SHP-1 Plays a Crucial Role in CD40 Signaling Reciprocity


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SHP-1 Plays a Crucial Role in CD40 Signaling Reciprocity

Tabish Hasan Khan,* Neetu Srivastava,* Ankita Srivastava,* Archana Sareen,* Ram K. Mathur,* Ajit G. Chande,† Krishnasastry V. Musti,* Somenath Roy,‡ Robin Mukhopadhyaya,† and Bhaskar Saha*

CD40 plays dual immunoregulatory roles in Leishmania major infection and tumor regression. The functional duality emerges from CD40-induced reciprocal p38MAPK and ERK-1/2 phosphorylations. Because phosphotyrosine-based signaling in hematopoietic cells is regulated by the phosphotyrosine phosphatase SHP-1, which is not implied in CD40 signaling, we examined whether SHP-1 played any roles in CD40-induced reciprocal signaling and anti-leishmanial function. We observed that a weaker CD40 stimulation increased SHP-1 activation. ERK-1/2 inhibition or p38MAPK overexpression inhibited CD40-induced SHP-1 activation. An ultra-low-dose, CD40-induced p38MAPK phosphorylation was enhanced by SHP-1 inhibition but reduced by SHP-1 overexpression. A reverse profile was observed with ERK-1/2 phosphorylation. SHP-1 inhibition reduced syk phosphorylation but increased lyn phosphorylation; lyn inhibition reduced but lyn inhibition enhanced CD40-induced SHP-1 phosphorylation. Corroborating these findings, in L. major–infected macrophages, CD40-induced SHP-1 phosphorylation increased and SHP-1 inhibition enhanced CD40-induced p38MAPK activation and inducible NO synthase expression. IL-10 enhanced SHP-1 phosphorylation and CD40-induced ERK-1/2 phosphorylation but reduced the CD40-induced p38MAPK phosphorylation, whereas anti–IL-10 Ab exhibited reverse effects on these CD40-induced functions, identifying IL-10 as a crucial element in the SHP-1-MAPK feedback system. Lenti- virally overexpressed SHP-1 rendered resistant C57BL/6 mice susceptible to the infection. Lenti- virally expressed SHP-1 short hairpin RNA enhanced the CD40-induced L. major parasite killing in susceptible BALB/c mice. Thus, we establish an SHP-1–centered feedback system wherein SHP-1 modulates CD40-induced p38MAPK activation threshold and reciprocal ERK-1/2 activation, establishing itself as a critical regulator of CD40 signaling reciprocity and mechanistically re-emphasizing its role as a potential target against the diseases where CD40 is involved. The Journal of Immunology, 2014, 193: 000–000.

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D40, a costimulatory molecule expressed on macrophages and dendritic cells, induces counteractive immune responses (1, 2). CD40 plays proleishmanial and antileishmanial roles (1), demonstrating its functional duality. CD40 has both antitumor and protumor functions that result from the expression of proinflammatory and anti-inflammatory cytokines, respectively, depending on the strength of CD40 stimulation (3, 4). In macrophages, a strong CD40 stimulation induces preferential phosphorylation of p38MAPK and proinflammatory cytokine IL-12 production, whereas a weaker CD40 stimulation induces ERK-1/2 phosphorylation and anti-inflammatory cytokine IL-10 production (5, 6). This CD40 signaling reciprocity emerges because of sustained p38MAPK and ERK-1/2 counterregulation effected by two trans-modular feedback loops (7). This emergent property of the CD40 signaling pathway implies that the threshold for activation of p38MAPK can be calibrated by regulating these MAPK-oriented feedback loops (8). Although these reports argued that the levels of activation are dependent on the extent of phosphorylation of p38MAPK and ERK-1/2, we argue that the observed reciprocity of MAPK phosphorylation is a function of a phosphatase that dephosphorylates these MAPKs differentially, as the cycle of phosphorylation and dephosphorylation of a kinase maintains the homeostasis of cellular signaling. Indeed, CD40-induced activation of MKP-1 and MKP-3, the dual-specific phosphatases, reciprocally regulates CD40 signaling through p38MAPK and ERK-1/2 and controls the host-protective antileishmanial immune response (9, 10).

Among the other phosphatases, Src homology-2 domain containing phosphotyrosine phosphatase SHP-1 was previously reported as a negative regulator of various phosphotyrosine–dependent signaling pathways in hematopoietic cells (11, 12). 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 (13–15). 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 inhibits innate inflammatory responses by modulating various signaling pathways (21–23). Therefore, it is possible that SHP-1 also plays a role in reciprocal CD40 signaling. Although CD40 plays important roles in Leishmania major infection (5–7, 9, 24–26), the possible roles for SHP-1 in CD40 signaling and, in particular, in the Leishmania-induced alteration of CD40 signaling remain unknown.

