LPS-induced down-regulation of signal regulatory protein α contributes to innate immune activation in macrophages

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Activation of the mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) cascades after Toll-like receptor (TLR) stimulation contributes to innate immune responses. Signal regulatory protein (SIRP) α, a member of the SIRP family that is abundantly expressed in macrophages, has been implicated in regulating MAPK and NF-κB signaling pathways. In addition, SIRPα can negatively regulate the phagocytosis of host cells by macrophages, indicating an inhibitory role of SIRPα in innate immunity. We provide evidences that SIRPα is an essential endogenous regulator of the innate immune activation upon lipopolysaccharide (LPS) exposure. SIRPα expression was promptly reduced in macrophages after LPS stimulation. The decrease in SIRPα expression levels was required for initiation of LPS-induced innate immune responses because overexpression of SIRPα reduced macrophage responses to LPS. Knockdown of SIRPα caused prolonged activation of MAPKs and NF-κB pathways and augmented production of proinflammatory cytokines and type I interferon (IFN). Mice transferred with SIRPα-depleted macrophages were highly susceptible to endotoxic shock, developing multiple organ failure and exhibiting a remarkable increase in mortality. SIRPα may accomplish this mainly through its association and sequestration of the LPS signal transducer SHP-2. Thus, SIRPα functions as a biologically important modulator of TLR signaling and innate immunity.

The Journal of Experimental Medicine

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Abbreviations used: CHX, cycloheximide; ERK, extracellular signal–related kinase; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase; ISRE, interferon-sensitive response element; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NO, nitric oxide; SIRP, signal regulatory protein; sh, short hairpin; SOCS, suppressor of cytokine signaling; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; Trif, TIR domain-containing adaptor inducing IFN-β.

The innate immune system is evolutionally conserved, and it is the first line of the defensive mechanisms for protecting the host from invading microbial pathogens (1). Innate immune cells, including macrophages and dendritic cells, express a series of receptors known as Toll-like receptors (TLRs), which bind to highly conserved sequences expressed by microorganisms (2, 3). LPS is an integral cell wall component of Gram-negative bacteria, and can provoke a life-threatening condition called endotoxic shock. The inflammatory response to LPS is mediated mainly by a receptor complex composed of LPS-binding protein CD14 and TLR4 (4). Upon activation of TLR4, the cytoplasmic domains recruit signal adaptor molecules, such as MyD88 and Trif, which, in turn, trigger a cascade of signaling events leading to the activation of MAPKs and IκB kinases (IKKs), as well as the downstream transcription factors AP-1, NF-κB, and IRF3. Activation of NF-κB and mitogen-activated protein kinases (MAPKs) by the MyD88-dependent pathway is essential for the transcription of a variety of proinflammatory cytokines, including TNFα and IL-6, whereas the initial induction of type I IFN, e.g., IFN-β, is largely dependent on Trif-mediated IRF3 activation (5). These proinflammatory cytokines can, in turn, stimulate the release of secondary mediators, which, if not properly controlled, may ultimately lead to dysfunction of multiple vital organs.

The optimal type and strength of the innate immune response can be regulated through a balance of activating and inhibitory signals that are delivered by receptors on the surface of cells of the innate immune system (6). One such group of cell-surface receptors is the signal regulatory protein (SIRP) family. The first and best-characterized member of the SIRP family,

X.-N. Kong and H.X. Yan contributed equally to this paper.

SIRPα, is especially abundant in innate immune cells, including macrophages and dendritic cells (7, 8). The extracellular region of SIRPα is heavily glycosylated, and it has been shown to bind to either widely expressed transmembrane ligand CD47 (9) or soluble ligands, such as the surfactant proteins A and D, which are present at high levels in the lungs (10). Interaction of CD47 with SIRPα negatively regulates phagocytosis of host cells by macrophages (11). Mice that lack the SIRPα cytoplasmic domain are thrombocytopenic, which apparently results from an increased rate of clearance of circulating platelets (12, 13). Nonopsonized erythrocytes from $CD47^{-/-}$ mice are recognized and rapidly eliminated in WT recipients, and they are phagocytosed by WT macrophages in vitro (14). This strongly suggests that SIRPα acts to negatively control innate immune effector function. In lungs, ligation of SIRPα on macrophages by surfactant proteins is required to keep the activity of alveolar macrophages in check, thus preventing damage to the airways caused by proinflammatory responses (10). Therefore, SIRPα induces well-characterized inhibitory signals in innate immune cells that can largely be reconciled by the association of tyrosine phosphatases with the cytoplasmic region (8, 15). The cytoplasmic region of SIRPα contains two immunoreceptor tyrosine-based inhibitory motifs with four tyrosine residues that are phosphorylated in response to a variety of growth factors and ligand binding. This phosphorylation enables recruitment of SHP-1 and -2 that, in turn, dephosphorylates specific protein substrates involved in mediating various physiological effects (8, 16, 17). SIRPα has been shown to negatively or positively regulate MAPK signaling initiated either by tyrosine kinase–coupled receptors for growth factors or by cell adhesion to extracellular matrix (8, 16). Moreover, the expression of the dominant-negative form of SIRPα stimulates NF-κB activity and makes the cells resistant to TNF-specific apoptosis (18). However, the role of SIRPα in TLR-mediated signaling during innate immune responses has not been defined. We show that SIRPα plays an essential role in negatively regulating LPS signaling at an early stage of TLR4 activation. LPS-induced SIRPα reduction is required for initiation of MyD88- and Trif-dependent intracellular signaling pathways. Knockdown of SIRPα leads to augmented production of proinflammatory cytokines and multiple organ failure, which is characteristic of severe sepsis after TLR activation. Thus, the expression level of SIRPα may represent a threshold for control of a magnitude of host inflammatory responses to microbial pathogens.

RESULTS

SIRPα knockdown enhances TLR signaling in macrophages

To examine SIRPα regulation of the innate immune responses during bacterial infection, we first constructed a short hairpin RNA (shRNA) vector that specifically down-regulated SIRPα and stably transfected it into RAW264.7 macrophage cells. RAW cells were also stably transfected with either WT SIRPα (Myc-tagged) or an empty vector. They are referred to here as SIRPα-KD (knockdown), -OV (overexpression), and -VT (vector) macrophages, respectively (Fig. 1 A). Compared with empty vector, introduction of shRNA or SIRPα gene had little effect on cell growth during the time period examined (Fig. 1 B). TLR stimulation activates IKKs and all three major subgroups of MAPK: c-Jun NH_2-terminal kinase (JNK), p38 MAPK, and extracellular signal–related kinase (ERK) (2, 3). In response to LPS, KD macrophages were found to exhibit enhanced phosphorylation of p38, JNK, ERK, and IκBα, whereas overexpression of SIRPα resulted in reduction of these signaling pathways in RAW cells (Fig. 1 C). Furthermore, transient transfection of siRNA directed against SIRPα into thioglycollate-elicited peritoneal macrophages from C57BL/6 mice (Fig. 1 D) also resulted in higher activation of p38, JNK, ERK, and IκBα compared with that in macrophages transfected with negative control siRNA upon LPS stimulation (Fig. 1 E). Because AP-1 and NF-κB transcription factors are known targets of LPS-activated MAPKs and IKKs, we then examined whether SIRPα affects AP-1 and NF-κB activities. As revealed by a luciferase reporter assay, there were substantial increases in AP-1 and NF-κB activities in SIRPα-KD macrophages after LPS stimulation (Fig. 1 F). Conversely, introduction of WT SIRPα attenuated activation of AP-1 and NF-κB reporter genes by LPS stimulation. These findings demonstrate that SIRPα negatively regulates LPS signaling by suppressing the MAPKs and NF-κB pathways in macrophages.

SIRPα knockdown alters the pattern of cytokine production in LPS-stimulated macrophages

Because MAPKs and NF-κB signaling are pivotal in modulating innate immune responses, we investigate whether SIRPα directly controls cytokine production in macrophages upon LPS treatment. SIRPα-KD, -OV, and -VT macrophages were stimulated with various concentrations of LPS for 12 h to determine the production of cytokines or for 24 h for the nitric oxide (NO) in the conditioned medium. In response to LPS stimulation, SIRPα-KD macrophages produced substantially more TNFα, IL-6, and NO than SIRPα-OV and -VT macrophages (Fig. 2 A). As expected, SIRPα-OV cells produced the least TNFα, IL-6, and NO at any of the doses examined. To exclude the possibility that SIRPα-KD macrophages might be compensatorily activated, thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were used to assess the role of SIRPα in cytokine production in primary macrophages. Similar to what was observed for SIRPα-KD macrophages, peritoneal macrophages transfected with SIRPα siRNA also mounted a more robust TNFα, IL-6, and NO production than did negative control siRNA upon various doses of LPS stimulation (Fig. 2 B), suggesting that no compensatory activation of macrophages occurs under SIRPα-KD conditions.

