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**Supplementary Material**

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Steroid Receptor Coactivator 3 Is Required for Clearing Bacteria and Repressing Inflammatory Response in Escherichia coli-Induced Septic Peritonitis

Qiang Chen,*† Tenghui Chen,* Yixiang Xu,* Jingwei Zhu,* Yuan Jiang,* Yang Zhao,* Jianming Xu,‡ and Chundong Yu*

Steroid receptor coactivator 3 (SRC-3) is a multifunctional protein that plays an important role in regulation of bacterial LPS-induced inflammation. However, its involvement in host defense against bacterial infection remains unclear. In this study, we used SRC-3 knockout mice to assess the role of SRC-3 in antibacterial defense in Escherichia coli-induced septic peritonitis. After E. coli bacteria were injected i.p., SRC-3–deficient mice exhibited excessive local and systemic inflammatory responses and more severe bacterial burdens, leading to a significantly higher mortality compared with wild-type mice. Peritoneal macrophages of SRC-3–deficient mice showed a decrease in bacterial phagocytosis in culture and an increase in apoptosis, which was consistent with the defective bacterial clearance observed in SRC-3–deficient mice. Accordingly, SRC-3 null macrophages expressed much lower levels of scavenger receptor A, the antioxidant enzyme catalase, and antiapoptotic gene Bel-2. Collectively, our data demonstrate that SRC-3 is important not only in modulating the local and systemic inflammation but also in intensifying bacterial clearance, which highlights a pivotal role of SRC-3 in the host defense system against bacterial infection. The Journal of Immunology, 2010, 185: 5444–5452.

Severe sepsis is a common, life-threatening infection resulting from a range of causative pathogens (1). The prevailing concept of the pathogenesis of sepsis is that mortality is a consequence of an uncontrolled hyperinflammatory response of the host mediated by proinflammatory cytokines (2, 3). Indeed, inhibition of proinflammatory cytokines such as IL-1β and TNF-α improves organ function and survival in animal models of sepsis (4, 5). However, deficiency in another proinflammatory cytokine IL-6 resulted in accumulation of live bacteria and increased mortality of mice infected by Escherichia coli (6). In addition, phagocytic cells and complements also play crucial roles in prevention of sepsis by clearing bacteria (7, 8). Therefore, precise control of proinflammatory cytokines and effective elimination of bacteria are crucial for host survival from infection.

Steroid receptor coactivator-3 (SRC-3/AIB1/ACTR/pCIP/RAC3/TRAM-1) is a member of the p160 coactivator family that also includes SRC-1 and SRC-2, which interacts with nuclear receptors and other transcription factors to enhance their effects on target gene transcription (9–13). SRC-3 was initially found to be amplified and overexpressed in breast cancer (9). Later studies demonstrated that SRC-3 played an important role in many developmental, physiological, and pathologic events, such as cell growth, oncogenesis (14–18), cancer metastasis (19), differentiation (20), and energy homeostasis (21). We recently reported that SRC-3–deficient mice are markedly more susceptible to LPS-induced endotoxic shock due to an increased production of proinflammatory cytokines including TNF-α, IL-1β, and IL-6 (22); in addition, SRC-3 is involved in the regulation of proinflammatory cytokine expression through suppressing cytokine translation from mRNA (22). These results indicate that SRC-3 has an essential function of suppressing LPS-induced inflammatory response. However, the biological role of SRC-3 in host defense against bacterial infection remains unclear.

Peritonitis is the second most common cause of sepsis (5) and a major abdominal emergency causing high rate of morbidity and mortality (23). Although different bacteria have been found responsible for peritonitis, E. coli remains one of the most common pathogens in i.p. infection (23). In the current study, we used SRC-3 knockout mice to determine the role of SRC-3 in antibacterial defense in E. coli-induced septic peritonitis. We found that SRC-3–deficient mice were more susceptible to peritonitis-induced lethality caused by excessive local and systemic inflammatory responses as well as bacterial outgrowth and disseminations. Our findings demonstrate that SRC-3 plays a pivotal protective role in the host defense against E. coli-induced peritonitis by enhancing bacterial clearance and modulating inflammatory responses.
Materials and Methods

**Mice**

SRC-3-deficient (SRC-3<sup>−/−</sup>) mice were generated as described previously (24). Wild-type littermates served as control mice. The average body weight of SRC-3<sup>−/−</sup> mice was ∼7.8% less than that of wild-type mice. Female mice (6–8 wk of age) were used in all experiments. Animal experiments were approved by the Laboratory Animal Center of Xiamen University, Xiamen, Fujian, China.

