting (FACS) buffer (PBS/EDTA plus 3% fetal bovine serum [FBS]) for flow cytometry analysis.

For western blotting (WB) assays and ChIP analysis, 3 × 10^6 BMDMs were plated in six-well plates (3 mL final volume; Corning) and stimulated with R10 or 200 μg/mL β-glucan (to maintain mass/cell ratio) for given times. ChIP was performed on day 5, without IFN-γ priming, as detailed below.

To address metabolic status, 3 × 10^6 BMDMs were plated in non-treated 6-well plates (3 mL final volume; Corning) and followed the training scheme described above but with 200 μg/mL β-glucan (to maintain mass/cell ratio). On day 4, without IFN-γ priming, cells were detached in PBS/EDTA, plated at 10^5 cells/well, and rested overnight in R10 prior to the Seahorse XF glycolysis stress test (Agilent Technologies). When glycolytic metabolism was evaluated after overnight stimulation with β-glucan, BMDMs (10^5) were directly plated in 96-well Seahorse cell culture plates (200 μL final volume; Agilent Technologies) and stimulated with R10 or 100 μg/mL β-glucan the following day.

PBMCs

Total PBMCs (5 × 10^5) were plated in 96-well plates (200 μL final volume) and stimulated with 100 μg/mL β-glucan for 24 hr. Then cells were washed and rested for 6 days in culture medium. On day 7, PBMCs were stimulated with 1 μg/mL LPS (EK). After 24 hr, supernatants were collected for IL-1β, IL-6, and TNFα measurement by ELISA. When required, β-glucan-trained PBMCs were pre-incubated for 30 min with 10 μM 3AC (toxic for non-trained cells). Inhibitor was also added together with the first wash-out, before the resting period.

To assess cell viability, 3 × 10^6 total PBMCs were plated in 24-well plates (1,200 μL final volume; Corning) and followed the training scheme described here. On day 7, prior to LPS stimulation, cells were collected in PBS/EDTA and stained on ice-cold FACS buffer for flow cytometry analysis.

For normalization of cytokine production, the fold cell number in each condition was calculated as follows: live cell number/live cell number in average non-trained WT, according to Figure S2. In case of SHIP-1 inhibition experiments, non-treated cells were used as reference. Thus, cytokine production was normalized per cell number as (absolute cytokine value/fold cell number) in each condition.

In Vivo Models

Mice were trained with either two intraperitoneal (i.p.) injections of 1 mg β-glucan particles on days −7 and −4 or 2 × 10^4 *Candida albicans* intravenously (i.v.) on day −7. Sterile PBS was used as control. One week later, mice were challenged with 5 μg *E. coli* LPS (serotype O55:B5; Sigma-Aldrich) i.p., and blood was collected 60 min later to assess serum TNFα (Mouse TNFα DuoSet; R&D Systems) or 90 min later to evaluate serum IL-1β and IL-6. Alternatively, mice were lethally infected with 2 × 10^6 *C. albicans* i.v. and monitored daily for weight, general health, and survival, following institutional guidance. For qPCR analysis of renal cytokines, RNA was purified from whole kidneys on day 2 post-secondary infection (p.i.). Kidney fungal burden at indicated time points p.i. was determined by plating organ homogenates obtained mechanically over 70 μm cell strainers (BD Biosciences) after slicing the tissue, in serial dilutions on YPD agar plates; colony-forming units (CFUs) were counted after growth at 30°C for 48 hr, and data are shown as CFUs in total kidney. When required, mice were i.p. treated with 0.11 mg 3AC on days −8 and −7. 3AC was diluted in PBS 0.3% hydroxypropylcellulose (Sigma-Aldrich), used as control.

Quantification and Statistical Analysis

The statistical analysis was performed using Prism (GraphPad Software). Unless specified, statistical significance for comparison between two sample groups with a normal distribution (Shapiro-Wilk test for normality) was determined using two-tailed paired or unpaired Student's t test. When groups were too small to estimate normality, a Gaussian distribution was assumed. Comparison of survival curves was carried out using the log rank (Mantel-Cox) test. Outliers were identified by means of Tukey's range test. Differences were considered significant at $p < 0.05$ as indicated. Except when specified, only significant differences are shown. As indicated in figure legends, either a representative experiment or a pool is shown, and the number of repetitions of each experiment and number of experimental units (either cultures or mice) is indicated. *In vitro* experiments are shown as a pool of experiments, in which linked WT-LysMΔSHIP-1 dots represent independent cultures that were processed within the same experiment. In this way, an internal comparison between genotypes can be visually done. Different conditions within the same genotype in a particular experiment, although not connected by a matter of clarity, were also pair-analyzed, and statistically significant differences are indicated by pound signs (#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.09.092.

