# Pre-Submission report of Ph.D.

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# Role of phosphatases in regulation of anti- leishmanial immune response in macrophage by tuning CD40 signaling.


**Submitted by:**

**Tabish Hasan Khan**

**Under the supervision of:**

**Dr. Somenath Roy,**
**Prof. Dept. of human physiology**
**with community health.**
**Vidyasagar University, Midnapore.**
**West Bengal, India**

**Dr. Bhaskar Saha, Scientist-F**
**Lab No. 5**
**National Centre for cell science**
**Pune-411007**
**Maharashtra. India.**

## Contents

## INTRODUCTION

CD40, a costimulatory molecule expressed on antigen presenting cells such as macrophages, B cells and dendritic cells can induce counteractive immune responses [1, 2]. CD40 has both host protective anti-tumor as well as pro-tumor functions which results from the expression of pro-inflammatory and anti-inflammatory cytokine respectively depending on the intensity of CD40 crosslinking [3-4]. In the experimental infection with Leishmania major, an obligatorily intracellular parasite in mammals, CD40-induced p38MAPK activation and IL-12 expression are suppressed whereas ERK-1/2 activation and IL-10 expression are enhanced [5-7]. In uninfected macrophages, a strong CD40 stimulation induces preferential phosphorylation of p38MAPK and pro-inflammatory cytokine IL-12 production whereas a weaker CD40 stimulation induces preferential phosphorylation of ERK-1/2 and anti-inflammatory cytokine IL-10 production. We argued that such reciprocal phosphorylation of these two MAP kinases was due to differential phosphorylation. As the cycle of phosphorylation and dephosphorylation maintains the homeostasis of cellular signaling and thereby regulates the cellular responsiveness to an external stimulus, averting extraordinarily prolonged activation or suppression of a particular signaling pathway, we propose that the observed reciprocity of MAPK phosphorylation can also be a function of a phosphatase that dephosphorylates these MAPKs differentially.

Phosphotyrosine phosphorylation is crucial for MAPK activation, which indicates the important role of phosphotyrosine phosphatases (PTPs) for their regulation. Src homology-2 (SH-2) domain containing phosphotyrosine phosphatase SHP-1 has been previously shown as a negative regulator of various phosphotyrosine–dependent signaling pathways in hematopoietic cells [6-10]. SHP-1 plays an important role in immune regulation, as the SHP-1-deficient mice or the catalytically inactive SHP-1 expressing mice develop severe motheaten (me/me) or viable motheaten (mev/mev) phenotype, respectively [12-15]. In these mice, hematopoietic cells have increased protein tyrosine phosphorylation in response to extracellular stimuli [16]. SHP-1 has also been implicated in the pathogenesis of various intracellular parasites including Leishmania [17-20]. Leishmania infection activates SHP-1, which modulates various signaling pathways and thus inhibits innate inflammatory response [21-25].

Therefore, it is possible that SHP-1 also plays a role in MAPK dephosphorylation and thereby influences the reciprocity in CD40 signaling. Although CD40 plays important roles in L. major infection, whether SHP-1 influences CD40 signaling and affects L. major infection or vice versa have never been examined.

In this report, we show that SHP-1 does contribute to the CD40 signaling reciprocity by differential p38MAPK and ERK-1/2 dephosphorylation regulating the threshold of CD40-induced p38MAPK/ERK-1/2 phosphorylation. In L. major infection, SHP-1 phosphorylation and activity are increased causing reciprocal down-regulation of CD40-induced p38MAPK phosphorylation. We also show that SHP-1 activation is associated with CD40-induced ERK-1/2 phosphorylation. SHP-1 overexpression renders the resistant C57BL/6 mice susceptible whereas its inhibition by an inhibitor or shRNA protects susceptible BALB/c mice to L. major infection. Thus, we demonstrate for the first time that SHP-1 has at least two functions: one, setting the threshold for CD40-induced MAPK activation at a lower dose and two, specifically regulating CD40-induced p38MAPK phosphorylation at a higher dose of CD40 stimulation, contributing to the CD40 signaling reciprocity. These properties are exploited by the parasite to design the SHP-1-targeted immune evasion strategy ensuring their survival in a mammalian host.

## Review of literature:

**Leishmaniasis**:

Leishmaniasis, an infection encountered predominantly in tropical regions of the world, is caused by protozoan parasites of the *leishmania* genus. *leishmania* parasites are propagated by different species of the sandfly vector (genus *Phlebotomus* or *Lutzomya*). Life cycle of *leishmania* involves two distinct developmental stages. Promastigotes are found within the sandfly and have an elongated shape and long flagellum. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective metacyclic promastigotes, which are found in the mouth parts and anterior gut and do not divide. Once inside the host these differentiate into round or oval amastigotes, which lack flagella (21). In its mammalian host, *Leishmania* enters the macrophages by phagocytosis and exists as an obligate intracellular pathogen. At present 20 species of leishmania are known which are charecterized by their tissue tropism . Visceral leishmaniasis, also known as kala-azar is caused by L. donovani. Its symptoms include fever, diarhhoea and enlargement of liver and spleen. Cutanoeus leishmaniasis is predominantly caused by L. major and L. mexicana. its symptoms include single or multiple localized lesions on skin.

**CD40 and CD40 ligand**:

CD40, a costimulatory molecule of 48 kDa is a member of the tumor necrosis factor receptor superfamily, found on the surface of B lymphocytes, dendritic cells, macrophages, hematopoietic progenitor cells and epithelial cells. CD40 interacts with CD40 ligand (CD154) expressed primarily by activated T-cells and also by activated B-cells and platelets. The interaction between CD40 and its ligand CD154 results in Th subset skewing to Th1 type and thus serves to protect the host organism against various infections (5). CD40 –CD40 ligand interaction also stimulate macrophages to produce a number of cytokines and inflammatory mediators which plays a key role in parasite killing.

**Mitogen-Activated Protein Kinases( MAPKs)**:

There are three groups of MAPKs in mammalian cells – the extracellular signal-regulated protein kinases (ERK), the p38 MAP Kinases, and the c-Jun $NH_2$ –terminal kinases (JNK). MAPK phosphorylate various intracellular proteins, including transcription factors, which subsequently regulate gene expression by transcriptional and posttranscriptional mechanisms

(28) . MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of a MAPK module. In general, ERK activity is induced by growth promoting mitogenic stimuli. By contrast, JNK and p38 activities are mainly induced by environmental stress and by pro-inflammatory stimuli and cytokines, such as lipopolysaccharide (LPS), interleukin-1 (IL-1), transforming growth factor-β (TGFβ) and tumour-necrosis factor (29-31.)