In this study, we show that SHP-1 contributes to the CD40 signaling reciprocity by differential regulation of the threshold for
CD40-induced phosphorylations of p38MAPK and ERK-1/2, which reciprocally regulate CD40-induced SHP-1 phosphorylation, evoking the concept of an SHP-1–centered CD40 signaling feedback system. SHP-1 overexpression inhibits the antileishmanial function of CD40, rendering the resistant C57BL/6 mice susceptible, whereas its inhibition by an inhibitor or short hairpin RNA (shRNA) enhances the antileishmanial functions of CD40 protecting susceptible BALB/c mice against 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, serving as a key element in the CD40 signaling feedback system, contributing to the CD40 signaling reciprocity. Leishmania exploits these properties to devise the SHP-1–targeted immune evasion strategy ensuring their survival in a mammalian host.

Materials and Methods

Constitutes and reagents

The pBluescript KS+ SHP-1 wild type (WT) construct was a gift from Dr. Ulrike Lorenz (University of Virginia). The SHP-1 insert was subcloned in pcDNA-6HisA at EcoRI site. The clone was confirmed by sequencing. bpV(phen), PD098059, and SB203580 were procured from Merck Millipore (Darmstadt Germany). Abs, namely anti–SHP-1, anti–phospho-p38MAPK, anti–p38MAPK, anti–phospho-ERK-1/2, anti–ERK-1/2, anti–phospho-Tyr, and anti–β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti–phospho-SHP-1 was obtained from ECM Biosciences (Versailles, KY). rIL-10 and ELISA Abs (IL-10, IL-12, IL-4, and IFN-γ) were obtained from BD Biosciences (San Diego, CA).

Mice, parasites, and infection

BALB/c and C57BL/6 mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were subsequently bred in the institute’s experimental animal facility in Thoren Caging systems. L. major (strain MHOM/Su/75/5ASKH) was maintained in vitro in RPMI 1640 medium supplemented with 10% FCS (Life Technologies BRL, Grand Island, NY), and the virulence was maintained by passage through BALB/c mice. L. major stationary phase promastigotes (2 × 10^6) were used to infect mice s.c. in the hind footpad. In experiments with lentivirus treatment, mice were injected s.c. in the hind footpad with 5 × 10^6 transduction units of lentivirus-expressed SHP-1 shRNA, control enhanced GFP (eGFP) shRNA, SHP-1 WT, or control vector 2 d after L. major infection. Some mice were treated with bpV(phen) (2.5 μmol/mouse) (27) or anti-CD40 (50 μg/mouse; clone 2/3) i.p. for three alternate days beginning from the fourth day after infection. Disease severity was assessed by measurement of footpad swelling using a digital micrometer (Mitituyo, Kawasaki, Japan) and parasite burden as described earlier (26). Mice were used per the animal use protocol approved by the Institutional Animal Care and Use Committee.

Macroculture phenotypes and L. major infection of macrophages

Thioglycolate-elicted BALB/c-derived peritoneal macrophages were infected with L. major promastigotes at a ratio of 1:10 for 6 h (26). After

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom)

CD40-induced SHP-1 activation self-limits the reciprocal regulation of p38MAPK and ERK-1/2 phosphorylation and effector functions. (A) BALB/c-derived thioglycolate-elicted peritoneal macrophages were treated with the indicated doses of anti-CD40 Ab or CD40-L for 15 min and analyzed for SHP-1 phosphorylation by Western blotting. The phosphorylation levels were quantitated using Quantity One analysis software and presented under the respective Western blot. In some experiments, kinetics of p38MAPK, ERK-1/2, and SHP-1 phosphorylation in uninfected and L. major–infected BALB/c-derived macrophages were studied (bottom panel). (B) CD40-induced activation of SHP-1 in peritoneal macrophages either untreated or stimulated with the indicated doses of anti-CD40 Ab for 15 min. Cells were harvested, lysed, and subjected to immunoprecipitation with anti–SHP-1 Ab, followed by phosphatase activity assay by pNPP hydrolysis. Data show relative fold change in the SHP-1 activity over the activity in untreated macrophages. (C) Immunoblot analyses of CD40 induced p38MAPK and ERK-1/2 phosphorylations in bpV (phen)-pretreated peritoneal macrophages. Macrophages pretreated with the indicated doses of bpV(phen) for 1 h were stimulated with anti-CD40 Ab (3 μg/ml) for 15 min and lysed, and the lysates were analyzed by Western blotting for p38MAPK and ERK-1/2 phosphorylations. (D) P388D1 cells were transfected with SHP-1 siRNA, or control siRNA were treated with anti-CD40 (3 μg/ml) or CD40-L (100 ng/ml), as indicated, for 15 min, followed by lysis and Western blot analysis for SHP-1, phospho-p38MAPK, and total p38MAPK. (E) Peritoneal macrophages were transduced with lentivirus-expressed SHP-1 shRNA or control eGFP shRNA for 48 h, followed by stimulation with anti-CD40 Ab (3 μg/ml) for 15 min. The relative levels of SHP-1 expression or p38MAPK and ERK-1/2 phosphorylation were assessed with Western blotting. (F) Immunoblot analysis of CD40-induced p38MAPK and ERK-1/2 phosphorylation in SHP-1–overexpressed cells. P388D1 cells were transfected with SHP-1 WT overexpression construct for 48 h followed by stimulation with anti-CD40 Ab (3 μg/ml). SHP-1, phospho-p38MAPK, and phospho-ERK-1/2 were assessed with Western blotting. Inset, SHP-1 expression in cells either nontransfected or transfected with SHP-1 WT or control empty vector construct. The experiments were repeated three times. The error bars represent mean ± SEM. The test of significance for the difference between the means were determined with one-way ANOVA. *p < 0.05, **p < 0.01.
washing of extracellular parasites, macrophages were treated with bpV (phen) for 1 h at the indicated doses. After treatment, fresh medium was added, and the cells were incubated with or without anti-CD40 Ab (3 μg/ml) for 72 h. Macrophages were fixed and Giemsa-stained, and amastigotes per 100 macrophages were enumerated, as described earlier (26).