**Cell culture and transfection**

The RAW 264.7 murine macrophages were grown in DMEM supplemented with 10% FBS. All plasmids were transfected by using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The SRC-3 small interfering RNA (siRNA) and control siRNA were purchased from Dharmacon (Lafayette, CO) and transfected into cells using TransIT-TKO transfection reagent (Mirus, Madison, WI).

**Bacterial strain and infection of mice**

*E. coli* (ATCC25922, American Type Culture Collection, Manassas, VA) was inoculated into tryptic soy broth (Oxoid, Basingstoke, Hampshire, U.K.) and incubated in a shaker incubator for 10 h at 37°C. Bacteria were washed twice with PBS prior to infection of mice. Mice were i.p. injected with 1 × 10<sup>3</sup> CFU *E. coli*. An initial estimate of *E. coli* numbers was performed by densitometry. Serial dilution was performed, and inoculum size was established by enumeration of CFU on Luria-Bertani (LB) plates after overnight incubation at 37°C.

**Chemical analyses and measurement of cytokines**

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase were determined by commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China) according to the manufacturer’s instructions. The concentrations of TNF-α, IL-1β, IL-6, MCP-1, MIP-2, and keratinocyte chemokine in either plasma or peritoneal fluid were determined using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Isolation of peritoneal macrophages**

Isolation of peritoneal macrophages from wild-type and SRC-3<sup>−/−</sup> mice were performed as described previously (22). Three days presolation, wild-type and SRC-3<sup>−/−</sup> mice were injected i.p. with 2 ml 4% thioacetamide (Sigma-Aldrich, St. Louis, MO) to elicit macrophages. Peritoneal macrophages were collected by peritoneal lavage with 10 ml cold PBS. Cells were incubated in DMEM supplemented with 10% FBS at 37°C for 2 h and then washed with PBS to eliminate nonadherent cells. Adherent cells were used as peritoneal macrophages.

**Phagocytosis of peritoneal macrophages**

*E. coli* strain ATCC25922 (American Type Culture Collection) and *Staphylococcus aureus* strain CMCC(B)26003 were labeled with FITC (Amresco, Cleveland, OH) according to Saresella et al. (25) (hereafter designated as *E. coli*-FITC or *S. aureus*-FITC). For phagocytosis, the bacteria were opsonized with sera obtained from syngeneic mice at 37°C for 30 min. Peritoneal macrophages were seeded in six-well plates (1 × 10<sup>5</sup> cells/well) for 2 h and subsequently cocultured with *E. coli*-FITC or *S. aureus*-FITC at 37°C for 1 h. For phagocytosis of fluorescens, fluorescens suspension (F8827, Molecular Probes, Eugene, OR) was added. At the 30-min time point, each well was washed in PBS to remove extracellular bacteria, and 1 ml 0.05% Triton X-100 per well was added in the first three wells to lyse cells, and then the cell lysates were collected and designated as t = 0. Other wells were incubated with RPMI 1640 supplemented with 10% FBS and 5 μg/ml gentamycin for 2 h and then washed with PBS and lysed with 0.05% Triton X-100 (26). Serial dilutions of the cell lysates were plated on the LB plates, and bacterial counts were enumerated after 24 h. Bactericidal activity was expressed as the percentage of killed bacteria in relation to t = 0 (percent killing = 1 − CFU of *E. coli* at 2 h/CFU of *E. coli* at time 0 × 100%).

**Acute-phase serum assay**

Acute-phase serum assay was performed as described (27). Briefly, mice were injected i.p. with heat-killed *E. coli*, and after 16 h, the mice were sacrificed and bled to collect sera. The sera were regarded as acute-phase serum. For in vitro growth measurements of *E. coli*, 5 × 10<sup>4</sup> CFU *E. coli* were grown in RPMI 1640 supplemented with 20% acute-phase serum or normal mouse serum at 37°C, and the number of viable bacteria in the supernatant at the indicated time was determined by plating serial dilutions onto LB plates.

**Apoptosis assay**

Peritoneal macrophages were plated on a six-well plate at 2 × 10<sup>6</sup> cells with RPMI 1640 supplemented with 10% FBS for 2 h as described (28), and medium was then removed and replaced with DMEM plus 10% FBS containing 100 μg/ml gentamycin. After 6 h, macrophage apoptosis was quantified by flow cytometry using sub–G0-G1 staining nuclei or Annexin V staining. For analysis of sub–G0-G1 phase cells, cells were collected, fixed overnight in 70% ethanol, and stained by incubation in PBS containing 50 μg/ml propidium iodide for 30 min. For Annexin V staining, cells were stained by the Annexin V-FLUOS Staining Kit (Roche) according to the manufacturer’s instructions.