To study the effects of SIRPα on the production of comprehensive inflammatory cytokines, SIRPα-KD and -OV cells were stimulated with LPS for 12 h, and the conditioned medium was subjected to an antibody array of 40 mouse inflammatory cytokines. As shown in Fig. 2 C, a substantial number of proinflammatory cytokines, including IL-6, TNFα, MCP-1, MIP-1α, RANTES, and GCSF, were dramatically

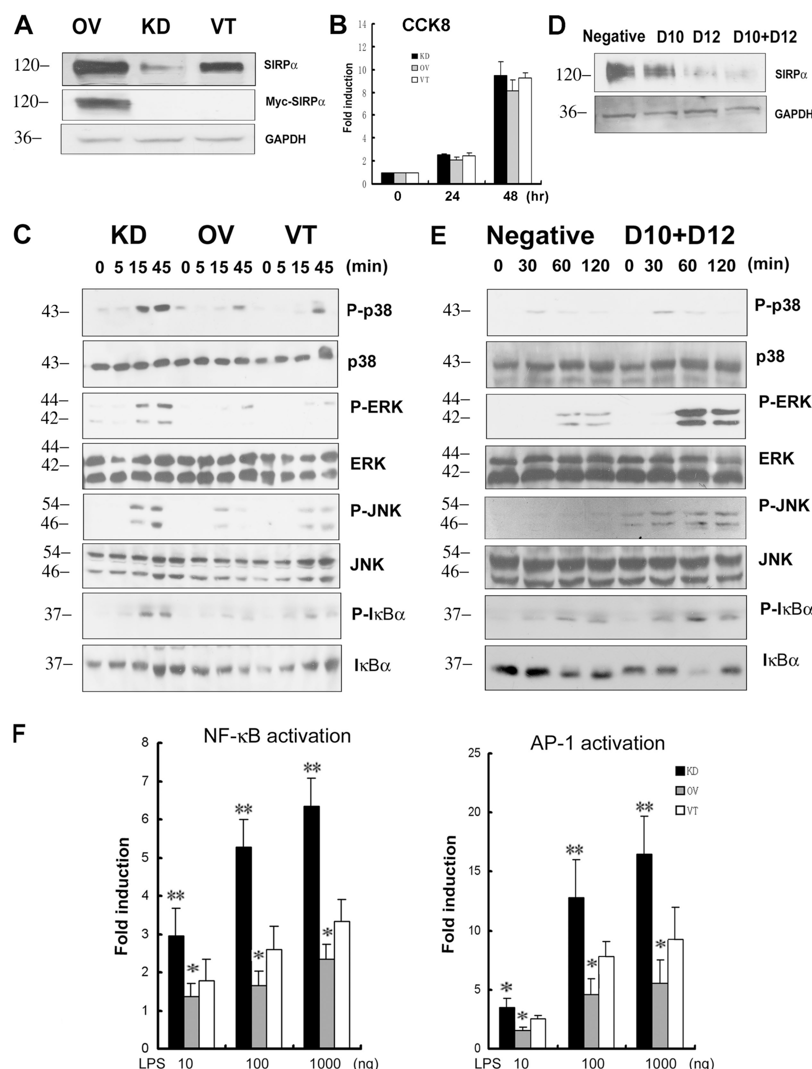

Figure 1. Increased signaling in SIRPα knockdown macrophages upon LPS stimulation. (A) RAW cells were stably transfected with empty vector or constructs containing shRNA specific for SIRPα or Myc-tagged SIRPα, and SIRPα expression levels were detected by Western blotting. (B) Cell proliferation of stable RAW cells was measured using CCK-8 assay at the indicated times. Data are the mean ± the SEM of triplicates from an experiment that was repeated a total of three times with similar results. (C) Strongly increased signaling in SIRPα-KD macrophage cell lines. 5×10^5 cells/well SIRPα-KD, -OV, and -VT RAW cells were stimulated with 10 ng/ml LPS for the indicated times. Cell lysates were prepared and blotted with the indicated antibodies. (D) Peritoneal macrophages were transiently transfected with siRNA (D10 and/or D12) targeting SIRPα or irrelevant control siRNA. The reduction of SIRPα expression was demonstrated by Western blotting. (E) 1.5×10^5 cells/well peritoneal macrophages from C57BL/6 mice were transfected with control or SIRPα siRNA, and then stimulated with 10 ng/ml of LPS for the indicated minutes. Cell lysates were blotted as mentioned in C. (F) 1×10^5 SIRPα-KD, -OV, and -VT cells were transfected with the NF-κB or AP-1 reporter plasmids (0.2 μg), together with the control plasmid pRL-TK (0.02 μg), and treated with various doses of LPS for 6 h, and then luciferase activities were detected. Data are expressed as relative fold activation to that of nonstimulated (–) sets. *, $P < 0.05$; **, $P < 0.01$ (OV or KD different from VT).

induced after LPS challenge. However, many antiinflammatory cytokines, such as IL-4 and -10, were not substantially altered. ELISA assays confirmed that the production of MIP-1α and RANTES in the LPS-stimulated SIRPα KD macrophages was, indeed, considerably higher than those in the LPS-stimulated SIRPα-OV and -VT macrophages (Fig. 2 D).

Interestingly, SIRPα knockdown also substantially raised LPS-induced IFN-β production and mRNA expression in either RAW cell line or mouse peritoneal macrophages (Fig. 2, E and F). In addition, the interferon-sensitive response element (ISRE) reporter activity was also increased in stably transfected RAW cell lines expressing shRNA for SIRPα (Fig. 2 G). Because initial induction of IFN-β is largely dependent on Trif-mediated IRF3 activation, we examined the phosphorylation of IRF3 upon LPS challenge and found that SIRPα siRNA treatment evidently led to the enhanced LPS-induced IRF3 activation in mouse peritoneal macrophages (Fig. 2 H). Collectively, our data clearly indicate that SIRPα regulates the expression profile of proinflammatory cytokines through both MyD88- and Trif-dependent pathways.