### Western blotting

Western blotting

Macrophages were washed twice with chilled PBS and lysed in NP-40 lysis buffer (200 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor mixture (Pierce, Rockford, IL). Protein quantification was performed with a BCA Kit (Pierce) and equal amount of protein was run on SDS-PAGE. Western blotting was performed as described previously (9).

### Immunoprecipitation

Immunoprecipitation

After treatment, macrophages were washed with ice-cold PBS and lysed in the lysis buffer (200 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, protease inhibitor mixture). Equal amounts of proteins were immunoprecipitated using anti–SHP-1 Ab and collected with protein A+G Agarose beads (Pierce) at 4˚C for 16 h. The immunoprecipitates were washed in cold lysis buffer and subjected to Western blot analysis (9).

### SHP-1 activity assay

SHP-1 activity assay

Cell lysates were subjected to immunoprecipitation with anti-SHP-1 Ab. Immunoprecipitates were washed with ice-cold lysis buffer (200 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40), and SHP-1 activity was assayed with a PTP Assay Kit 2 (Upstate, Lake Placid, NY) following the manufacturer’s protocol. Immunoprecipitates 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-nitrophenyl phosphate (pNPP) and incubation for 2 h at 37˚C. Absorbance of the samples was measured at 410 nm in a spectrophotometer.

### RT-PCR and quantitative real-time PCR

RT-PCR and quantitative real-time PCR

RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) and reverse transcribed using M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA) (9). The reactions were performed in thin-wall, 0.2-ml strip tubes (Axygen, Union City, CA) in 10 μl reaction mixture containing 10 ng cDNA, 2 ng forward primer, 2 ng reverse primer, and 5 μl 2× IQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed on the Eppendorf RealPlex® Mastercycler under the following conditions: 95˚C for 2 min, 40 cycles of 95˚C for 1 min, 60˚C for 30 s, and 72˚C for 35 s. Reactions were performed in duplicates. Relative quantitation was performed using the ΔΔ comparative threshold (ΔΔCt) method. mRNA expression levels of the target genes were normalized against those of GAPDH levels and expressed as relative fold change compared with untreated controls. RT-PCR was performed using primers specific for mouse IL-12p40, IL-4, IFN-γ, and inducible NO synthase (5, 6) (Table I). Transcripts encoding GAPDH were used for normalization.

### Transient transfection studies

Transient transfection studies

P388D1 macrophage-like cell line (70–75% confluence) was transfected using Lipofectamine 2000 (Life Technologies). SHP-1 WT construct (4 μg) or empty vector were used for transfection at a DNA:lipofectamin ratio of 1:3. Transfection was performed in serum-free medium (optiMEM; Life Technologies). After 6 h of transfection, medium was changed with RPMI 1640 supplemented with 10% FCS. After 48 h, cells were stimulated for the indicated time with anti-CD40 Ab (3 μg/ml) and harvested for Western blot or real-time PCR analyses. SHP-1 small interfering RNA (siRNA), ERK1 siRNA, control siRNA, transfection medium, and transfection reagents were procured from Santa Cruz Biotechnology. siRNA transfection was performed according to the manufacturer’s protocol.

### Cytokine ELISA

Cytokine ELISA

Peritoneal macrophages were stimulated with anti-CD40 (3 μg/ml) or lymph node cells isolated from different groups of mice were stimulated with anti-CD3 (0.5 μg/ml) and anti-CD28 (2 μg/ml) for 48 h. Culture supernatants were assayed for IL-12, IL-10, IL-4, and IFN-γ contents using respective ELISA kits, following the manufacturer’s protocol.

### Generation of SHP-1 shRNA and SHP-1 WT lentiviral constructs

Generation of SHP-1 shRNA and SHP-1 WT lentiviral constructs

U6 promoter-driven, SHP-1-specific shRNA cassette was generated by PCR amplification of positions 913–935 of the SHP-1 gene (GenBank accession no. NM_013545; http://www.ncbi.nlm.nih.gov/genbank/), selection of shRNA target sequences was based on published guidelines (28, 29). The shRNA cassette was cloned into the third-generation lentivirus vector pCRI.LV-Neo. Vector carrying GFP-specific shRNA and empty vector were used as controls. The SHP-1 gene was PCR amplified from the pcDNA-6His plasmid and cloned in the T/A vector pTZ57R (MBI Fermentas, Vilnius, Lithuania) and further subcloned at SalI and NotI sites of pCRI.LV-Neo under a constitutive EF1α promoter. Virus particles containing the shRNA transgene were generated by multiplasmid transfections in HEK-293T cells (Life Technologies), and virus was collected and concentrated as described earlier (29).