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AUTHOR CONTRIBUTIONS

P.S.-L., C.d.F., P.B., and S.M.-C. performed the experiments. W.G.K. shared reagents. O.M.D. and J.D.C. prepared 3AC for the pharmacological targeting to SHIP-1. P.S.-L., C.d.F., and D.S. conceived and designed experiments, analyzed data, and wrote the manuscript. All authors discussed the results and the manuscript.

DECLARATION OF INTERESTS

C.d.F., P.S.-L., J.D.C., W.G.K., and D.S. have patents pending and issued on the use of SHIPi in modulating immune function. W.G.K. is chief scientific officer at Alterna Therapeutics, which is devoted to developing SHIPi for therapeutic purposes. J.D.C. serves on the Scientific Advisory Board of Alterna Therapeutics. Both J.D.C. and W.G.K. hold equity in Alterna Therapeutics. All other authors declare no competing interests.

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Cell Reports, Volume *25*

Supplemental Information

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

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SUPPLEMENTAL INFORMATION

Figures S1-S4

Supplemental Experimental Procedures

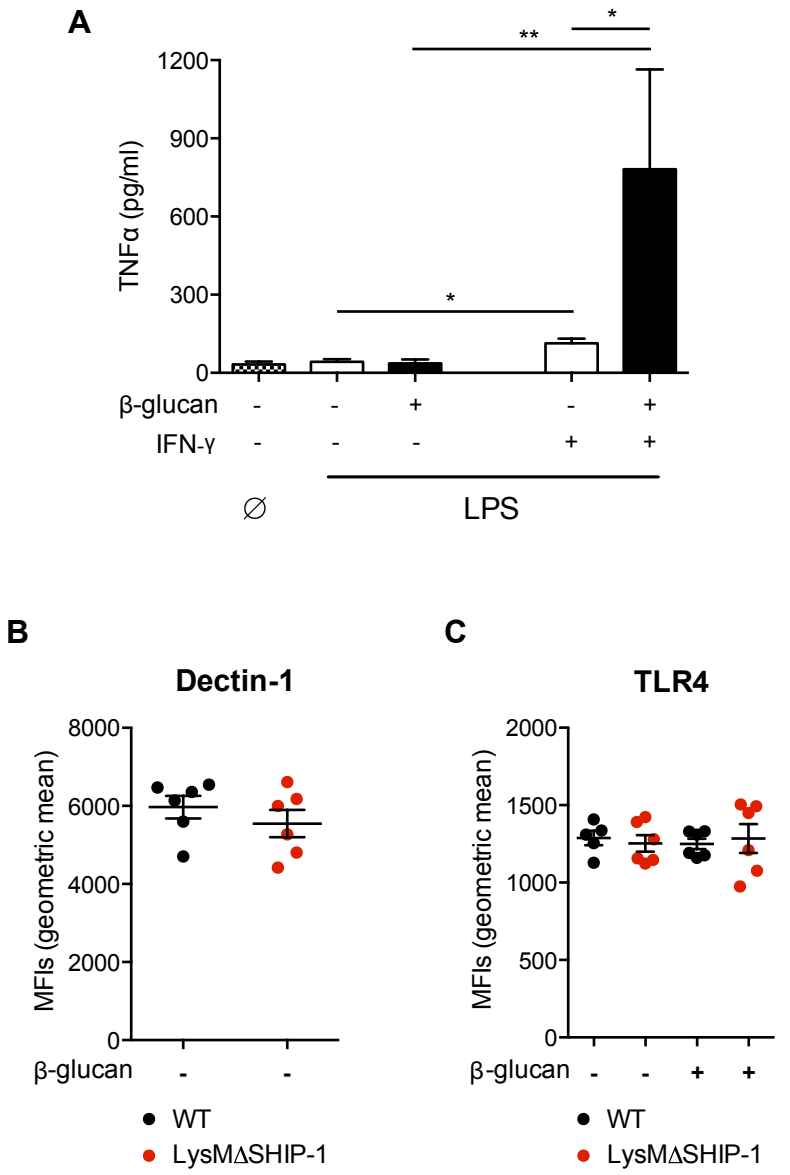

Figure S1 Experimental set-up of trained immunity *in vitro* model applied to BMDMs, related to Figure 1.