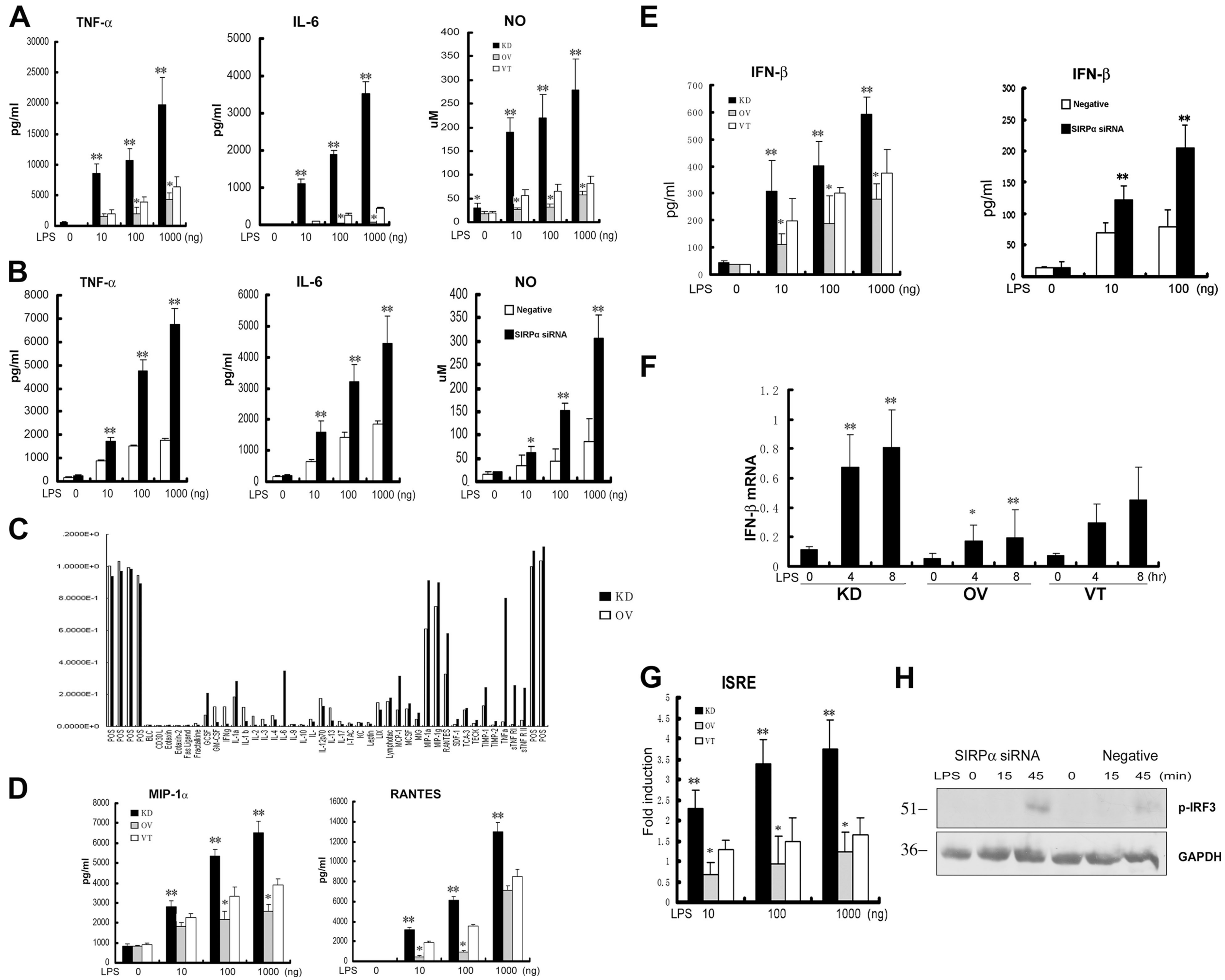

Figure 2. Increased cytokine production of SIRPα knockdown macrophages upon LPS stimulation in vitro. (A) 5 × 10^{5} cells/well SIRPα-KD, -OV, and -VT cells were treated with different amounts of LPS for 12 or 24 h, after which culture supernatants were harvested for measurement of TNFα, IL-6, and NO production. (B) Cytokine production by LPS-challenged peritoneal macrophages. 3 × 10^{5} cells/well transfected with SIRPα siRNA or negative control oligonucleotides. (C) Cytokine antibody array analysis of LPS-treated cytokine production in SIRPα-KD and -OV cells. Cells were primed with 100 ng/ml LPS for 12 h, after which culture supernatants were harvested for the cytokine antibody array analysis. The density value of each test sample was normalized to control spots on the same membrane graphed. (D) 5 × 10^{5} cells/well SIRPα-KD, -OV, and -VT cells were primed with different amount of LPS for 12 h, after which culture supernatants were harvested for measurement of MIP-1α and RANTES by ELISA. (E) IFN-β production after LPS challenge in stable RAW cell lines (left) or in peritoneal macrophages transfected with SIRPα siRNA (right) were analyzed as mentioned above. (F) Cells were incubated with 100 ng/ml of LPS for the indicated time, and IFN-β mRNA levels were determined by quantitative real-time PCR. (G) Cells were transfected with the ISRE reporter plasmid (0.2 μg), together with the control plasmid pRL-TK (0.02 μg), and treated with various doses of LPS for 6 h, and then luciferase activities were detected. The data are representative of three independent experiments with similar results. (H) Peritoneal macrophages from C57BL/6 mice were transfected with control or SIRPα siRNA and then stimulated with 10 ng/ml of LPS for the indicated minutes. Cell lysates were blotted with anti–phospho-IRF3 and anti-GAPDH antibodies. Data are the mean ± the SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (OV or KD different from VT in A, D, F, and G).

Transferring with SIRPα-KD macrophages increases susceptibility of mice to lethal LPS shock

Because SIRPα-KD macrophages exhibited a marked elevation in TNFα, which plays an important role in the pathogenesis of septic shock (19), we asked whether mice transfered with SIRPα-KD macrophages were more susceptible to LPS-induced toxicity. To determine whether RAW cells were capable of reconstituting macrophage-depleted allogeneic BALB/c mice ($GdCl_3$ pretreatment), RAW264.7 cells were stained with a fluorescent vital dye, SP-DiI, which was systemically delivered in vivo. The cells were analyzed by light and fluorescent microscopy in frozen sections 24 h after injection. As shown in Fig. 3 A, a large number of the injected RAW cells were found in the normal spleen, liver, and lung tissues, which is a typical distribution pattern for host-derived macrophages. Furthermore, the reconstituted mice showed a similar survival rate to the control normal mice after lethal dose challenge with LPS (Fig. 3 B). These results may support

the feasibility of this model system. Next, age- and sex-matched cohorts of mice pretreated with $GdCl_3$ for 24 h were injected i.v with SIRPα-KD, -OV, or -VT cells; after an additional 24 h, they were injected i.p. either with vehicle (PBS) or LPS. Survival of these animals was monitored over 4–5 d. As shown in Fig. 3 C, none of the SIRPα-KD, -OV, and -VT mice injected with PBS showed a difference on survival. However, at a LPS dose of 20 mg/kg body weight, mortality was observed within 14 h after LPS challenge for KD mice. By 24 h, >50% of KD mice had died, whereas the first mortality for VT mice occurred at 24 h. Although 85% death was noted for KD mice by the end of the experiment (120 h), only 26 and 35% of the OV and VT mice had died. Therefore, KD mice were more susceptible to LPS-induced lethality.

Endotoxic shock is mediated by an overproduction of proinflammatory cytokines, including TNFα and IL-6. To examine whether SIRPα controls cytokine release in response to LPS stimulation in vivo, we measured levels of various cytokines in the serum from mice after 3 h of LPS challenge. In the absence of LPS challenge, serum levels of TNFα and IL-6 were at a very low level. In response to LPS challenge, serum TNFα and IL-6 levels in KD mice were substantially elevated by >3–4 and 1.5–2 times, respectively, of those in OV and VT mice (Fig. 3 D).

To examine the effects of SIRPα on the function of key organs, SIRPα-KD, -OV, and -VT mice were challenged with LPS at a dose of 10 mg/kg body weight and killed 24 h after LPS injection. As shown in Fig. 3 E, KD mice challenged with LPS showed gross organ failure and severe inflammatory infiltrates in lung and liver, compared with OV and VT mice. Collectively, our results indicate that SIRPα-KD mice are highly susceptible to the development of multiple organ failure syndromes after LPS administration, and that SIRPα is required for the restriction of macrophage responses to LPS in vivo.

Endotoxin tolerance is SIRPα independent

The aforementioned results, finding that SIRPα down-regulated LPS-induced biological responses, prompted us to investigate whether SIRPα is also important for mediating endotoxin tolerance, a transient state of LPS refractoriness after the initial, nonlethal exposure to LPS. To determine whether SIRPα regulates the production of proinflammatory cytokines after LPS pretreatment, we first treated SIRPα-KD, -OV, and -VT macrophages with LPS for 24 h, after which we assessed IL-6 and TNFα production during a subsequent 12-h LPS treatment, or 24-h treatment for NO. SIRPα-KD cells substantially elaborated increased amounts of TNFα, IL-6, and NO during the first 12 h after exposure to LPS (Fig. 4 A). Their production by all SIRPα-KD, -OV, and -VT macrophages was considerably reduced after 24 h of LPS pretreatment, indicating that SIRPα is not required for LPS-induced down-regulation of cytokine production. Similarly, stimulation of cells with CpG or LPS for 24 h, followed by a second 12 or 24 h of LPS or CpG treatment, also resulted in decreased IL-6, TNFα, and NO production by all cells (Fig. 4 B), suggesting that cross-tolerance between TLR4 and TLR9 apparently occurred independently of SIRPα expression. We also performed these studies with peritoneal macrophages transiently transfected with synthetic SIRPα siRNA and obtained similar results (Fig. 4 C). Thus, SIRPα seems to regulate the magnitude and duration at the early "time window" of TLR-induced responses independently of previously described endotoxin tolerance mechanisms (20).

LPS-induced SIRPα reduction contributes to macrophage activation

To understand the cellular mechanisms by which SIRPα controls LPS signaling, we first examined whether SIRPα could modulate the expression of TLR4, which is a functional receptor for LPS. By using immunoblot assay, we measured TLR4 expression in SIRPα-KD, -OV, and -VT macrophages, or in peritoneal macrophages from C57BL/6 mice transfected with SIRPα siRNA or negative control siRNA with or without LPS exposure for 12 h, and we found no detectable difference in TLR4 levels between the cells examined (Fig. 5 A). We next analyzed SIRPα protein and RNA levels after an initial LPS treatment in mouse macrophage cell lines (RAW 264.7 and J774A.1), as well as in mouse peritoneal macrophages. Fig. 5 B shows a pattern of rapid and persistent down-regulation of SIRPα protein after LPS treatment in a time- and dose-dependent manner. Quantitative real-time PCR analysis further showed that there was an approximately threefold reduction of SIRPα RNA expression after 15 min of exposure to LPS, and that SIRPα mRNA remained lower after 24 h (Fig. 5 C). To determine whether the reduction in SIRPα expression induced by LPS was also attributable to an increased rate of degradation, we examined the effects of inhibitors of protein degradation by lysosomes or the proteasome. MG132, which is a reversible peptide aldehyde that blocks proteasomal activity, had no marked effect on the ability of LPS to suppress SIRPα expression. In contrast, prior treatment of RAW cells with chloroquine or NH_4Cl, both of which inhibit lysosomal function, substantially attenuated the effect of LPS. On the other hand, treatment with cycloheximide (CHX) alone considerably suppressed the levels of SIRPα protein, and LPS stimulation in the presence of CHX further down-regulated SIRPα expression (Fig. 5 D). Collectively, these data clearly indicate that, in addition to transcriptional repression of SIRPα expression, LPS also induces the degradation of SIRPα by the lysosome, thus contributing to the loss of SIRPα protein in macrophages after LPS stimulation.