### In vitro lentiviral transduction

In vitro lentiviral transduction

Peritoneal macrophages were transduced with a third-generation lentiviral vector encoding SHP-1 shRNA, eGFP control shRNA, SHP-1 WT, or an empty vector using lentivirus vector pCRI.LV-Neo. Vector carrying GFP-specific shRNA and empty vector were used as controls. The SHP-1 gene was PCR amplified from the pcDNA-6His plasmid and cloned in the T/A vector pTZ57R (MBI Fermentas, Vilnius, Lithuania) and further subcloned at SalI and NotI sites of pCRI.LV-Neo under a constitutive EF1α promoter. Virus particles containing the shRNA transgene were generated by multiplasmid transfections in HEK-293T cells (Life Technologies), and virus was collected and concentrated as described earlier (29).

### FIGURE 2.

SHP-1 reciprocally modulates CD40-induced IL-10 and IL-12 production. (A) CD40-induced IL-10 and IL-12 production in cells transfected with SHP-1 siRNA. P388D1 cells were transfected with SHP-1 siRNA or control siRNA for 36 h, followed by stimulation with anti-CD40 Ab (3 μg/ml) for 48 h. IL-10 and IL-12 production were assessed in culture supernatants by ELISA. The experiments were repeated twice. The error bars represent mean ± SEM. The test of significance for the difference between the means were determined with one-way ANOVA. *p < 0.05, **p < 0.01.
empty vector for 12 h in complete media supplemented with 8 μg/ml polybrene. Cells were washed to remove residual virus, and cells were stimulated 48 h later 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 in vivo experiments comprised at least five mice per group. The statistical significance of the differences between the means was deduced with one-way ANOVA. The results were plotted as mean ± SE of means representing three individual experiments.

Results

CD40-induced SHP-1 activation self-limits the reciprocal regulation of p38MAPK and ERK1/2 phosphorylation and effector functions

SHP-1 is implicated in intracellular signaling triggered by several membrane receptors (11–13); however, SHP-1 is not tested for its roles in signaling from CD40, a transmembrane receptor. Therefore, we tested CD40 modulated SHP-1 activation in BALB/c-derived peritoneal macrophages, which were stimulated with different doses of an agonistic anti-CD40 Ab or CD40-ligand (CD40-L). We observed that a lower dose of the Ab or the ligand induced SHP-1 phosphorylation, which was reduced with the increase in their doses (Fig. 1A, upper panels). Comparison of the kinetics of CD40 (3 μg/ml) induced SHP-1, p38MAPK, and ERK1/2 phosphorylation in uninfected and L. major–infected macrophages showed that SHP-1 and ERK-1/2 phosphorylation increased, but that of p38MAPK decreased in infected macrophages; the phase of peak activation remained unaltered (Fig. 1A, bottom panel). Likewise, SHP-1 activity was significantly increased at a lower dose of the anti-CD40 Ab (Fig. 1B), suggesting that SHP-1 activation is reciprocally related to the strength of CD40 stimulation. Because p38MAPK and ERK-1/2 phosphorylations are controlled reciprocally (5), we investigated whether altered SHP-1 activation or expression would influence the CD40-induced p38MAPK and ERK1/2 phosphorylations. With the increasing doses of bpV (phen), a phosphotyrosine phosphatase inhibitor, the CD40-induced p38MAPK phosphorylation was enhanced but reciprocally, and ERK-1/2 phosphorylation was reduced (Fig. 1C).

Similarly, SHP-1 siRNA augmented both anti-CD40 Ab–induced

FIGURE 3. p38MAPK and ERK1/2 reciprocally regulate CD40-induced SHP-1 phosphorylation. (A) BALB/c-derived peritoneal macrophages were pretreated with the indicated doses of SB203580 and PD098059, the inhibitors for p38MAPK and ERK-1/2, respectively, for 1 h, followed by treatment with anti-CD40 Ab (3 μg/ml). Cells were harvested 15 min after the anti-CD40 Ab treatment. Phosphorylations of SHP-1, p38MAPK, and ERK-1/2 were assessed with Western blotting. (B and C) P388D1 cells, a macrophage-like cell line, were transfected with either ERK-1 siRNA or control siRNA for 36 h or were pretransduced with p38MAPK WT vector, followed by treatment with anti-CD40 Ab (3 μg/ml) and Western blot analyses of the CD40-induced SHP-1 phosphorylation. (D) P388D1 cells were transfected with p38MAPK siRNA or control siRNA were treated with anti-CD40 (3 μg/ml) or CD40-L (100 ng/ml), as indicated, for 15 min, followed by lysis and Western blot for total p38MAPK, phospho–SHP-1, and SHP-1. (E and F) Association of SHP-1 with p38MAPK was assessed by immunoblotting with anti–SHP-1 antibody. Data from one representative experiment are shown. Immunoblot analyses of SHP-1 association with p38MAPK (E), ERK-1, syk, and lyn (F) were performed after SHP-1 immunoprecipitation from BALB/c-derived peritoneal macrophages—either untreated or treated with the indicated doses of anti-CD40 Ab. Whole cell lysates from the macrophages stimulated with anti-CD40 Ab served as a positive control. Normal rabbit IgG was used as isotype for SHP-1 Ab. The phosphorylations were quantified with Quantity One analysis software. Data from one representative experiment are shown. The error bars represent mean ± SEM of multiple sets. The significance of the differences between the means were calculated with one-way ANOVA. *p < 0.05, **p < 0.01.
and the CD40-L–induced p38MAPK phosphorylation (Fig. 1D). Lentivirally expressed SHP-1 shRNA but not the control eGFP shRNA reduced the anti-CD40 Ab–induced or CD40-L–induced SHP-1 phosphorylation, but enhanced p38MAPK phosphorylation in BALB/c–derived peritoneal macrophages (Fig. 1E), whereas SHP-1 overexpression in P388D1, a macrophage-like cell line, resulted in a reverse profile of CD40-induced p38MAPK and ERK-1/2 activation (Fig. 1F). These results suggest that as SHP-1 expression and activity increases in L. major–infected macrophages, CD40-induced phosphorylation of p38MAPK decreases but that of ERK-1/2 diminishes.