**Protein Phosphatases**:

Protein phosphorylation is a reversible modification catalysed by protein kinases and reversed by protein phosphatases. The balance of protein phosphorylation is maintained by the action of protein tyrosine kinases (PTKs) and PTPs, which thus keeps a tight regulation of PTP and PTK activities crucial for proper signal transduction (32). Protein phosphatases are divided according to their substrate specificity- protein serine/threonine Phosphatases (PSTPs), protein tyrosine Phosphatases (PTPs) and Dual specificity phosphatases(DUSPs).

**Src-2 homology domain containing phosphatase-1(SHP-1):**

*Leishmania* infection causes activation of various phosphatases that inhibit intracellular signaling cascades. It causes the induction of a macrophage PTP, SHP-1 also called SHPTP-1, which is expressed principally in hematopoietic (21). It contains two SH2 domains in its N-terminal portion, a phosphatase domain conserved in a central position and a C-terminal tail . SHP-1 interacts with its substrates through the two –SH2 domains (24). SHP-1 is involved in negative regulation of many signaling pathways. Macrophages infected with *Leishmania* parasite shows elevated levels of SHP-1 activity (25). The SH2 domains of SHP-1 bind to receptors causing selective dephosphorylation of tyrosyl residues and inhibition of IFN-γ inducible JAK2 and MAPK signaling pathways and thus helps in the installation and progression of *Leishmania* infection in vitro and in vivo. SHP-1 also plays a vital role in limiting the activation of the JAK/STAT pathways following cytokine receptor stimulation. SHP-1 is a negative regulator of p38MAPK and Erk1/2. At high dose of CD40 stimulation, activity of SHP-1 is reduced resulting in activation of p38 MAPK which induces IL-12 production causing activation of anti-leishmanial immune response . However, at low dose of CD40 interaction, SHP-1 is induced resulting in inhibition of p38MAPK hence the pathway is skewed towards the activation of ERK1/2 which causes IL-10 secretion that helps in the progression of the *Leishmania* infection.

**AIMS AND OBJECTIVES:**

1. To check the role of phosphatase SHP-1 in CD40 signaling in uninfected and Leishmania infected macrophages.
2. To check the regulation of different MAPKinases by phosphatase SHP-1 in CD40 signaling.
3. To check the role of phosphatase SHP-1 in CD40 induced effector functions

## MATERIAL AND METHODS

**Mice, parasites and infection**: BALB/c and C57BL/6J mice were originally obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, ME) and were subsequently bred in the Institute's experimental animal facility in Thoren Caging systems. Leishmania major (strain MHOM/Su73/5ASKH) was maintained in vitro in RPMI-1640 medium, supplemented with 10% FCS (GIBCO BRL; Grand Island, NY) and the virulence was maintained by passage through BALB/c mice. $2x10^6$ L. major promastigotes in the stationary phase, were used to infect mice subcutaneously in the hind footpad. In experiments with lentivirus treatment, mice were injected subcutaneously in the hind footpad with $5x10^6$ transduction units (TU) of lentivirus expressed SHP-1 shRNA, control eGFP shRNA, SHP-1WT or control vector 2 days after L. major infection. Some mice were treated with bpV(phen) (2.5 μmol/mouse) or anti-CD40 (50μg / mice; clone 3/23) i.p for three alternate days beginning $4^{th}$ day after infection. Disease severity was assessed by measurement of footpad swelling using a digital micrometer (Mitituyo, Japan) and parasite burden.

**Macrophage culture and L. major infection of macrophages**: Thioglycolate-elicited BALB/c-derived peritoneal macrophages were maintained in RPMI-1640 medium, supplemented with 10% FCS. Macrophages were infected with Leishmania promastigotes at a ratio of 1:10 for 6h (5). After washing of extracellular parasites, macrophages were treated with bpV (phen) for 1h at the indicated doses. Following treatment fresh media was added and cells were incubated with or without anti CD40 antibody (3μg/ml) for 72 h. Macrophages were then fixed, Giemsa-stained and counted to calculate the number of amastigotes per 100 macrophages, as described earlier (5-7).

**Western blotting**: Macrophages were washed twice with chilled PBS and lyse in NP-40 lysis buffer (20mM Tris (pH 7.4), 150mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA, protease inhibitor cocktail (Roche Applied Science; Mannheim, Germany) and phosphatase inhibitor cocktail (Pierce; Rockford, IL). Protein was quantified by BCA kit (Pierce; Rockford, IL) and equal amount of protein was run on SDS–PAGE. Resolved proteins were blotted to PVDF (Millipore; Bedford, MA) and then blocked with 5% nonfat dried milk in TBST (25mM Tris, pH 7.6, 137mM NaCl, and 0.2% Tween20). Membranes were incubated with primary antibody at $4^oC$ overnight, washed with TBST, incubated with HRP-conjugated secondary antibody. Immunoreactive bands were visualized with the Luminol reagent, purchased from

Santa Cruz Biotechnology. Densitometric analysis of bands were performed using Quantity One 4.6.1(Basic) software (Bio-Rad; Hercules, CA)

**Immunoprecipitation:** After treatment cells were washed twice with ice-cold PBS and harvested at 4°C in an immunoprecipitation lysis buffer (20mM Tris (pH 7.4), 150mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA, protease inhibitor cocktail and phosphatase inhibitor cocktail). Equal amounts of proteins were immunoprecipitated using anti-SHP-1 antibody and collected with protein A+G Agarose (Pierce; Rockford, IL) beads at 4 °C for 16 h. The immunoprecipitates were then washed three times in cold lysis buffer and subjected to Western blot analysis.

**SHP-1 activity assay**: Cell lysates were subjected to immunoprecipitation with anti SHP-1 antibody, immunecomplexes were washed three times with ice-cold lysis buffer (20mM Tris (pH 7.4), 150mM NaCl, 1% NP-40) and SHP-1 activity was assayed by using PTP assay Kit 2 (Upstate; Lake Placid, NY) according to manufacturer's protocol. Briefly, immunecomplex pellets were resuspended in pNPP Tyr assay buffer (1 mg/ml BSA, 25 mM HEPES pH 7.2, 50 mM NaCl, 2.5 mM EDTA, and 10 mM DTT. The reaction was initiated by the addition of p-nitro phenyl phosphate (pNPP) and incubated for 2h at $37^0$C, absorbance of the samples was measured at 410 nm in a spectrophotometer.