**Determination of reactive oxygen species**

Intracellular H<sub>2</sub>O<sub>2</sub> was measured by 2′, 7′-dichlorofluorescein (DCF) fluorescence (29). In brief, peritoneal macrophages were incubated with 10 μM DCF diacetate (Sigma-Aldrich), which is taken up and oxidized to the fluorescent DCF by intracellular H<sub>2</sub>O<sub>2</sub> at 37°C for 30 min. After that, the cells were immediately measured by flow cytometry.

**Catalase activity assay**

The activity of catalase was measured according to Sinha et al. (30). Briefly, dichromate/acetic acid was prepared by mixing a 5% solution of potassium dichromate with acetic acid (1:3 by volume). The assay mixture containing 2 ml H<sub>2</sub>O<sub>2</sub> (400 μmol) and 2.5 ml PBS was rapidly mixed with 0.5 ml cell sample, and the reaction was run for 2 min, then a 1-ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid for measuring the hydrogen peroxide contents. The catalase activity was calculated according to the content of H<sub>2</sub>O<sub>2</sub> remained.

**Quantitative real-time PCR**

Total RNA was prepared from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram total RNA was converted to cDNA by SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR reactions were performed using SYBR Premix ExTaq (TaKaRa, Dalian, China). Relative quantification was achieved by normalization to endogenous TBP. The primers used for real-time PCR are listed in Supplemental Table I.

**Luciferase reporter assay**

The −494 to +68 fragment of the catalase promoter was inserted into the pGL3-basic vector to generate the catalase reporter plasmid according to the method reported previously (31). The pCRII-Blu vector that constitutively expresses Renilla luciferase was used to normalize transfection efficiency. Luciferase activity was assayed using a dual luciferase reporter assay system (Promega, Madison, WI).

**Chromatin immunoprecipitation assay**

RAW264.7 cells were used for chromatin immunoprecipitation (CHIP) assay and processed according to the method described by Abcam (Cambridge, MA). The following primers were used: catalase promoter sense strand, 5′-ATTGACCTGACGCTTGGAC-3′; and antisense strand, 5′-GACTT-CAGGCTCCACCAATC-3′. Anti-SRC-3 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Determination of scavenger receptor A expression by flow cytometry**

Scavenger receptor A (SR-A) protein on macrophages was stained with SR-A Ab (Santa Cruz Biotechnology). Then, secondary FITC-labeled Ab F(ab)′<sub>2</sub> goat anti-rat IgG (Pierce, Rockford, IL) was added, and the expression was analyzed by flow cytometry.
Statistical analysis

The Kaplan-Meier and log-rank methods were used to analyze mortality. Data were collected from several independent experiments, with triplicates per experiment. All data were expressed as mean ± SD. Statistically significant effects were examined using two-tailed Student t test in SPSS 11.0 (SPSS, Chicago, IL).

Results

**SRC-3−/− mice are ultrasausceptible to E. coli-induced peritonitis**

To determine whether SRC-3 plays roles in antibacterial defense in *E. coli*-induced septic peritonitis, we first examined the survival results of *SRC-3−/−* and wild-type mice in *E. coli*-induced septic peritonitis. Postinfection with 1 × 10^7 CFU *E. coli*, *SRC-3−/−* mice displayed more severe symptoms of sepsis such as a crouched position, shivering, and ruffled fur as compared with wild-type controls. *SRC-3−/−* mice showed a significantly accelerated lethality compared with wild-type mice. Within 24 h, 42% (5 out of 12) of *SRC-3−/−* mice died, whereas no (0 out of 12) wild-type mice died at this time point. All *SRC-3−/−* mice (12 out of 12) died within 48 h postinfection, but 58% of the wild-type controls (7 out of 12) still survived and recovered by the end of experiment (Fig. 1A). Therefore, *SRC-3−/−* mice are hypersensitive to *E. coli* infection compared with wild-type mice.

Multiple organ damage occurs frequently during sepsis (32). Therefore, we examined whether the liver and kidney were damaged upon *E. coli* infection. The levels of AST and ALT (indicators of liver damage) as well as BUN (an indicator of kidney damage) in plasma of *SRC-3−/−* mice were significantly higher than that of wild-type mice postinfection (Fig. 1B–D). Histopathological examination further showed that the liver and kidney of *SRC-3−/−* mice had more necrotic areas (Supplemental Fig. 1). Thus, consistent with more lethality in *SRC-3−/−* mice, the degree of multiple organ damage in *SRC-3−/−* mice is much more severe than that in wild-type mice after *E. coli* infection.