SHP-1 reciprocally modulates CD40-induced IL-10 and IL-12 production

Because SHP-1 modulated CD40-induced phosphorylations of p38MAPK and ERK1/2, which upregulate the production of IL-12 and IL-10, respectively (5), we examined whether modulation of SHP-1 expression by its siRNA in P388D1 cells or by its lentiviral overexpression in BALB/c–derived peritoneal macrophages would alter the CD40-induced IL-12 and IL-10 productions. It was observed that the inhibition of SHP-1 expression increased IL-12 but reduced IL-10 production (Fig. 2A), whereas the overexpression reduced IL-12 but enhanced IL-10 production (Fig. 2B). These data indicate that the CD40-activated SHP-1 self-limits the reciprocally regulated p38MAPK and ERK1/2 phosphorylations and IL-10 and IL-12 production.

p38MAPK and ERK-1/2 reciprocally regulate CD40-induced SHP-1 phosphorylation

Because SHP-1 inhibition or overexpression reciprocally modulated CD40-induced p38MAPK and ERK-1/2 activation, and because CD40-induced p38MAPK and ERK-1/2 are reciprocally regulated, we examined whether inhibitors of p38MAPK and ERK-1/2 would reciprocally regulate CD40-induced SHP-1 activation. We observed that the inhibition of p38MAPK by SB203580 increased CD40-induced SHP-1 activation, whereas ERK1/2 inhibition by PD098059 reduced SHP-1 activation (Fig. 3A). Consistent with the CD40-induced reciprocal control of p38MAPK and ERK-1/2, ERK-1 silencing (Fig. 3B) but p38MAPK overexpression reduced SHP-1 activation (Fig. 3C). Corroborating the effects of p38MAPK inhibitor SB203580 or p38MAPK overexpression on CD40-induced SHP-1 activation, p38MAPK siRNA increased the anti-CD40 Ab or CD40-L induced SHP-1 phosphorylation in macrophages (Fig. 3D). It was observed that SHP-1 binds more to p38MAPK (Fig. 3E) and lyn (Fig. 3F) in macrophages treated with a low dose of the anti-CD40 Ab than with a higher dose. Therefore, SHP-1 is an important factor

FIGURE 4. SHP-1 regulates the threshold of ultra-low CD40-induced p38MAPK and ERK1/2-mediated signaling and effector functions. (A) BALB/c–derived thioglycolate-elicited macrophages were pretreated with 10 μM bpV(phen) for 1 h, followed by stimulation with the indicated doses of the anti-CD40 Ab for 15 min. The SHP-1 expression and the CD40-induced p38MAPK and ERK-1/2 phosphorylations were assessed in the cell lysates with Western blotting. (B and C) The macrophages were transduced with lentivirally expressed SHP-1 shRNA (left panel) or control shRNA (right panel) for 48 h, followed by treatment with the indicated doses of anti-CD40 Ab for 15 min and the expressions of SHP-1. The CD40-induced phosphorylations of p38MAPK and ERK-1/2 (B) or syk and lyn (C) were assessed in the cell lysates with Western blotting. (D) P388D1 cells, a macrophage-like cell line, were transfected with either syk siRNA or control siRNA (left panel) or with either lyn siRNA or control siRNA (right panel) for 36 h, followed by treatment with anti-CD40 Ab (3 μg/ml). SHP-1, syk, and lyn phosphorylations were assessed with Western blotting. (E and F) The anti-CD40 Ab (3 μg/ml) induced IL-10 and IL-12 production in the presence of bpV(phen) (E) or in the presence of SHP-1 shRNA (F) or control shRNA were assessed by ELISA. The experiments were repeated minimum two (A–D) or three times (E, F). The data from one representative experiment are shown. The significance of the differences between the means were calculated with one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.005.
SHP-1 regulates the threshold of ultra-low CD40-induced p38MAPK- and ERK-1/2-mediated signaling and effector functions