***Quantitative real time PCR:*** RNA was isolated using TRI reagent (Sigma Aldrich; St. Louis, MO) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen Life Technologies ; Carlsbad, CA ), as described previously (5-7). The reactions were performed in thin wall 0.2ml strip tubes (Axygen; Union city, CA ) in 10µl reaction mixture containing 10ng cDNA, 2ng forward primer, 2ng reverse primer and 5µl of 2x IQ$^{TM}$ SYBR Green supermix (BIO-RAD; Hercules, CA). Quantitative real-time PCR was performed on the Eppendorf RealPlex$^4$ mastercycler under following conditions: $95^0$C for 2min, 40 cycles of $95^0$C for 1min, $60^0$C for 30 sec, $72^0$C for 35 sec. Reactions were performed in duplicates. Relative quantitation was done using ΔΔ comparative threshold ($\Delta C_t$) method. mRNA expression levels of the target genes were normalized against those of GAPDH levels and expressed as relative fold change compared to untreated controls. Reverse-transcriptase (RT) PCR was performed as described previously (6,7) using primers specific for mouse IL-12p40, IL-10 and inducible nitric oxide synthase-2. Transcripts encoding β-actin were used for normalization.

**Transient transfection studies**: P388D1 macrophage cell line (70–75% confluence) was transfected using LipofectAMINE 2000 (Invitrogen Life Technologies; Carlsbad, CA). 4µg of SHP-1 WT construct or empty vector were used for transfection at DNA:Lipofectamine ratio of 1:3. Transfection was carried out in serum free media (optiMEM, Invitrogen Life Technologies; Carlsbad, CA), after 6h of transfection media was changed with RPMI-1640 supplemented with 10% FCS. After 48h, cells were stimulated for indicated time with anti-CD40 antibody (3µg/ml) and harvested for western blot analysis or Real Time PCR analysis. SHP-1 siRNA, ERK1 siRNA, control siRNA, transfection medium and transfection reagent were procured from Santa Cruz Biotechnology (Santa. Cruz, CA). siRNA transfection was carried out according to manufacturer's protocol.

**Cytokine ELISA**: Peritoneal macrophages were stimulated with anti-CD40 (3µg/ml) or lymph node cells isolated from different groups of mice were stimulated with anti-CD3 (0.5µg/ml) and anti- CD28 (2µg/ml) for 48 h. Culture supernatants were assayed for IL-12, IL-10 and IL-4, IFN-γ content respectively by ELISA according to manufacturer's protocol. Briefly ELISA plates were coated overnight at $4^0$C with purified capture antibody (BD Bioscience; San Diego, CA). Plates were washed three times (0.05% (vol/vol) Tween20 in PBS) and were blocked for 2h at RT with blocking buffer (1% (wt/vol) BSA and 0.05% (vol/vol) Tween20 in PBS). Plates were washed three times and then incubated overnight at $4^0$C with standards or culture supernatants. Plates were then washed and the biotin-conjugated detection antibodies (BD Bioscience; San Diego, CA ) were added for 1h at RT. Plates were then washed and peroxidase-conjugated streptavidin (Roche Applied Science; Mannheim, Germany) was added for 45 min at RT. Thereafter the plates were washed six times, TMB substrate (BD Bioscience; San Diego, CA) was added and color was allowed to develop for 15 min in the dark. Reaction was then stopped by the addition of 1N $H_2SO4$ in distilled water and absorbance was measured at 450 nm.

**RT-PCR**: BALB/c derived macrophages were infected with Leishmania and then both uninfected and infected macrophages were cultured for different time points, after that total RNA was extracted using Tri reagent (Sigma). For cDNA synthesis, 2 µl g of total RNA from each sample was incubated with random primer, 0.1M dithiothreitol, 500µM dNTPs, 40U RNase inhibitor, and 1µl of MMLV-reverse transcriptase (Promega) per 25 µl of reaction mixture. Samples were then incubated at $37^0$C for 1h followed by 5min. incubation at $68^0$C. cDNA from each sample was amplified with Taq DNA Polymerase (Invitrogen) in 25 µl under following conditions: $95^0$C for 2min., appropriate annealing temperature for 1 min and

$72^0$C for 1min, for a total of 30 cycles. Each sample was amplified for mouse GAPDH to ensure equal cDNA. Amplified PCR products were run in 1.2% agarose gel.

**In vitro lentiviral transduction**: Peritoneal macrophages were transduced with a third generation lentiviral vector encoding either SHP-1 shRNA, eGFP control shRNA, SHP-1 WT or an empty vector for 12h in complete media supplemented with 8μg/ml polybrene. Cells were washed to remove residual virus and 48h later, cells were stimulated with anti-CD40 (3 μg/ml) for indicated time and harvested for western blot analysis or ELISA.

**Statistical analyses**: The in vitro experiments were always set in triplicates whereas the in vivo experiments comprised at least five mice per group. The experiments were repeated at least three times. The statistical significance of the differences between the means was deduced by student's t-test. The results were plotted as mean±standard error of means.

**Result and Discussion:**

**SHP-1 activation is differentially modulated by CD40 in L. major infection and it reciprocally regulates CD40-induced p38 and ERK1/2 phosphorylation.**

Macrophage expressed CD40 has reciprocal outcome in *L. major* infection, a lower dose of anti-CD40 leads to disease progression whereas a higher dose leads to disease suppression (6). Because SHP-1 is a key negative regulator of macrophage signaling in *Leishmania* infection, we tested CD40 modulated activation of SHP-1 in *L. major* infection. BALB/c-derived peritoneal macrophages- uninfected or infected with *L. major*- were stimulated with different doses of anti-CD40 antibody. Maximum SHP-1 phosphorylation was observed at a lower dose (1μg/ml) of anti-CD40 antibody treatment in uninfected as well as in *L. major* infected macrophages. With increasing doses of anti-CD40, phosphorylation of SHP-1 was decreased (Fig. *1A*). However, at any given dose of anti-CD40, compared to uninfected macrophages, SHP-1 phosphorylation was higher in infected macrophages(Fig. *1A*).

Similar results were observed for uninfected or infected macrophages stimulated with different doses of CD40 ligand (CD40L) (Fig. *1B*). These data indicate that activation of SHP-1 is reciprocally related to the strength of CD40 signal. Because phosphorylation of p38MAPK and ERK-1/2 are reciprocally controlled by CD40 stimulation and *L. major* infection, we investigated whether altered SHP-1 activation in *L. major* infection can influence the CD40-induced p38MAPK and ERK-1/2 phosphorylation in vivo. Peritoneal macrophages from *L. major* infected BALB/c mice showed enhancement of CD40-induced SHP-1 and ERK1/2 phosphorylation and reduction in p38MAPK activation, as compared to naïve macrophages(Fig. *1C*). Altogether, the data suggest that as a function of CD40 stimulation dose SHP-1 is activated at a lower dose but inhibited at a higher dose. In *L. major* infection, its phosphorylation is enhanced and show similar profile with ERK1/2 activation but reciprocal with p38MAPK activation implying a selective association with these two MAP kinases.