(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.

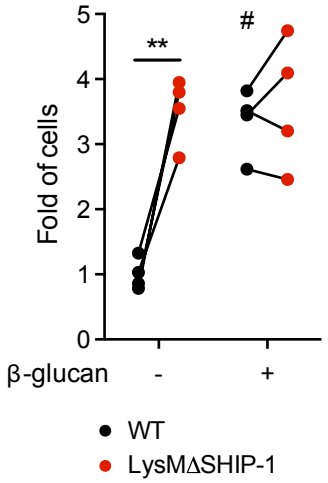

Figure S2. Relative amount of BMDMs recovered before LPS stimulation, related to Figure 1. WT and LysMΔSHIP-1 BMDMs were exposed (+) or not (-) to β-glucan according to model in Figure 1C. At day 5 and before LPS stimulation, the number of viable BMDMs was determined. Fold cell number was calculated 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.

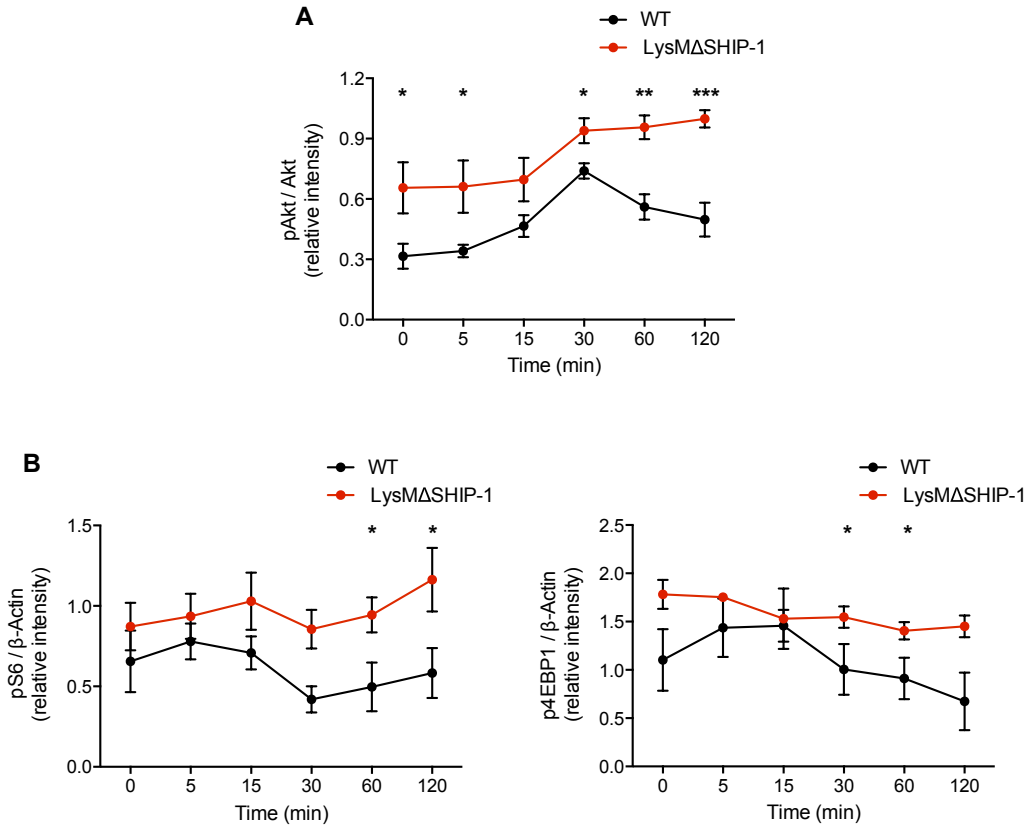

Figure S3. Quantification of WB kinetics, related to Figure 2. WT and LysMΔSHIP-1 BMDMs were exposed to β-glucan for the indicated time and phospho-Akt, Akt (A), phospho-S6, phospho-4EBP1 and β-Actin (B) analyzed by WB and quantified by ImageJ software. Relative band intensity is shown. Mean ± SEM from 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.