The results described thus far imply that the low phospho-p38MAPK observed in lower doses of CD40 stimulation could be due to enhanced p38MAPK dephosphorylation. Therefore, we tested whether SHP-1 inhibition would enhance p38MAPK phosphorylation at lower doses of CD40 stimulation. Indeed, bpV(phen)-mediated or lentivirally expressed SHP-1 shRNA-mediated SHP-1 inhibition resulted in enhanced p38MAPK phosphorylation and reciprocal decrease in ERK-1/2 phosphorylation (Fig. 4A, 4B), even at ultra-low doses of the anti-CD40 Ab. As lyn and syk were shown to be the first kinases mediating CD40 signaling and were also parts of two feedback loops (7), we assessed the role of SHP-1 in modulating ultra-low-dose anti-CD40-induced lyn and syk phosphorylation. We observed that SHP-1 inhibition reduced syk but increased lyn phosphorylation (Fig. 4C). As SHP-1 and ERK-1/2 or p38MAPK were regulating each other’s activation, we examined whether lyn or syk could also modulate SHP-1 phosphorylation. It was observed that syk siRNA reduced CD40-induced SHP-1 phosphorylation, whereas lyn siRNA increased CD40-induced SHP-1 activation (Fig. 4D). Corroborating these data, SHP-1 inhibition by bpV or SHP-1 shRNA reduced IL-10 but increased IL-12 productions at these ultra-low doses of anti-CD40 Ab (Fig. 4E, 4F). These data indicate that SHP-1 plays a crucial role in determining the threshold for CD40 signaling through p38MAPK and ERK-1/2 pathways and IL-10 or IL-12 production.

Leishmania major upregulates SHP-1 phosphorylation to interfere with reciprocal CD40 signaling via p38MAPK and ERK-1/2

We tested CD40 modulated SHP-1 activation in L. major infection. BALB/c-derived peritoneal macrophages—uninfected or infected with L. major—were stimulated with different doses of CD40-L. We observed that in L. major–infected macrophages, the CD40-induced SHP-1 phosphorylation was upregulated (Fig. 5A). We transduced these macrophages with SHP-1 shRNA Lv (Fig. 5B) or SHP-1 WT Lv (Fig. 5C) and treated with anti-CD40 Ab (8 μg/ml) for 15 min. The lysate subjected to Western blot analysis. CD40 induced iNOS expression in P388D1 transfected with either SHP-1 WT construct–control vector or SHP-1 siRNA–control siRNA. Thirty-six hours after transfection, cells were stimulated with the anti-CD40 Ab (3 μg/ml) for 8 h, iNOS expression was assessed with quantitative RT PCR. iNOS mRNA expression levels were normalized against GAPDH mRNA levels and expressed as relative fold change compared with nontransfected controls (Medium). Data show mean ± SEM (D, E). All the experiments were performed three times. The significance of differences between the mean values is deduced with one-way ANOVA. *p < 0.05, **p < 0.01.

Table I. RT-PCR and real-time PCR primers used

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induced SHP-1 phosphorylation (although increased in the infected macrophages) decreased with the increasing doses of CD40-ligand (Fig. 5A). Because SHP-1 regulates CD40-induced p38MAPK and ERK-1/2 activation, we next examined whether inhibited or enhanced SHP-1 altered CD40-induced p38MAPK or ERK-1/2 phosphorylations. It was observed that SHP-1 shRNA, which reduced SHP-1 expression, reduced the ERK-1/2 but increased p38MAPK phosphorylations (Fig. 5B); a reverse profile was observed in the case of SHP-1 overexpression (Fig. 5C). As inducible NO synthase (iNOS) is required for CD40-induced elimination of L. major but its expression is decreased in L. major-infected macrophages (5, 26), we examined whether SHP-1 modulated the CD40-induced iNOS expression in macrophages (Table I). It was observed that SHP-1 shRNA increased, but SHP-1 overexpression decreased CD40-induced iNOS expression in macrophages (Fig. 5D, 5E). These data show that CD40-induced SHP-1 activation regulates the CD40-induced p38MAPK and ERK-1/2 phosphorylation and iNOS expression.

**IL-10 induces SHP-1 activation to alter CD40-induced signaling and antileishmanial functions**

Because the antitumor or antileishmanial effects are significantly self-limited by CD40-induced IL-10 (4, 5), we examined whether CD40-induced ERK-1/2-mediated production of IL-10 could re-