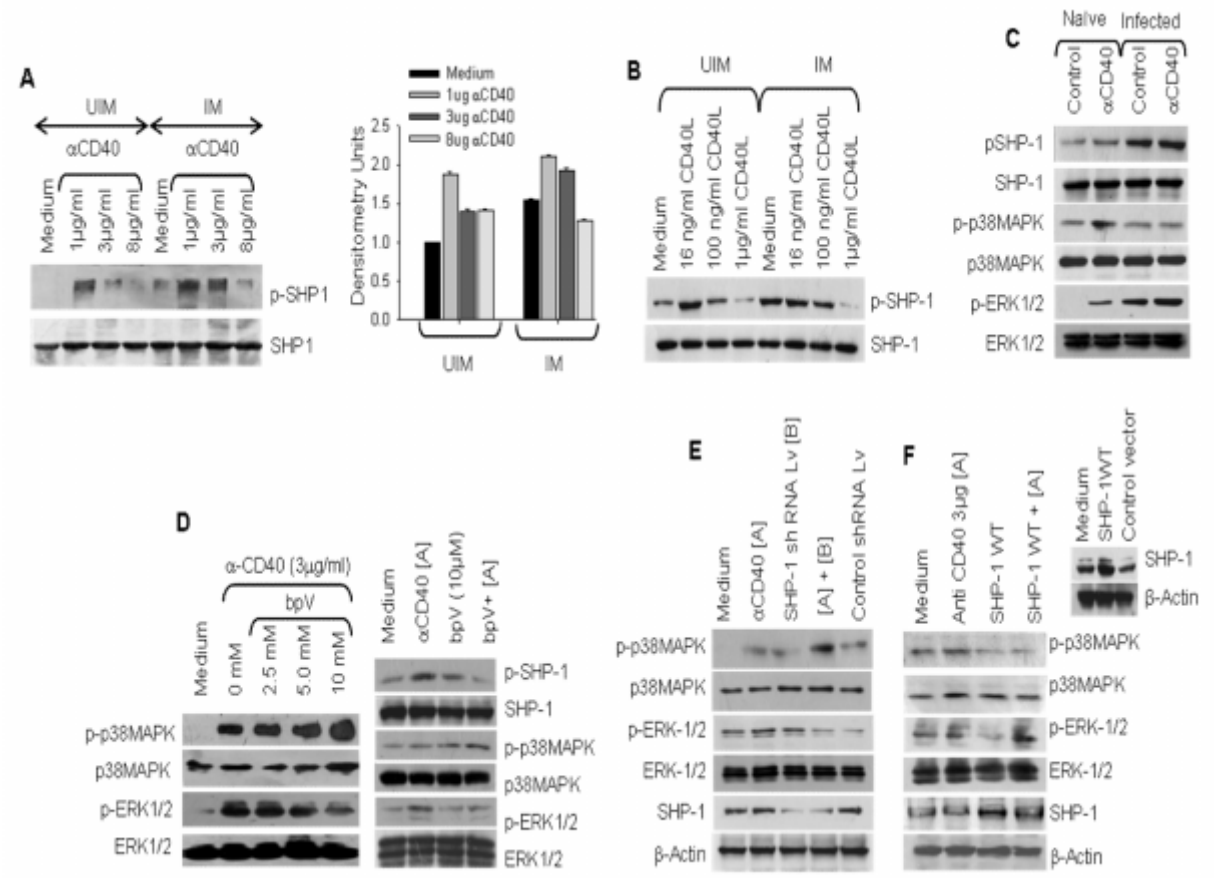


Fig 1: SHP-1 phosphorylation is differentially modulated by CD40 in *L. major* infection (A-B) and SHP-1 reciprocal regulation of CD40 induced p38 MAPK and ERK1/2 phosphorylation (C-F)

We next examined the role of SHP-1 in regulation of CD40-induced p38MAPK and ERK-1/2 phosphorylation. It was observed that with the increasing doses of bpV(phen) a pharmacological inhibitor of SHP-1 activation [26], CD40-induced p38MAPK phosphorylation was enhanced but reciprocally, ERK-1/2 activation was reduced (Fig. 1*D* left panel) however bpV (phen) alone does not have significant effect (Fig. 1*D* right panel). To address the role SHP-1 in CD40 signaling more specifically, peritoneal macrophages were transduced with lentivirally expressed SHP-1 shRNA and control eGFP shRNA. Recapitulating the effect of bpV (phen), lenti-virally expressed SHP-1 shRNA significantly enhanced CD40-induced p38MAPK phosphorylation but decreased ERK-1/2 phosphorylation (Fig. 1E). In contrast, SHP-1 overexpression in macrophage cell line P388D1 resulted in a reverse profile of CD40-induced p38MAPK and ERK-1/2 activation (Fig. 1F). Taken together, all these data suggest that SHP-1 negatively regulate CD40-induced p38MAPK phosphorylation.

**ERK1/2 directly influences CD40-induced SHP-1 activation**. To investigate the role of p38 and ERK1/2 on CD40 induced SHP-1 phosphorylation, peritoneal macrophages were pre-treated with different doses of SB203580, a p38MAPK inhibitor and PD098059, an inhibitor of MEK1/2, an upstream kinase of ERK-1/2 signaling pathway. It was observed that anti-CD40 antibody induces SHP-1 phosphorylation, which was further enhanced with p38MAPK inhibition by SB203580, whereas reciprocally its phosphorylation was completely abrogated with PD098059 (Fig. 2A). SB mediated increased SHP-1 phosphorylation with anti-CD40 stimulation coincided with reduced p38MAPK but it significantly enhanced ERK-1/2 phosphorylation. In contrast, PD mediated inhibition of SHP-1 phosphorylation res ulted in a reverse profile of p38 MAPK and ERK-1/2 phosphorylation (Fig. 2A).