**SRC-3−/− mice produce more proinflammatory cytokines than wild-type mice during *E. coli*-induced peritonitis**

Because uncontrolled inflammatory response is considered to play a major role in the pathogenesis of sepsis (3, 33), and *SRC-3−/−* mice are markedly susceptible to LPS-induced endotoxic shock due to increased production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (22), we hypothesized that the increased mortality and multiple organ damage observed in *SRC-3−/−* mice during *E. coli*-induced peritonitis are caused by an overwhelming inflammatory response. We therefore measured the levels of proinflammatory cytokines after *SRC-3−/−* and wild-type mice were infected by *E. coli*. The concentrations of TNF-α and IL-6 in plasma of both types of mice were low at 6 h postinfection, and they remained low in wild-type mice at 20 h postinfection, but the concentrations of TNF-α and IL-6 in plasma of *SRC-3−/−* mice at 20 h postinfection were increased 12- and 30-fold, respectively (Fig. 2A, 2C). The concentrations of IL-1β in plasma of both types of mice were increased to a similar degree at 6 h. However, sustained levels of IL-1β were observed in *SRC-3−/−* mice in contrast to the substantial decrease of IL-1β in wild-type mice at 20 h (Fig. 2B). The concentrations of TNF-α, IL-1β, and IL-6 in peritoneal fluid of both types of mice were relatively low at 6 h postinfection, but their concentrations in *SRC-3−/−* mice were markedly increased at 20 h compared with wild-type mice (Fig. 2D–F). The higher levels of proinflammatory cytokines observed in *SRC-3−/−* mice at 20 h indicate that *SRC-3* deficiency is responsible for the much more robust local and systemic inflammatory responses.

**SRC-3−/− mice exhibit impaired bacterial clearance during *E. coli*-induced peritonitis**

It is suggested that the increased local inflammation may be beneficial to bacterial clearance during *E. coli*-induced peritonitis (3). To determine whether *SRC-3* deficiency, which augments inflammation, can enhance bacterial clearance, we assessed the number of *E. coli* in peritoneal fluid, blood, and tissue homogenates of *SRC-3−/−* and wild-type mice at 6 h and 20 h postinfection. Bacterial loads had no differences in both groups of mice at 6 h postinfection, and unexpectedly, *SRC-3−/−* mice had significantly more bacteria in peritoneal fluid compared with wild-type mice at 20 h postinfection (Fig. 3A). Similarly, blood, liver, spleen, and lung of *SRC-3−/−* mice had more bacterial loads than wild-type mice at 20 h postinfection (Fig. 3B–E). These data demonstrate that *SRC-3* deficiency impairs bacterial clearance during *E. coli*-induced peritonitis.

Hosts possess many kinds of defensive systems against bacterial infection. Infection-induced acute-phase serum represents an iron-restricted environment for bacteria and contains many substances related to antibacterial activity, such as antimicrobial peptides, complements, and cytokines (27). Therefore, it plays an important role in host defense against bacterial infection. Bacteriostatic effects of serum were determined by measuring the growth of *E. coli* in sera from *SRC-3−/−* and wild-type mice. Both types of acute-phase sera effectively inhibited bacterial growth as compared with normal sera, but no differences were observed, indicating that the differences in bacteria outgrowth and dissem- inations between infected SRC-3−/− and wild-type mice are not due to any differences in bacteriostatic effects of acute-phase serum (Fig. 3F). Consistent with this, there were no significant differences in the expression of lipocalin-2, an iron-sequestering bacteriostatic peptide expressed in the liver and spleen but secreted into the blood circulation (27), between SRC-3−/− and wild-type mice.
wild-type mice in the absence and presence of E. coli infection (Fig. 3G, 3H).

Phagocytosis of E. coli by SRC-3−/− peritoneal macrophages and neutrophils is decreased

Because no significant differences were detected in the peritoneal leukocyte counts between SRC-3−/− and wild-type mice during E. coli-induced peritonitis (Table I), we speculated other defects than leukocyte recruitments may be responsible for the impaired bacterial clearance observed in SRC-3−/− mice. Phagocytosis of microorganisms by macrophages is a key component of the host defense against bacterial infections (34, 35). To determine whether there are any differences in phagocytosis between SRC-3−/− and wild-type macrophages, we examined the phagocytosis of E. coli

**FIGURE 2.** SRC-3−/− mice produce more proinflammatory cytokines than wild-type mice during E. coli-induced peritonitis. ELISA of TNF-α, IL-1β, and IL-6 in plasma (A–C) and peritoneal fluid (D–F) from SRC-3−/− and wild-type mice at 6 h and 20 h postinfection. Data are the means + SD of five mice per time point. **p < 0.01.