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**FIGURE 6.** IL-10 induces SHP-1 activation to alter CD40-induced signaling and antileishmanial functions. (A) BALB/c-derived macrophages were treated with the indicated doses of rIL-10, followed by SHP-1 phosphorylation analyses by Western blots. The Quantity One analyses of the phosphorylations are shown in the bottom panel. (B) Uninfected and L. major–infected macrophages were treated with rIL-10 (10 ng/ml), followed by treatment with anti-CD40 Ab (3 mg/ml) for 15 min. The cell lysates were probed for p38MAPK and ERK-1/2 phosphorylations by Western blots. (C and D) In a similar experimental setup where anti-CD40 treatment was for 8 or 48 h, the effect of rIL-10 on the CD40-induced iNOS (C) and IL-12 (D) expressions were assessed with real-time PCR (8 h) or nitrite estimation or IL-12 assessment by ELISA (48 h). (E) Uninfected and L. major–infected macrophages were cultured in the presence or absence of anti–IL-10 Ab (10 μg/ml) during the last 24 h of L. major infection (total 72 h infection), followed by the treatment with anti-CD40 Ab (3 μg/ml) for 15 min. The macrophages were lysed, and the lysates were assessed for p38MAPK, ERK-1/2, and SHP-1 phosphorylations. (F) In a similar experimental setup where anti-CD40 treatment was for 8 h, the effect of anti–IL-10 on the CD40-induced iNOS and IL-12 expressions were assessed with real-time PCR. (G) BALB/c-derived macrophages were infected with L. major promastigotes, followed by treatment with the indicated doses of bpV(phen) and anti-CD40 Ab (5 μg/ml). The top panel shows that IL-10 production was tested by RT-PCR. The bottom panel shows the amastigote counts in macrophages. The experiments were repeated three times. The error bars, representing the mean ± SEM, were from multiple sets, and the significance of differences between the means was deduced with one-way ANOVA. *p < 0.05, **p < 0.01.
sult in the observed feedback effect. First, rIL-10–induced SHP-1 phosphorylation (Fig. 6A) reduced CD40-induced p38MAPK but enhanced ERK-1/2 activation (Fig. 6B) and decreased the CD40-induced iNOS expression (Fig. 6C) and IL-12 production (Fig. 6D). Second, anti–IL-10 Ab restored CD40-induced p38MAPK activation (Fig. 6E) and increased the CD40-induced iNOS and IL-12 production (Fig. 6F). Third, increasing doses of bpV(phen) enhanced CD40-induced parasite killing that correlated with dose-dependent inhibition of IL-10 expression in macrophages (Fig. 6G, inset). These data suggest that CD40 activates ERK-1/2, which triggers IL-10 production. IL-10 inhibits p38MAPK that results in SHP-1 activation. Thus, CD40-induced IL-10 might be a crucial link in the MAPK–SHP-1 feedback loop.

**SHP-1 reduces the CD40-induced antileishmanial functions**

Because SHP-1 overexpression resulted in enhanced CD40-induced proparasitic cytokine IL-10 and reduced antileishmanial cytokine IL-12 and iNOS expression, we tested whether its overexpression (Fig. 7A) would render resistant C57BL/6J mice susceptible to *L. major* infection. It was observed that the mice injected with lentivirally overexpressed SHP-1 had thicker footpads (Fig. 7B, inset), higher parasite load (Fig. 7C), and higher IL-4 but reduced IFN-γ productions (Fig. 7D, Table I), as compared with the control mice.

As SHP-1 expression was increased in *L. major* infection and was accompanied by enhanced CD40-induced IL-10 production, we examined whether inhibition of SHP-1 expression by the lentivirally expressed SHP-1 shRNA (Fig. 7E) would enhance the CD40-induced antileishmanial functions in susceptible BALB/c mice. Coadministration of anti-CD40 Ab and the lentivirus–expressed SHP-1 shRNA but not the lentivirus–expressed control eGFP shRNA resulted in significantly less disease severity as evidenced by reduced parasite load (Fig. 7F, inset). As compared with the cells from infection alone or control eGFP shRNA–treated mice, the lymph node cells from the mice treated with anti-CD40 together with SHP-1 shRNA produced significantly more IFN-γ but less IL-4 (Fig. 7G).

The data described in this report reveal that SHP-1 increases the threshold for CD40-induced p38MAPK activation, which is associated with the production of IL-12, an antileishmanial cytokine. Once SHP-1 is inhibited, CD40 induces p38MAPK activation at a weak stimulation. On the other hand, *Leishmania* enhances SHP-1 activation, leading to enhanced ERK-1/2 activation, which is associated with the production of IL-10, a proleishmanial cytokine that enhances SHP-1 and ERK-1/2 activation but reduces CD40-induced p38MAPK activation. Thus, the CD40 signaling threshold appears to be an emergent property of the receptor that results from the feedback interactions between these MAPKs, lyn and syk, SHP-1, and IL-10 (Fig. 8). The knowledge helps to refine the CD40-based antileishmanial therapy.

**Discussion**

The quality and amplitude of a cell’s response to various stimuli are regulated within a range so that hyperresponsiveness or hyporesponsiveness to a given stimulus is averted. The quality of the response pertains to its counteractively functioning elements. Using CD40 as a model receptor with crucial roles in diverse immune functions, we previously showed that such counteractive...
functions or the quality of the response—proinflammatory or anti-inflammatory—are regulated by a signaling system with signal strength calibrating feedback loops (7, 10). The CD40 signaling system consists of cascades of kinases in two modules: one module transmits CD40 signal through lyn and p38MAPK while the other module transmits CD40 signal through syk and ERK-1/2. The reciprocity between these two modules is maintained by a minimum of two trans-modular feedback loops: ERK-1/2 \textarrow{inhibition} lyn \rightarrow p38MAPK and p38MAPK \textarrow{activation} syk \rightarrow ERK-1/2 (7) (\textarrow{indicates inhibition, and \textarrow{indicates eventual activation on the same signaling module}). Thus, the reciprocal functions of the bimodal CD40 signaling were represented only as a function of differential phosphorylations of kinases.