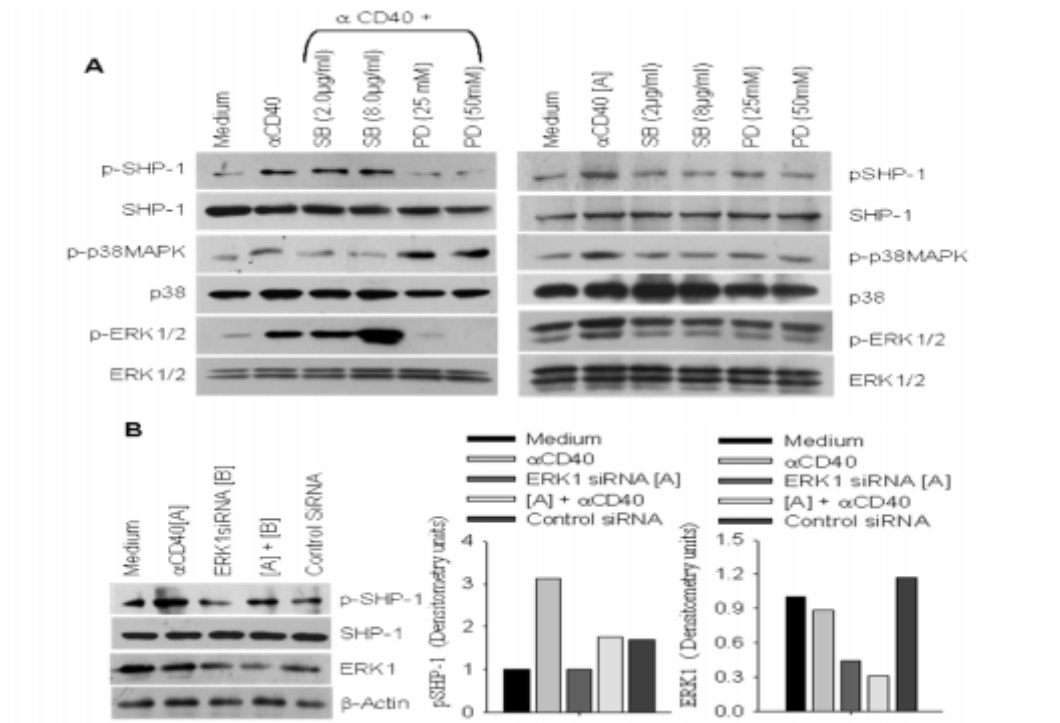


Fig 2: The effect of inhibition of MAP Kinases on SHP-1 phosphoryalation and activation

Because CD40-induced SHP-1 activation is inhibited with ERK-1/2 inhibition, it indicates that SHP-1 activation may be mediated through ERK-1/2 pathway. To confirm it further, macrophage cell line P388D1 was transfected with ERK-1 siRNA and stimulated with anti-CD40 to check the activation of SHP-1. Indeed ERK-1 inhibition significantly reduced CD40-induced SHP-1 phosphorylation (Fig. 2B) suggesting that SHP-1 activation is directly mediated through ERK-1/2 activation.

**SHP-1 functions as a threshold determinant for CD40-induced p38 MAP Kinase activation**. All these observations suggested that SHP-1 and p38 activation are connected reciprocally such that activation of SHP-1 results in dephosphorylation of p38MAPK. We have previously reported that CD40 induces low p38MAPK phosphorylation at lower doses of anti-CD40 stimulation in uninfected or infected macrophages [6]. Because SHP-1 achieves maximum activation at lower doses of anti-CD40 stimulation, we next tested, whether it suppresses p38MAPK phosphorylation at lower doses. Indeed, inhibition of SHP-1 expression by lentivirally expressed shRNA against SHP-1 (Fig. 3A) or inhibition of its activity by bpV (phen) (Fig. 3B) resulted in enhanced p38MAPK phosphorylation even at a very low dose of anti-CD40 stimulation, which resulted in reciprocal decrease in ERK1/2 phosphorylation.

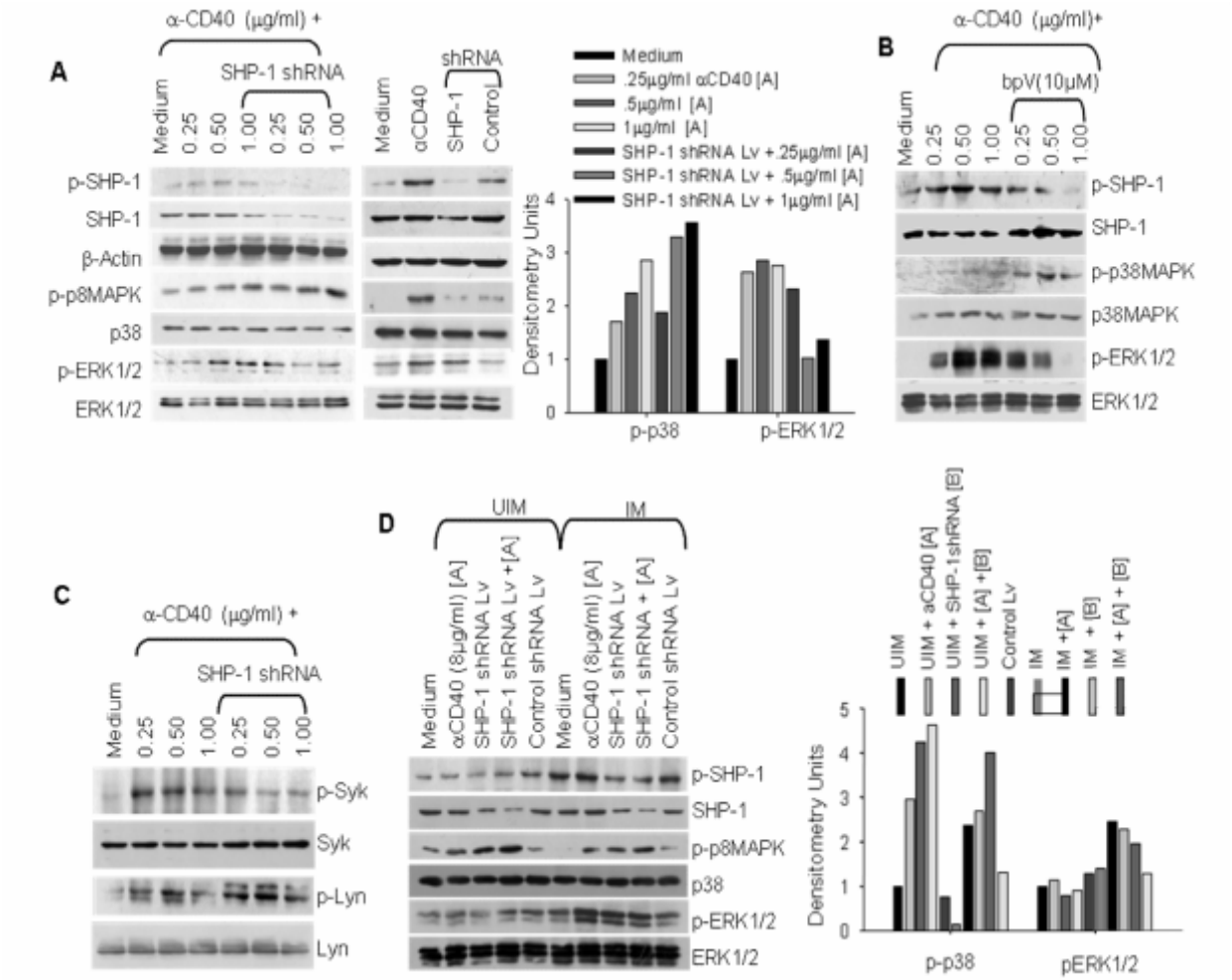


Fig 3: The role of SHP-1 as a threshold determinant for CD40-induced p38 MAP Kinase activation