To determine the upstream signals responsible for the reduction of SIRPα, we examined the expression of SIRPα in mice lacking TLR4 (C57BL/10ScCr) or in mice with a missense mutation of TLR4 (C3H/HeJ) (21). After LPS treatment, the reduction of SIRPα expression was not observed in peritoneal macrophages from either TLR4 knockout or mutant mice, suggesting that TLR4 is required for LPS-induced SIRPα down-regulation (Fig. 5 E). Interestingly, SIRPα protein levels were constitutively higher in peritoneal macrophages from C57BL/10ScCr or C3H/HeJ mice than in cells from control mice, indicating that basal TLR4 signaling activity is necessary for controlling the expression level of SIRPα.

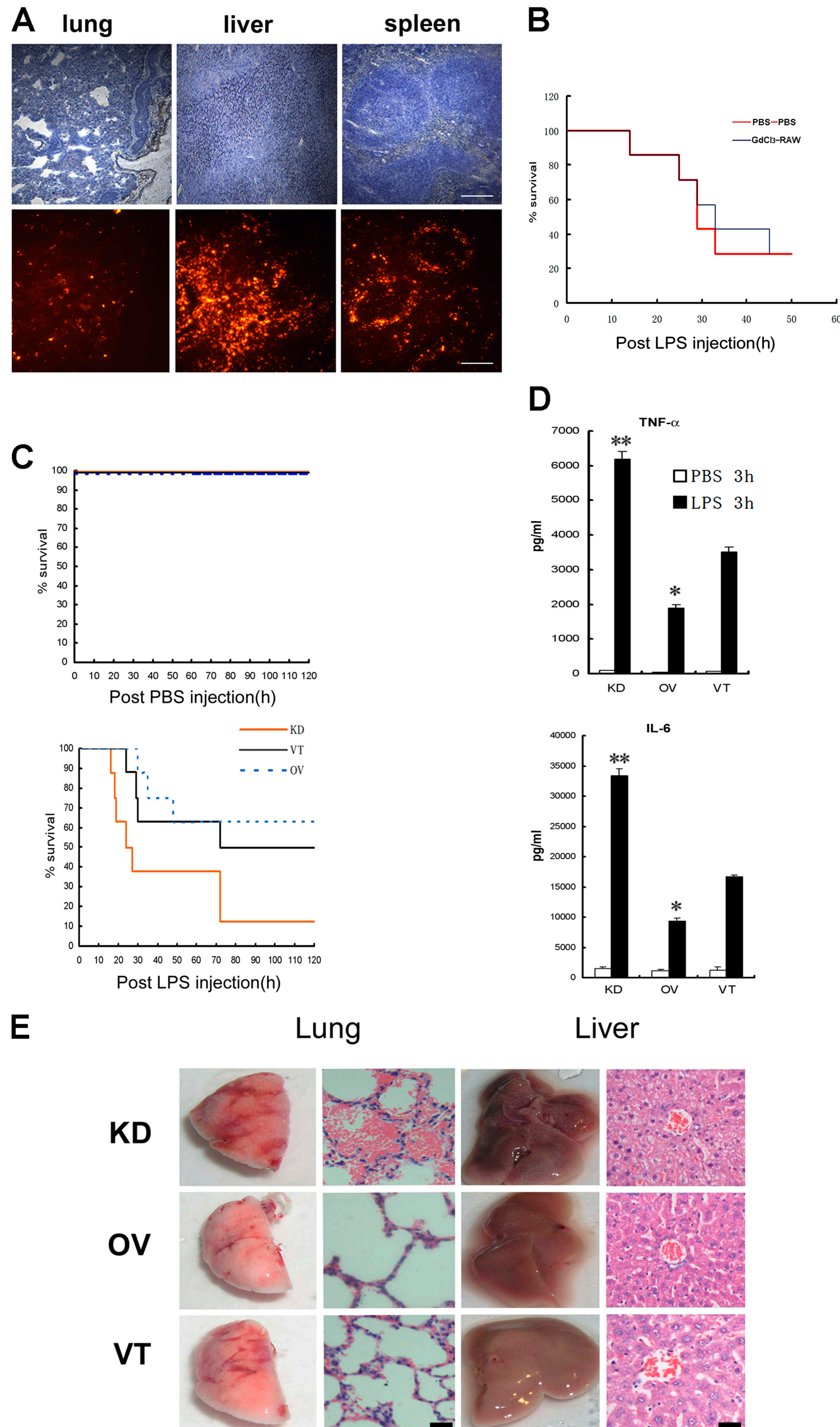

Figure 3. Increased response in mice transfered with SIRPα knockdown macrophages upon LPS stimulation in vivo. (A) Reconstitution of macrophage-depleted mice with RAW264.7 cells. Fluorescent dye-labeled RAW264.7 cells were injected i.v. into $GdCl_3$-treated Balb/C mice. Mouse organs (spleen, liver, and lung) were preserved for fluorescence microscopy analysis. Numerous injected cells are detectable in lung, spleen, and liver. Tissue morphology is visualized by hematoxylin staining. (B) Similar lethality in mice reconstituted with RAW264.7 cells challenged with LPS. Age- and sex-matched cohorts of mice (n = 6) were pretreated with $GdCl_3$ (10 mg/kg of body weight) or PBS and, 24 h later, were i.v. injected with RAW264.7 cells (10^7/each) or PBS, respectively. Another 24 h later, mice were i.p. administered with 25 mg LPS/kg of body weight, and lethality was observed over 60 h after this challenge.