In biologic systems, the reciprocity is maintained by kinases and phosphatases such as the reciprocity between CD28 and CD152—positive and negative costimulators for T cells, respectively (30–33). Whereas CD28 activates the kinases and potentiates TCR-driven T cell proliferation, CD152 activates the phosphatases and downregulates T cell proliferation. The CD152-deficient T cells show hyperproliferation in response to TCR activation and CD28 costimulation. A similar role for SHP-1 can be envisaged in regulating the state of CD40-induced macrophage activation and function. As phosphatases dephosphorylate the active kinases, a receptor’s signaling and effector functions can be modulated accordingly. Thus, the sensitivity of a cell to a stimulus appears to be expressed in terms of threshold of activation of a given signaling pathway and its downstream effector function. Using CD40 and SHP-1 as a model receptor and a model phosphotyrosine phosphatase, respectively, we have shown how SHP-1 determines the threshold of a receptor’s signaling and modulates the receptor-regulated, host-protective, antileishmanial functions. Because SHP-1 inhibition restored CD40-induced p38MAPK phosphorylation, reciprocally inhibiting ERK-1/2 phosphorylation, but SHP-1 overexpression inhibited the CD40-induced p38MAPK phosphorylation, we argue that the CD40-activated SHP-1 negatively regulates p38MAPK but positively regulates ERK-1/2. How SHP-1 activation leads to enhanced ERK-1/2 phosphorylation and vice versa is not known, but it is possible that as SHP-1 reduces p38MAPK dephosphorylation, the CD40 signal is adjusted through the trans-modular feedback loop to reciprocally enhance ERK-1/2 phosphorylation. The increased SHP-1 phosphorylation and suppressed CD40-induced p38MAPK phosphorylation were observed in virulent but not in avirulent L. major–infected macrophages (N. Srivastava and T.H. Khan, unpublished data), suggesting an association between the virulence of the parasite and its ability to induce SHP-1 phosphorylation and its phosphatase activity (34, 35). The study by Gomez et al. (34) demonstrated that the Leishmania-expressed membrane protease GP63, the key virulence factor, interacted with SHP-1, phosphotyrosine phosphatase (PTP)-1B, and T-cell PTP (TCPTP) in macrophages. SHP-1 was activated by its GP63 catalyzed cleavage, which is abrogated after lipid raft disruption (34). We previously reported that lipid raft disruption reduced p38MAPK, but enhanced ERK-1/2 activation (6), implying that SHP-1 might not be solely responsible for reduced p38MAPK phosphorylation. By contrast, as SHP-1 inhibition or silencing enhances p38MAPK phosphorylation and as SHP-1 coimmunoprecipitates with p38MAPK, we argue that SHP-1 activated at a low strength of CD40 stimulation reduces CD40-induced p38MAPK phosphorylation. It is plausible that in L. major infection, SHP-1 might be activated in a lipid raft-independent manner, or there are other phosphatases that work in concert with SHP-1 to modulate the CD40-induced MAPK activation. Indeed, as compared with the reciprocal regulation of CD40 signaling by the dual-specific phosphatases, MKP-1 and MKP-3 (9), SHP-1 presents a particularly intriguing case because the same phosphatase SHP-1 associates with only p38MAPK but not with ERK-1/2, but it regulates the CD40 signaling reciprocity. Thus, a detailed independent study is required to determine whether SHP-1 has a reciprocally working partner, such as PP-2A, and whether these two phosphatases are reciprocally regulated by CD40 signaling and vice versa. As the role of phosphatases in CD40 signaling reciprocity is not well studied, the current and the future studies would help to build a complete view of the CD40 signaling network, constituted of adaptors, kinases, and phosphatases.

We propose that at least three mechanisms operate in tandem to impose the SHP-1 effect on the reciprocal CD40 signaling through MAPKs. First, as a lower dose of anti-CD40 Ab stimulation results in less p38MAPK phosphorylation (5) but maximum SHP-1 activity and phosphorylation, it is possible that SHP-1 determines the threshold of p38MAPK phosphorylation. This is evident from the following two observations: 1) increased p38MAPK phosphorylation at an ultra-low dose of anti-CD40 in SHP-1 inhibited macrophages and 2) a stronger CD40 stimulation resulted in

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reduced SHP-1 phosphorylation and activity, but increased p38MAPK phosphorylation (5). The reduced SHP-1 will relieve the negative effects on CD40 signaling feedback and eventual effector functions. Second, SHP-1 contributes to the reciprocity through its physical association with the p38MAPK pathway signaling intermediates. It is possible that at lower doses of CD40 stimulation, higher syk phosphorylation and reciprocally less lyn phosphorylation increases ERK-1/2 activity that reciprocally regulates p38MAPK phosphorylation. SHP-1 can thus bind to lyn to result in reduced p38MAPK phosphorylation (5).

10 SHP-1 MODULATES CD40 SIGNALING RECIPROCITY


Disclosures

The authors have no financial conflicts of interest.

References