As reported earlier that low doses of CD40 stimulation induces upstream kinase Syk which in turn activates ERK1/2 and promotes IL-10 production and reciprocally inhibits Lyn, p38MAPK activation and thereby IL-12 production (7). We next investigated the effect of SHP-1 inhibition on Lyn and Syk phosphorylation at lower doses of CD40 stimulation. SHP-1 inhibition by a

lenti-virally expressed shRNA resulted in decreased phosphorylation of Syk but increased Lyn phosphorylation at very low doses of CD40 stimulation (Fig. 3C). It has been shown previously that L. major infection leads to suppression of p38MAPK phosphorylation even at higher doses of anti-CD40 stimulation [6], we next tested whether SHP-1 inhibition can restore p38MAPK phosphorylation in infected macrophages at a higher dose of anti-CD40 stimulation. Indeed, SHP-1 inhibition by a lentivirus-expressed shRNA resulted in partial restoration of p38MAPK phosphorylation at a higher dose of anti-CD40 stimulation in infected macrophages (Fig. 3D). These data suggests that SHP-1 can in fact work as a threshold determinant for the CD40-induced p38MAPK activation. This may happen through selective regulation of Lyn and Syk phosphorylation. Whether this is due to differential recruitment of these tyrosine kinases to CD40 or due to differential phosphorylation or dephosphorylation is not known, nevertheless, SHP-1 does have an initial impact in regulating CD40-induced MAPK activation that controls cellular responsiveness to a variety of stimuli.

***SHP-1 differentially regulates CD40-induced effector function.*** CD40-induced p38MAPK and ERK-1/2 phosphorylations are associated with expression of pro-inflammatory molecules like IL-12, iNOS2 and anti-inflammatory cytokine IL-10 respectively. Because SHP-1 regulates CD40-induced p38MAPK and ERK-1/2 activation, we next examined its role in the regulation of these effector functions. P388D1 cells transfected with SHP-1 siRNA (Fig. 4A, Left panel) show CD40-induced decreased production of anti-inflammatory cytokine IL-10 but increased production of pro-inflammatory cytokine IL-12 (Fig. 4A, Right panel). However, lentivirus-mediated SHP-1 overexpression significantly enhanced CD40-induced IL-10 production (Fig. 4B, Left panel) but reciprocally reduced IL-12 production (Fig. 4B, Right panel), but control vector fails to do so. Because iNOS2 mediated NO is required for the killing of intracellular parasite, we next investigated the role of SHP-1 in CD40-induced iNOS2 expression. It was observed that SHP-1 siRNA enhanced (Fig. 4C Left panel) whereas lenti-virus mediated SHP-1 overexpression significantly reduced CD40-induced iNOS2 expression (Fig. 4C right panel), in comparison to control siRNA or control Lv. Overall, these data indicate that SHP-1 acts as a negative regulator of CD40-induced pro-inflammatory cytokine and IL-10-mediated autocrine regulation of anti-leishmanial functions.