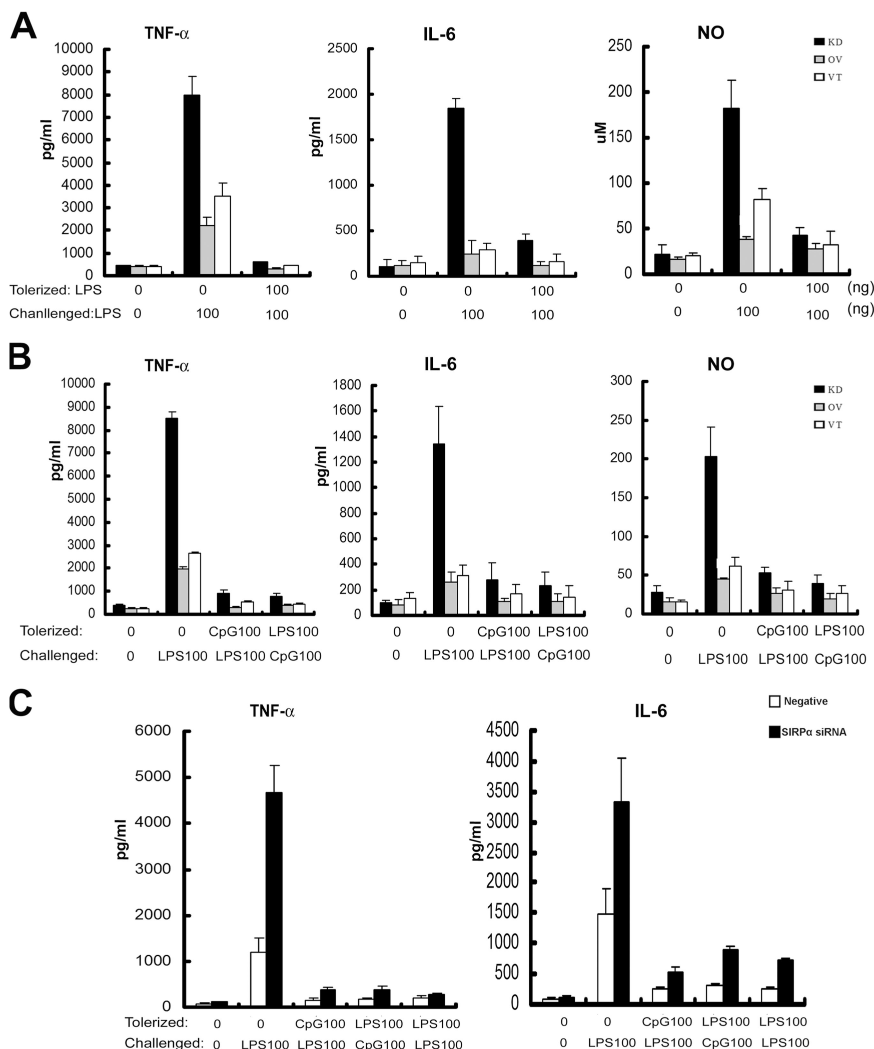

Figure 4. SIRPα is not required for endotoxin tolerance. (A) 5×10^5 cells/well SIRPα-KD, -OV, and -VT cells were untreated or tolerized with 100 ng/ml LPS for 24 h, washed, and rechallenged with 100 ng/ml LPS. After 12 h of incubation, the media were assessed for TNFα and IL-6, and for NO levels after 24 h. Data are presented as the mean ± the SEM of 3–8 independent experiments. (B) 5×10^5 cells/well SIRPα-KD, -OV, and -VT cells were tolerized with LPS or CpG for 24 h, washed, and rechallenged with the indicated doses of LPS or CpG. TNFα, IL-6, and NO levels were determined as described in A. (C) 3×10^5 cells/well peritoneal macrophages from C57BL/6 mice were transfected with either control or SIRPα siRNA. 24 h later, cells were tolerized with LPS or CpG for 24 h, washed, and rechallenged with the indicated doses of LPS or CpG. TNFα, IL-6, and NO levels were determined as described in A.

SIRPα is activated by LPS and binds constitutively to SHP-1 and -2

SIRPα is a surface receptor containing immunoreceptor tyrosine–based inhibitory motif domains that are known to exert inhibitory functions through the recruitment of phosphatase enzymes SHP-1 and -2 (22). Thus, we investigated whether LPS treatment affected tyrosine phosphorylation of SIRPα and its association with SHP-1 and -2 in macrophages. RAW264.7 cells were stimulated with LPS, and the endogenous SIRPα was immunoprecipitated and subjected to immunoblot with respective antibodies. As shown in Fig. 6 A, LPS treatment induced tyrosine phosphorylation of SIRPα, but failed

The data are representative of two independent experiments with similar results. (C) More lethality in mice transfered with SIRPα-KD cells challenged with LPS. Age- and sex-matched cohorts of mice ($n = 7$) were pretreated with $GdCl_3$ (10 mg/kg of body weight), and 24 h later were i.v. injected with SIRPα-KD, -OV, and -VT cells (10^7/each). Another 24 h later, mice were i.p. administered with PBS or 20 mg LPS/kg of body weight, and lethality was observed over 120 h after this challenge. The data are representative of two independent experiments with similar results. (D) Sera from mice pretreated as described in A and injected with PBS or LPS (10 mg/kg of body weight) were collected at 3 h after the challenge, and IL-6 and TNFα levels were measured by ELISA. Data show the mean ± the SEM for three mice from each group. *, $P < 0.05$; **, $P < 0.01$ (OV or KD different from VT). (E) Severe multiple organ failure in mice transfered with SIRPα-KD cells challenged with LPS. Representative images of lung and liver with histological sections from mice pretreated as described in C and killed at 24 h after LPS administration (10 mg/kg weight body). Bars, 100 μm.

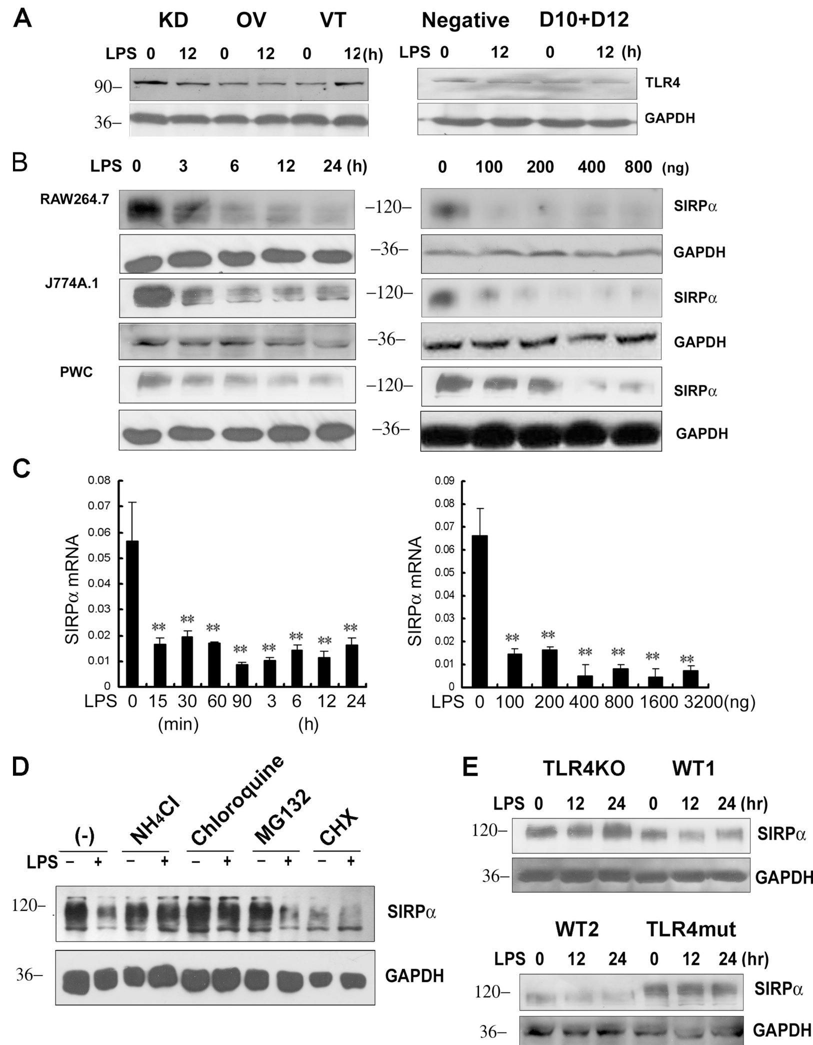

Figure 5. LPS-induced SIRPα reduction contributes to macrophage activation. (A) Stable RAW-derived cell lines (left) and peritoneal macrophages transiently transfected with either control or SIRPα siRNA (right) were incubated with or without 100 ng/ml LPS for 12 h. Immunoblots with a TLR4-specific antibody were performed. (B) Down-regulation of SIRPα protein levels in macrophages after LPS treatment. Mouse macrophage cell lines RAW264.7 and J774A.1, as well as peritoneal macrophages from C57BL/6 mice, were incubated with 100 ng/ml LPS for the indicated times (left) or with the indicated LPS doses for 12 h (right), and SIRPα protein expression was determined by Western blotting. PWC, peritoneal macrophages. (C) Down-regulation of SIRPα mRNA levels in macrophages after LPS treatment. RAW264.7 macrophages were incubated with 100 ng/ml of LPS for the indicated time (left), or with the indicated LPS doses for 12 h (right), and SIRPα mRNA levels were determined by quantitative real-time PCR. (D) The reduction of SIRPα protein level involves protein degradation. RAW264.7 cells were incubated for 2 h at 37°C in the absence or presence of 100 μM chloroquine, 10 mM NH_4Cl, 40 μM MG132, or 50 μg/ml CHX, after which they were treated with or without 100 ng/ml LPS for 5 h and subjected to Western blot analysis using SIRPα-specific antibody. (E) TLR4 is essential for the down-regulation of SIRPα. Peritoneal macrophages from TLR4 KO (C57BL/10ScCr) and littermate WT1 (C57BL/10snj) mice or TLR4 mutant (C3H/HeJ) and littermate WT2 (C3H/Heouj) mice were incubated with 100 ng/ml LPS for the indicated times, and SIRPα protein expression was determined by Western blot analysis.

to enhance SHP-1 and -2 recruitment. Interestingly, SHP-1 and -2 were found to be constitutively associated with SIRPα in macrophages (Fig. 6 A), which coincided with the results of previous studies (23, 24). However, treatment of RAW cells with either the Src-specific inhibitor PP_2 or universal tyrosine kinase inhibitor Genistein nearly abrogated the constitutive association of SIRPα with SHP-1 or -2, which suggests that the basal association of SHP proteins is dependent on the undetectable phosphorylation of SIRPα (Fig. 6 B). In addition, PP_2 also blocked LPS-induced SIRPα phosphorylation, indicating that such phosphorylation of SIRPα is mainly mediated by Src kinases (Fig. 6 C).