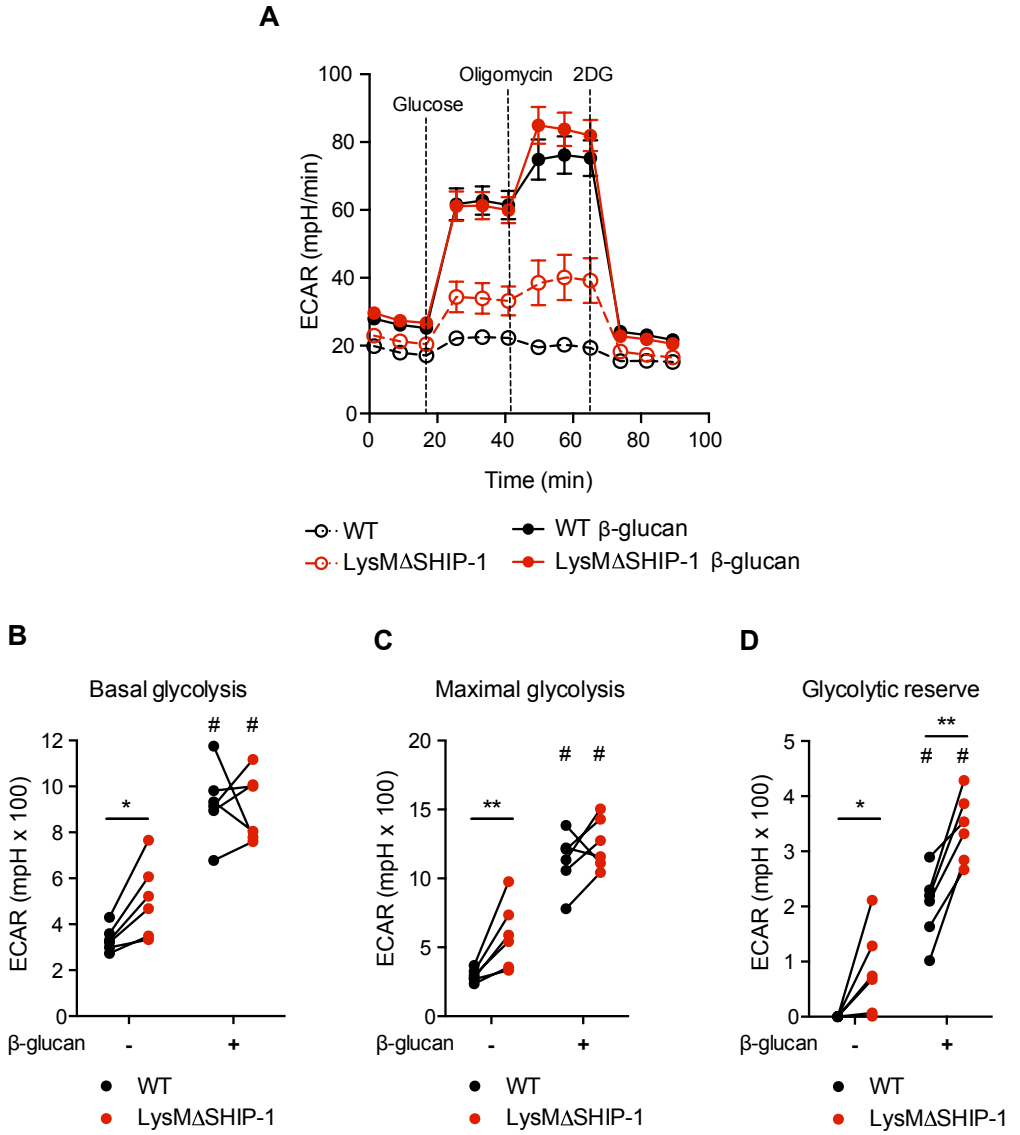

Figure S4. SHIP-1 controls the extent of the early glycolytic metabolism, related to Figure 2. (A-D) WT and LysMΔSHIP-1 BMDMs were left untreated (dashed lines, A) or treated overnight with β-glucan (solid lines, A) and extracellular acidification rate (ECAR) was determined. ECAR in a glycolysis stress 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).