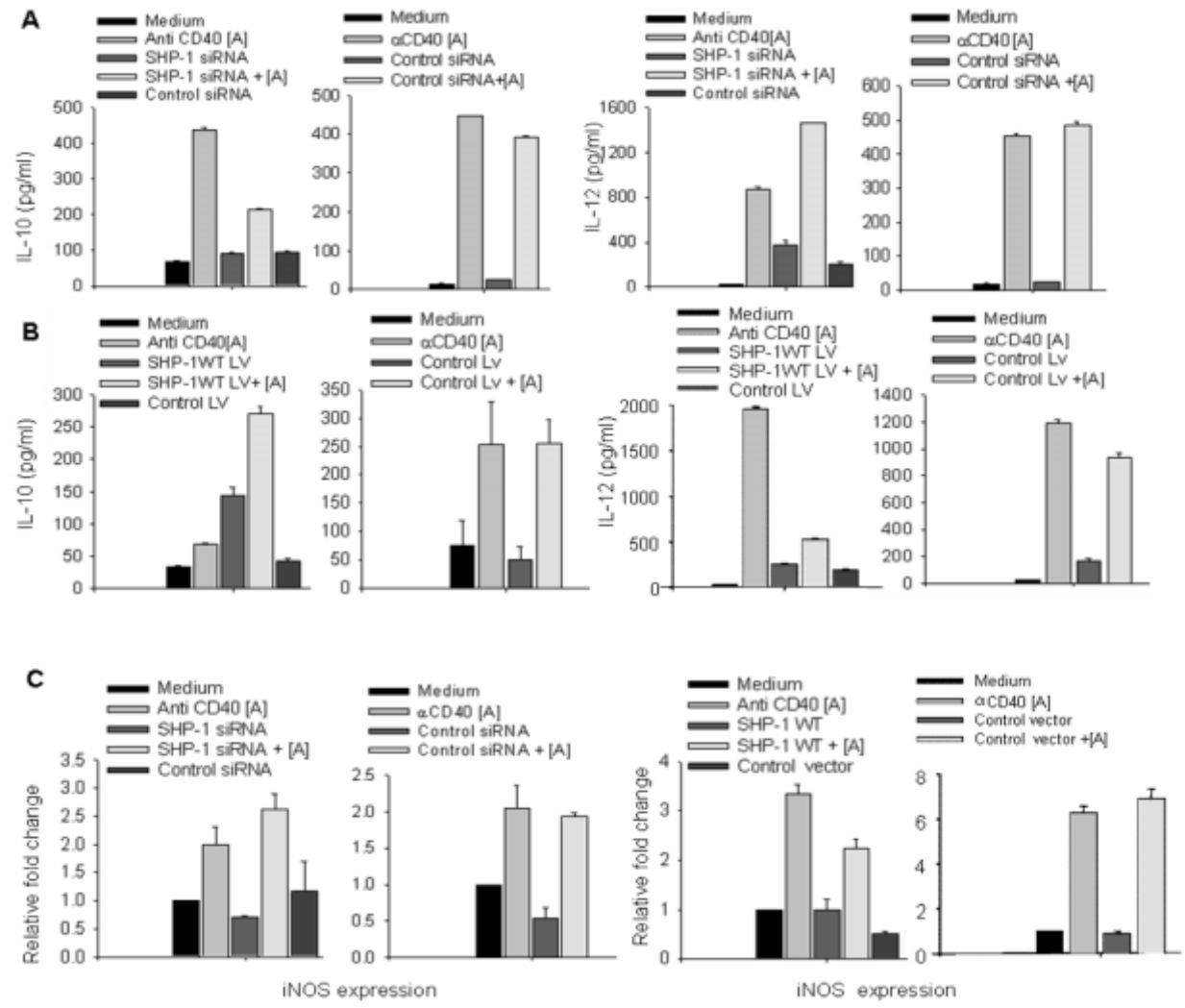


Fig 4: SHP-1 differentially regulates CD40-induced effector function

**SHP-1 over-expression renders resistant B6 mice susceptible to L. major infection.** Because SHP-1 overexpression resulted in enhanced CD40-induced proparasitic cytokine IL-10 and reduced anti-leishmanial cytokine IL-12 and iNOS2 expression, we tested whether its overexpression can make resistant mice susceptible to *L. major* infection. C57BL/6J mice were injected with *L. major* parasite and then injected with either lentivirally overexpressed-SHP-1 or control Lv. Four weeks later footpad lesions and lymph node cells were tested for SHP-1 overexpression. A significant enhanced expression was observed in the footpad lesion as well as lymph node cells of SHP-1 Lv treated mice as compared to control treated mice (Fig 5A). C57BL/6J mice were infected with *L. major* and then injected with lentivirally overexpressed-SHP-1 or control vector followed by treatment with anti-CD40 antibody. It was observed that mice injected with lentivirally overexpressed SHP-1 show increased disease progression (Fig. 5B) and enhanced parasite burden (Fig. 5C) compared to mice either treated with control virus or anti-CD40 antibody alone. Consistent with the disease progression, lymph node cells from the

mice injected with Lv-SHP-1 show increased CD3e and CD28 induced IL-4 and decreased IFNγ expression (Fig5D) compared to cells from mice treated infection alone or control virus.

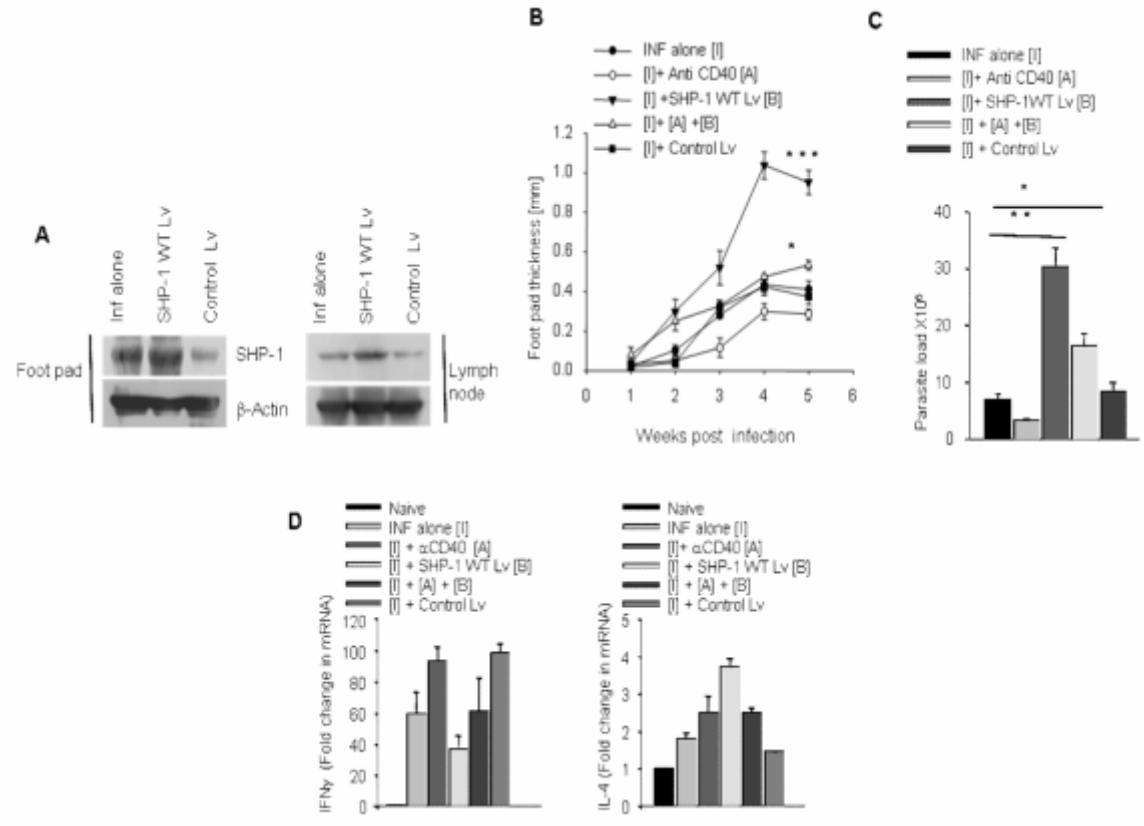


Fig 5: SHP-1 over-expression makes resistant B6 mice susceptible to L. major infection

**SHP-1 inhibition protects susceptible BALB/c mice from L. major infection.**

Because SHP-1 expression and activity was increased in *L. major* infection, we next tested whether inhibition of SHP-1 would enhance the CD40-induced anti-leishmanial functions in susceptible BALB/c mice. A pharmacological inhibitor bpV (phen) was used as described else where (26) .Co-treatment of BALB/c mice with anti-CD40 and bpV (phen) significantly reduced disease progression (Fig. 6A) and parasite burden (Fig. 6B) in a susceptible BALB/c host. Because bpV (phen) is a general PTP inhibitor and as *Leishmania* also expresses other cellular phosphatase, which may be important for its virulence, it thus raises the possibility that pharmacological inhibitor may target both host as well pathogen PTPs. To rule that possibility out, a lentivirus expressed SHP-1-specific short hairpin RNA (shRNA) was used to specifically knock down SHP-1 expression. We investigated the disease progression in *L. major* infected BALB/c mice treated with SHP-1 shRNA or control shRNA. Co-administration of anti-CD40 antibody and lentivirus-expressed SHP-1 shRNA resulted in significant lower disease severity (Fig. 6C) and parasite burden (Fig. 6D); mice injected with

lentivirus-expressed eGFP shRNA failed to rescue *L. major* infection. The efficacy of SHP-1 shRNA was tested in the footpad lesion and lymph node cells in *L. major* infected and Control shRNA or SHP-1 shRNA mice. Significant reduction of SHP-1 was observed in SHP-1 shRNA Lv treated but not in control treated mice (Fig 6E)