SHP-1 is generally considered a negative signal transducer, whereas SHP-2 is considered a positive one (25). However, the precise role of each enzyme in shared signaling pathways during innate immune activation is not well defined. To examine their roles in LPS-mediated signaling, SHP-1 and -2 expression were separately knocked down by siRNAs in

peritoneal macrophages (Fig. 6 D) with subsequent LPS treatment, and the production of TNFα and IL-6 was analyzed using ELISA assay. As expected, knockdown of SHP-1 caused an ~20% increase in cytokine production, whereas knockdown of SHP-2 resulted in a nearly twofold lower production of TNFα and IL-6 (Fig. 6 E). Further analysis of IFN-β production in SHP-1 and -2 siRNA-transfected cells reveals that SHP-2 plays a critical role in positive regulation of LPS-induced Trif signaling, whereas SHP-1 has no marked effect on the pathway (Fig. 6 F). In addition, knockdown of SHP-1 enhanced the activation of ERK and p38, whereas knockdown of SHP-2 slightly reduced their activities (Fig. 6 G).

To elucidate whether SHP-1 and -2 contribute to the inhibitory function of SIRPα in macrophage activation, peritoneal macrophages were cotransfected with SIRPα and SHP-1 siRNAs or SIRPα and SHP-2 siRNAs to eliminate both proteins simultaneously. After LPS stimulation, the conditioned medium was analyzed for cytokine production. As shown in Fig. 6 H, knockdown of SHP-1, together with SIRPα, slightly increased the production of TNFα and IL-6 more than knockdown of SIRPα alone, suggesting a synergetic role of

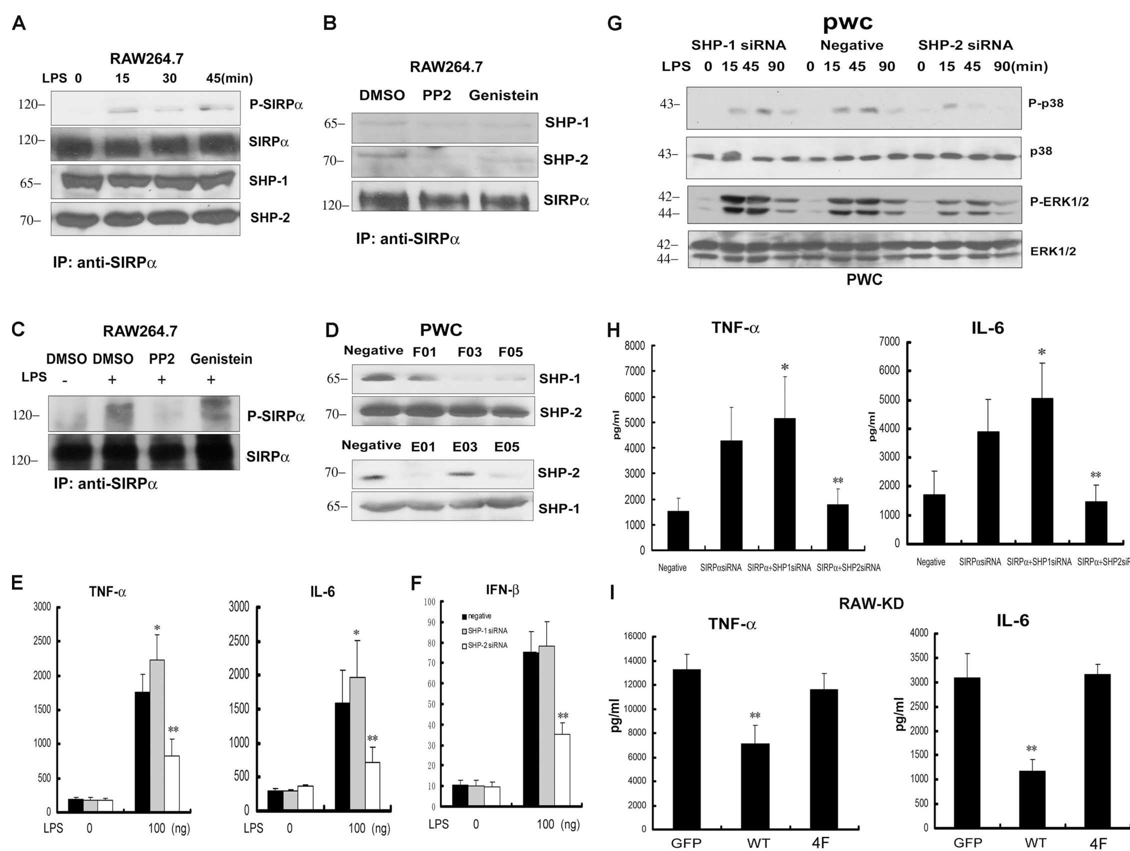

Figure 6. SIRPα inhibits LPS signaling mainly through sequestration of SHP-2. (A) RAW264.7 cells were stimulated with LPS for the indicated time, immunoprecipitated for endogenous SIRPα, and probed with an antiphosphotyrosine antibody. Immunoblots were also probed with anti-SIRPα, –SHP-1, and –SHP-2 antibodies. (B) Basal association of SHP proteins is dependent on SIRPα phosphorylation. RAW264.7 cells were treated with vehicle (DMSO), 10 μM PP2, or 50 μM genistein for 1 h, and then subjected to immunoprecipitation and immunoblot, as in A. (C) TLR4-induced SIRPα phosphorylation is mediated by Src kinases. RAW264.7 cells were stimulated with LPS for 15 min in the absence or presence of DMSO, PP2, or genistein, and then subjected to immunoprecipitation and immunoblotting as in A. (D) Western blot analysis demonstrates the effects of siRNAs that specifically down-regulate SHP-1 or -2 expression in peritoneal macrophages. (E) Peritoneal macrophages transfected with SHP-1– or -2–specific siRNAs were stimulated with 100 ng/ml LPS for 12 h, and the production of TNFα and IL-6 was determined using ELISA. Data are presented as the mean ± the SEM of 3–6 independent experiments. *, $P < 0.05$; **, $P < 0.01$ (SHP-1 or SHP2 siRNA different from negative control siRNA). (F) IFN-β production after LPS challenge in peritoneal macrophages. 3×10^5 cells/well transfected with SHP-1– or -2–specific siRNAs was analyzed as in E. *, $P < 0.05$; **, $P < 0.01$ (SHP-1 or -2 siRNA different from negative control siRNA). (G) Peritoneal macrophages transfected with SHP-1 or -2 siRNAs or negative control oligonucleotides were stimulated with 10 ng/ml of LPS for the indicated times. Cell lysates were blotted with the indicated antibodies. (H) Peritoneal macrophages were transfected with SIRPα-specific siRNA alone or together with SHP-1– or -2–specific siRNAs with subsequent LPS stimulation. The production of TNFα and IL-6 were determined as described in E. *, $P < 0.05$; **, $P < 0.01$ (SIRP1α/SHP-1 or SHP-2 double-knockdown is different from SIRP1α knockdown alone). (I) Rescue of cytokine production by reintroduction of WT SIRPα. 2×10^6 cells/well SIRPα-KD cells transfected with GFP, WT SIRPα (WT), or its mutant form SIRPα-4F (4F) were treated with 100 ng/ml LPS. The amounts of secreted TNFα and IL-6 in supernatants were determined by ELISA as described in E. Data are the mean ± the SEM of three independent experiments. **, $P < 0.01$ (WT different from GFP).

SIRPα and SHP-1 in suppressing LPS signaling. Conversely, knockdown of SHP-2 nearly reversed the hyperresponsive effects induced by SIRPα depletion in response to LPS, which indicate that SIRPα mainly functions through its association and sequestration of SHP-2 to prevent LPS-induced macrophage activation. In addition, transient overexpression of WT SIRPα into SIRPα-knockdown RAW cells restrained LPS-induced cytokine production, whereas its mutant form, SIRPα-4F, which is incapable of binding to SHP-2, was unable to restore SIRPα-mediated repression of LPS-inducible cytokine genes (Fig. 6 I). These data further indicate that SIRPα acts via SHP-2 sequestration to negatively regulate TLR4 signaling.

SIRPα prevents LPS responses through sequestration of SHP-2 from IKKs

As a previous study indicates that SHP-2 is an integral component of the IKK complex, and a functional SHP-2 is required for efficient phosphorylation of IκB by the IKK complex in cellular response to IL-1/TNF (26), we next searched for physical evidence for SHP-2 involvement in the MyD88 and Trif pathways by coimmunoprecipitation experiments. As expected, LPS induced interaction of endogenous SHP-2 with IKKβ and the IKK-like kinase TANK-binding kinase 1 (TBK1)/NAK (Fig. 7 A), both of which are critically important for activation of MyD88- and Trif-dependent signaling, respectively (27, 28). In contrast, SHP-1 could not be found in a complex with IKK (not depicted). Interestingly, compared with empty vector–transfected RAW cells, SIRPα knockdown drastically enforced LPS-induced SHP-2–IKK association (Fig. 7 B), which strongly suggests that SIRPα may compete with IKKs to bind SHP-2, thus compromising the essential role of SHP-2 in LPS signaling activation.

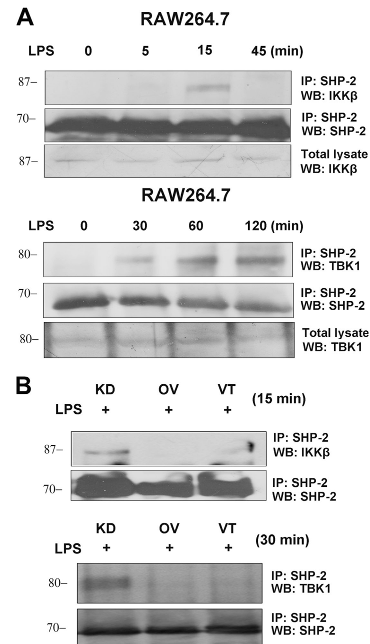

Figure 7. SIRPα prevents LPS-induced SHP-2–IKK complex formation. (A) RAW264.7 cells were treated with 100 ng/ml LPS for the indicated time period. Equal amounts of cell lysates were immunoprecipitated with SHP-2–specific antibody. Precipitated proteins and cell lysates were blotted with anti–SHP-2, anti-IKKβ, and anti-TBK1 antibodies. (B) SIRPα-KD, -OV, and -VT RAW cells were stimulated with 100 ng/ml LPS for the indicated minutes, immunoprecipitated for endogenous SHP-2, and probed with anti-IKKβ, -TBK1, and –SHP-2 antibodies.

DISCUSSION

A dynamic balance between activation and repression of the innate immunity is of critical importance in the host immunological defenses. Under conditions of LPS exposure, multiple feedback mechanisms exist for restraining the strength and duration of the transduced signals, and the production of inflammatory cytokines, which include the down-regulation of surface TLR expression, transcriptional induction of negative regulators such as IL-1 receptor–associated kinase (IRAK-M), suppressor of cytokine signaling 1 (SOCS1), SH2-containing inositol phosphatase, MyD88s, and antiinflammatory cytokines, mainly IL-10 and TGF-β (20). Although these mechanisms probably play a prominent role in termination of TLR signals, the factors that restrict initiation of TLR-mediated responses remain largely unknown. Our results demonstrate that SIRPα also acts as a crucial negative regulator of the innate immune responses both in vivo and in vitro. However, unlike the induction of other inhibitory proteins, SIRPα is rapidly down-regulated in response to LPS, which places it in the field of the hitherto poorly understood negative regulatory mechanisms dictating the activation of innate immune responses. Multiple lines of evidence support such an early inhibitory role of SIRPα during TLR activation. First, LPS-induced SIRPα down-regulation is required for the initiation of macrophage responses because introduction of SIRPα induces inability to respond to LPS in RAW cells, whereas depletion of SIRPα results in hypersusceptibility to LPS. Second, the SIRPα-mediated inhibition of TLR signal functions independently of endotoxin tolerance mechanisms, which is consistent with the rapid and sustained decrease in SIRPα levels after LPS challenge. Thus, it appears that SIRPα is not a component of a feedback regulatory system of innate immunity. Instead, SIRPα more likely operates at an early "time window" of TLR-induced responses. Third, depletion of SIRPα causes a substantial induction of proinflammatory cytokine expression upon LPS exposure, whereas most antiinflammatory cytokines are not considerably affected. Compared with the release of proinflammatory cytokines, which occurs rapidly after TLR stimulation, production of antiinflammatory cytokines is considerably slower. This further indicates that SIRPα-mediated repression is an early signaling event during TLR activation. Finally, LPS-induced activation of the MAPKs and NF-κB pathways is reversely proportional to SIRPα expression level, which strongly suggests that the negative regulation of TLR signals by SIRPα may occur at a receptor-proximate level upstream of multiple signaling pathways. Given all the evidence obtained so far, our results raise the possibility that the expression level of SIRPα may represent a threshold factor for septic shock.

A notable feature of SIRPα is its ability to recruit and signal via the tyrosine phosphatases SHP-1 and -2. Therefore, it is likely that this feature is important for the negative regulatory role of SIRPα in LPS-activated signaling. Although structurally very similar, these two phosphatases play quite different cellular roles. SHP-1 has been generally considered as a negative signal transducer, essentially as an antagonist of SHP-2. The data presented here suggests that SHP-1 plays a relatively minor role in suppressing LPS-induced TLR activation, which is consistent with a recent study that SHP-1 modestly inhibits LPS-mediated TNFα and iNOS production in mouse macrophages (29). As double-knockdown of SHP-1 and SIRPα induced a little more cytokine production than knockdown of SIRPα alone, SHP-1 may represent a collaborating event in SIRPα-mediated negative regulation of TLR signaling. On the other hand, our data provide direct evidence that SHP-2 plays a largely positive-signaling role in macrophage activation by LPS. By analyzing immune responses in macrophages lacking both SHP-2 and SIRPα expression, we propose a model in which SIRPα plays reciprocating roles in the temporal regulation of both pro- and antiinflammatory responses. In the initial phase of activation, macrophages have relatively high levels of SIRPα protein, which probably acts as a scaffolding molecule to recruit SHP-2 in the vicinity of the cell membrane, thus preventing SHP-2 from activating downstream signaling pathways. Subsequently, the decrease in SIRPα levels causes a disassociation of SHP-2 with SIRPα, thus making SHP-2 available to mediate TLR signaling activation.

All TLRs are known to elicit conserved inflammatory pathways, culminating in the activation of two major kinase-mediated signaling pathways: the MAPK and IKK complexes, which transduce various upstream signals to the activation of AP-1, NF-κB, and IRF3 transcription factors. Both SHP-1 and -2 have been shown to be necessary for growth factor–induced MAPK activation (30). However, whether SHP-1 and -2 regulate LPS-induced MAPK activation remains unknown. By knocking down the expression of SHP-1 and -2, we demonstrate that SHP-1 exerts inhibitory effects on MAPK activation, which is inconsistent with the observation that ERK activation occurred normally in mev/mev cells in response to LPS (17). It is likely that experimental variations in these experiments explain the differing results. In contrast, SHP-2 is seemingly indispensable for MAPK activation, as depletion of SHP-2 reduces macrophage responses to LPS. Previously, the effects of SIRPα on growth factor–induced MAPK activation in epithelial or fibroblast cells have been controversially reported (15). We clearly show that SIRPα negatively regulates LPS-induced MAPK activation in macrophages. Given that SHP-1 is highly expressed in macrophages and, at a much lower level, in epithelial or fibroblast cells, whereas SHP-2 is expressed in the opposite manner, it is possible that the dynamic interaction of SIRPα with SHP-1 and -2 is crucial for proper regulation of the MAPKs by LPS or some growth factors in cells derived from different tissues. In addition to MAPKs, the NF-κB pathway is also regulated either positively by SHP-2 (26, 31) or negatively by SHP-1 (18, 32). However, the molecular basis for SHP's activities in the NF-kB pathway is not yet fully understood. We show that SHP-2, but not SHP-1 (not depicted), forms an inducible complex with IKKβ or TBK1 upon stimulating TLR4 with LPS, which appears to be required for NF-κB and IRF3 activation, as the enforced SHP-2–IKK associations after SIRPα knockdown are accompanied by the increased phosphorylation of IκBα and IRF3. Because SIRPα preferentially associates with, and most probably sequesters, SHP-2, which is required for both MAPK and IKK activities, it most likely acts at the level of this phosphatase to negatively regulate TLR signaling.