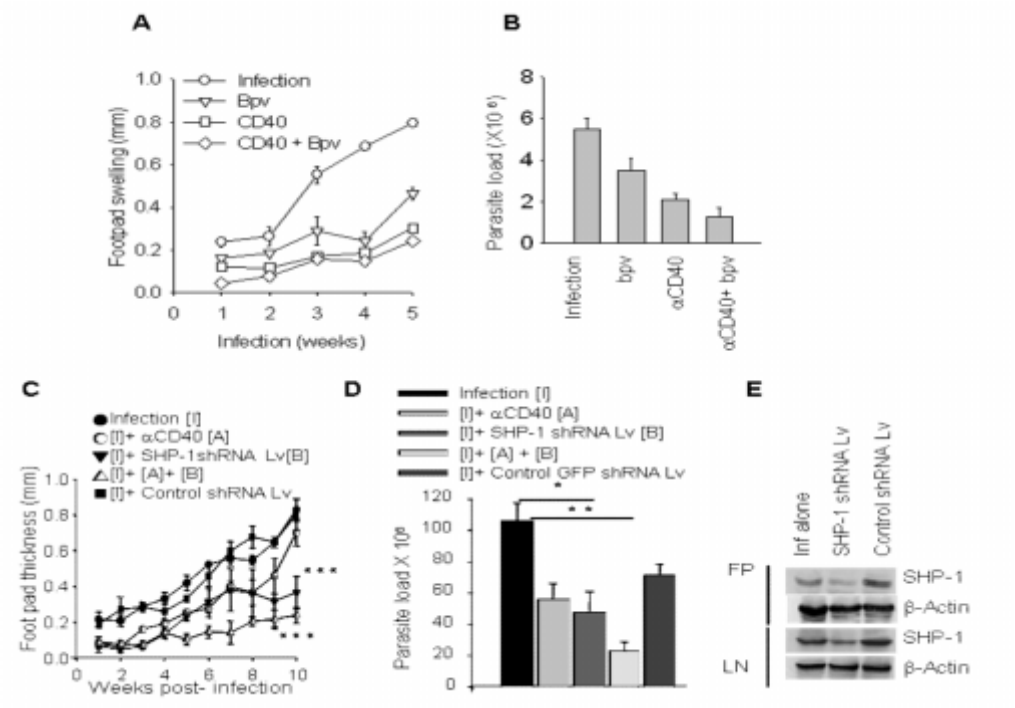


Fig 6(A-E): SHP-1 inhibition protects susceptible BALB/c mice from L. major infection

Lymph node cells from the mice treated with anti-CD40 together with SHP-1shRNA produced significantly more IFNγ and less IL-4 as compared to the cells from infection alone or control eGFPshRNA treated mice (Fig. *6F*). Some of the mice after 5 weeks of infection were injected with thioglycollate and peritoneal macrophages were isolated and stimulated with anti-CD40 antibody. Enhanced phosphorylation of p38MAPK (Fig. 6G) and IL-12 production but reduced IL-10 production (Fig 6Hs) with anti-CD40 stimulation was observed in SHP-1 shRNA treated mice compared to the control shRNA treated mice. Altogether, it suggests that it is the functional concentration but not necessarily compensated activity may be regulatory factor in SHP-1- controlled MAPK activation.

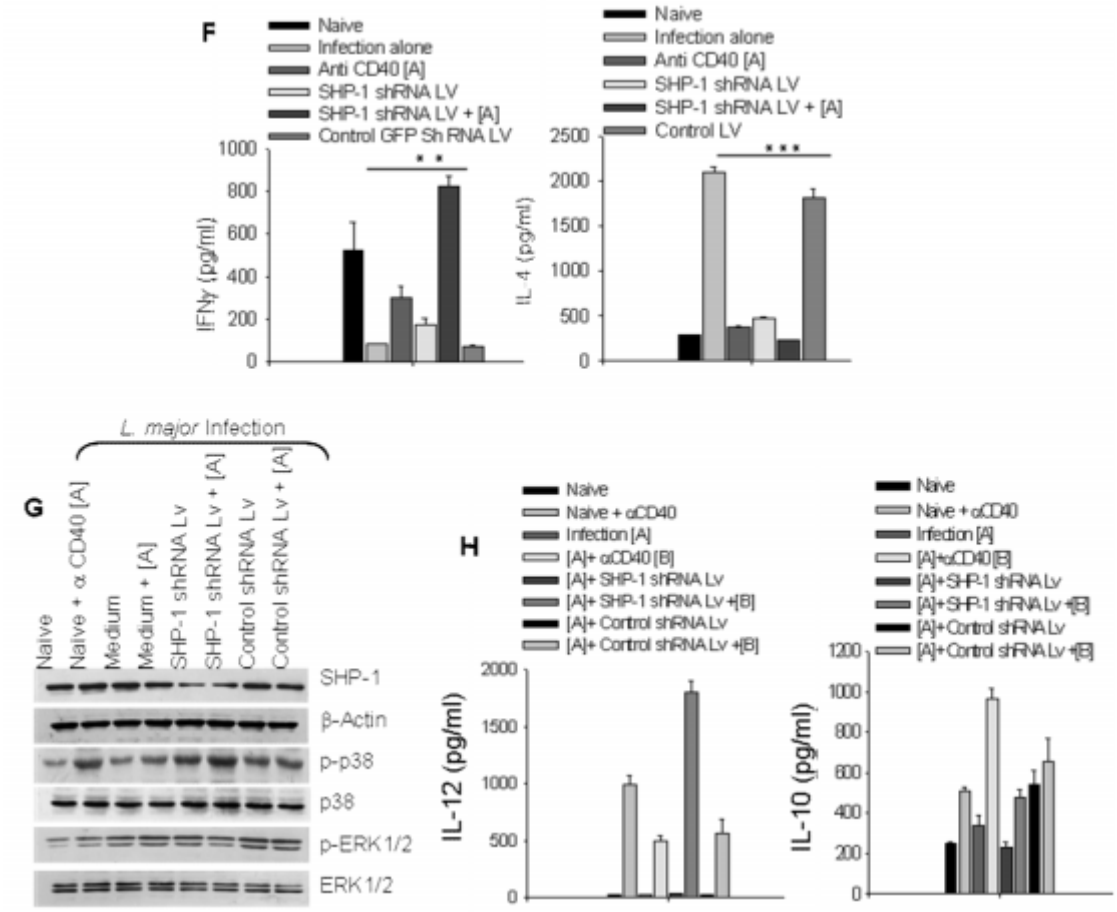


Fig 6(F-H): The effect of SHP-1 inhibition on CD40 induced effector functions in BALB/c mice.

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