TLR-induced cytokine production in innate immune cells is mediated mainly by MAPK- and IKK-dependent signals. By inactivating these two pathways, SIRPα determines the window of synthesis of a variety of proinflammatory cytokines, including TNFα and IL-6. However, unlike other negative regulators, such as IRAK-M or SOCS1, depletion of SIRPα has no significant effect on IL-1 and IL-12p40 production after LPS challenge (Fig. 2 C), and both IL-1 and IL-12p40 are known to contribute to the lethal outcome of endotoxin shock. Furthermore, it appears that LPS induces a much more dramatic release of TNFα and IL-6 in SIRPα-KD macrophages than in IRAK-M– or SOCS1-deficient macrophages (33, 34). These results perhaps reflect the different mechanisms on which these regulators operate. Interestingly, it has been shown that ligation of SIRPα with monoclonal antibody results in modest reduction of TNFα production, possibly via inducing intracellular retention of the cytokine, but has no effect on other cytokine induction in response to LPS stimulation (35), which is inconsistent with the broad and drastic augmentation of proinflammatory cytokines in SIRPα-depleted macrophages after LPS challenge. Considering that both MAPKs and IKKs positively regulate TNFα synthesis (36, 37), knockdown of SIRPα may mainly enhance both the stability and the translation of TNFα mRNA, rather than modulate its secretion. It has been suggested that SIRPα ligation may trigger signal cascades other than MPAKs or IKKs; e.g., the PI3K pathway to specifically reduce TNFα release. In addition, SIRPα ligation has also been shown to induce macrophages to produce NO, thus indicating a positive effect of SIRPα (23). However, the level of H_2O_2 is increased after SIRPα ligation, and is found to be essential for the SIRPα-induced production of NO, so it is difficult to distinguish between effects that result from a direct activation of SIRPα signaling or an indirect activation as a consequence of increased ROS production, or both. Future studies should elucidate more precisely whether and how these pathways integrate and translate into the various cellular functions that are regulated by SIRPα in macrophages and other cells.

In summary, we have shown that SIRPα plays a critically negative role in macrophage activation. LPS-induced SIRPα down-regulation is required for inducing optimal strength and duration of the TLR signaling. SIRPα therefore functions as a "homeostatic" effector in innate immunity. The basal level of SIRPα in macrophages may represent a threshold for maintaining a non-/antiinflammatory environment. SIRPα is likely to become a rational pharmacological target for

treatment of patients with autoimmunity, chronic inflammation, or infectious diseases.

MATERIALS AND METHODS

Antibodies and reagents. Antibodies specific for phosphorylated JNK, p38, and ERK, as well as total ERK, JNK, p38, and Myc-tag, were all purchased from Cell Signaling Technology; anti–phospho-IκBα, IκBα, SHP-1, and SHP-2 antibodies were obtained from Santa Cruz Biotechnology. Rabbit polyclonal SIRPα antibody was generated in our laboratory against residues in the cytoplasmic domain. Mouse inflammation cytokine antibody arrays were purchased from RayBiotech, Inc. LPS was purchased from Sigma-Aldrich. The CpG oligodeoxynucleotides1826 was purchased from InvivoGen. Vector-based shRNAs containing target sequences of AAGTGAAGGTGACTCAGCCTG and AATCAGTGTCTGTTGCTGCTG of SIRPα were constructed using the pSUPER-neo vector (OligoEngine) according to the manufacturer's protocol. Predesigned phosphorothioate-modified stealthTM RNA targeted against SIRPα, SHP-1, and SHP-2 were provided by Invitrogen, with the following sequences: SIRPα: D10, 5′-AAGUGAAGGUGACUCAGCCUGAGAA-3′, D12, 5′-CAAAGCCGGCUGUUGAUCUACAGUU-3′; SHP-1: F01, 5′-CCCUCUCAGUCAGGGUGGAUGAUCA-3′, F03, 5′-GAGAUGGCACCAUCAUCCACCUUAA-3′; F05, 5′-GAGAACACUCGUGUCAUCGUCAUGA-3′; SHP-2: E01, 5′-GAGGGAAGAGCAAAUGUGUCAAGUA-3′, E03, 5′-CAGACAGAAGCACAGUACCGGUUUA-3′, E05, 5′-AAGUAUUCCUUGGUGGACCAGACAA-3′.

Animals. Male C57BL/6 and BALB/c mice (6–8 wk old, weighing 16–20 g) were obtained from the Shanghai Experimental Center, (Chinese Science Academy, Shanghai, China) and maintained at an animal facility under pathogen-free conditions. Male WT (C3H/HeOuj and C57BL/10SnJ), TLR4 mutant (C3H/HeJ; Pro712-His712), and TLR4-deficient (KO; C57BL/10ScCr) mice were obtained from the Model Animal Research Center of Nanjing University (Nan Jing, China; 8–12 wk old). All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, which was prepared by the National Academy of Sciences and published by the National Institutes of Health (publication 86–23; revised 1985).

Cell lines and isolation of peritoneal macrophages. Mouse macrophage cell lines RAW264.7 and J774A.1 were obtained from the Shanghai Cell Bank (Shanghai, China). Cells were cultured in RPMI 1640 with 10% FBS within a humidified incubator containing 5% CO_2 at 37°C and passed every 2–3 d to maintain logarithmic growth. Approximately 6×10^5 cells/well were seeded into 6-well plates and transfected 24 h later with the pSUPER-shRNAs, pcDNA3.1-Myc-SIRPα constructs and empty vector using PEI (Polyplus; AFAQ) according to the manufacturer's instructions. Stable RAW cell transformants were selected with 300 μg/ml G418. Peritoneal macrophages were isolated from mice (6–8 wk old, weighing 16–20g). Mice were i.p. injected with 2 ml of 4% thioglycollate (Sigma-Aldrich). 3 d after thioglycolate injection, cells in the peritoneal exudates were isolated by washing the peritoneal cavity with ice-cold HBSS. Collected cells were incubated for 4 h, and adherent cells were taken as peritoneal macrophages. Transfection into peritoneal macrophages was performed using GENEPORTER (Genlantis) according to the manufacturer's instructions. The measurement of viable cell mass was performed with a Cell Counting Kit-8 (Dojin Laboratories) to count living cells by WST-8.

Adoptive transfer of macrophages. BALB/c mice were injected i.v. with $GdCl_3$ (Sigma-Aldrich; 10 mg/kg of body weight) to eliminate macrophages in vivo. 24 h after $GdCl_3$ injection, SIRPα-KD/-OV/-VT RAW264.7 macrophages (1×10^7 cells) suspended in 100 μl of pyrogen-free PBS were injected i.v. into the mice. Another 24 h later, mice were injected i.p. with PBS or LPS (20 mg/kg of body weight) and the resulting lethality was observed. Cytokine levels in sera were measured at 3 h after LPS injection (10 mg/kg of body weight).

Cytokine assay. Cytokine levels in culture supernatants or in sera were determined using commercial ELISA kits for TNFα, IL-6 (R&D Systems and BD Biosciences), RANTES, MIP-1α, and IFNβ (Biosource) according to the manufacturer's instructions. Each value represents the mean of triplicate values. Comprehensive analysis of cytokine levels were performed by using commercially available RayBio Mouse Inflammation Antibody Array 1.1 (RayBiotech, Inc.) according to manufacturer's protocol.

Nitrite oxidant detection. Cells plated at 1.5×10^5 cells/well in 24-well culture dishes were incubated overnight before stimulation. After the cells were treated with 100 ng/ml LPS for 24 h, culture medium was collected for analysis by the Griess Reagent kit. Nitrite concentrations were determined by the measurement of the optical density at 570 nm.

Luciferase assay. SIRPα-KD/-OV/-VT RAW264.7 macrophages were plated at 5×10^4 cells/well in 48-well culture dishes and were transfected with 0.2 μg NF-κB-Luc, AP-1-Luc, or ISRE-Luc reporter plasmids together with 0.02 μg of pRL-TK (Promega) by GENEPORTER (Genlantis). Luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was normalized with Renilla luciferase activity.

RT-PCR. RNA was extracted from RAW264.7 cells by using Trizol Reagent (Invitrogen). 2 μg cellular RNA was used for cDNA synthesis. For real-time PCR, we used the specific SYBR-Fluo from TaKaRa Biotechnology Co. Ltd. PCR primers for detecting mRNA of SIRPα and β-actin were synthesized by TaKaRa (Biotechnology Co., Ltd.). PCR reaction consisted of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, ×35 cycles for both β-actin and SIRPα. Primer sequences were as follows: β-actin, sense, 5′-GGACTCCTATGTGGGTGGCGAGG-3′, antisense, 5′-GGGAGAGCATGCCCTCGTAGAT-3′; and SIRPα, sense, 5′-TCGAGTGATCAAGGGAGCAT-3′, antisense, 5′-CCTGGACACTAGCATACTCTGAG-3′.

We thank Dr. Axel Ullrich and Dr. Gensheng Feng for kindly providing antibodies or cDNAs.

This work was supported by grants from China Key Basic Research Program grant (2004CB518703) and National Natural Science Foundation of China (30371301, 30620130434, and 30530790).

The authors have no conflicting financial interests.

Submitted: 14 December 2006
Accepted: 28 September 2007

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