**FIGURE 3.** SRC-3−/− mice exhibit more severe bacterial outgrowth and dissemination during E. coli-induced peritonitis. Bacterial loads in peritoneal fluid (A), blood (B), liver (C), spleen (D), and lung (E) at 6 and 20 h postinfection with E. coli. Data are the means + SD of five mice per time point. F, SRC-3 deficiency had no effect on antibacterial activity of serum. Expression of antimicrobial protein lipocalin-2 (Lcn2) in the liver (G) or spleen (H) from both types of mice with or without infection of E. coli was analyzed by real-time PCR. Data are the means + SD of four mice per group. **p < 0.01.
and fluospheres by peritoneal macrophages from SRC-3<sup>−/−</sup> and wild-type mice. Phagocytosis of *E. coli* by SRC-3<sup>−/−</sup> macrophages was significantly decreased compared with that by wild-type macrophages (Fig. 4A, 4B), whereas phagocytosis of fluospheres by both types of macrophages was comparable (Supplemental Fig. 2). Furthermore, we found that phagocytosis of *E. coli* by SRC-3<sup>−/−</sup> peritoneal neutrophils was also significantly decreased (Fig. 4C, 4D). To determine whether phagocytosis of other types of bacteria by SRC-3<sup>−/−</sup> macrophages is impaired, we investigated the phagocytosis of Gram-positive bacteria *S. aureus* and found that phagocytosis of *S. aureus* by SRC-3<sup>−/−</sup> macrophages was significantly decreased (Supplemental Fig. 3). These results suggest that SRC-3<sup>−/−</sup> leukocytes have defects in phagocytosis of bacteria.

Macrophages express several surface receptors, such as SR-A and complement receptor C3 (CR3), to aid in the recognition of microorganisms (36). The expression of SR-A and CR3 mRNAs in

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<th>20 h</th>
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<td>10.3 ± 1.5</td>
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<tr>
<td>Macrophages</td>
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<td>1.5 ± 0.1</td>
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<tr>
<td>Neutrophils</td>
<td>7.3 ± 0.4</td>
<td>8.7 ± 1.6</td>
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Data are the means ± SD of five mice per time point at 6 and 20 h postinfection.

**FIGURE 4.** Phagocytosis of *E. coli* by peritoneal macrophages and neutrophils is decreased in SRC-3<sup>−/−</sup> mice. *A* and *B*, Phagocytosis of *E. coli* by peritoneal macrophages. Macrophages were incubated with *E. coli*-FITC at an moi of 100. FACS histogram is a representative of three independent experiments. *C* and *D*, Phagocytosis of *E. coli* by peritoneal neutrophils. FACS histogram is representative of three independent experiments. Relative mean fluorescence intensity (MFI) was expressed in fold change relative to wild-type (+/+ values assigned a unit of 1. Data are the means ± SD of four mice per group. The expression of SR-A (*E*) and CR3 (*F*) in macrophages was determined by real-time PCR. Data are the means ± SD of four mice per group. *G*, The expression of SR-A protein on the surface of macrophages was analyzed by flow cytometry. Data shown are representative of three independent experiments. *H*, Bactericidal activity of macrophages from SRC-3<sup>−/−</sup> and wild-type mice. Data are the means ± SD of four mice per group. *p* < 0.05; **p** < 0.01.
macrophages was analyzed by real-time PCR. SR-A expression was significantly lower in SRC-3−/− macrophages than in wild-type macrophages, but CR3 expression had no difference (Fig. 4E, 4F). In agreement with mRNA expression data, the expression of SR-A protein on the surface of SRC-3−/− macrophages was lower than that of wild-type macrophages (Fig. 4G). These results suggest that the SRC-3 deficiency-caused decrease in SR-A expression may be partially responsible for the impaired phagocytosis of bacteria observed in SRC-3 null macrophages. Once engulfing bacteria, SRC-3−/− and wild-type macrophages displayed a similar bactericidal activity (Fig. 4H), suggesting that SRC-3−/− macrophages have no defect in bacterial killing.

SRC-3 deficiency increases apoptosis of peritoneal macrophages

Bacterial pathogens are ingested and destroyed by macrophages, which is a mechanism used by host to combat bacterial infections. Although most bacteria are killed by macrophages, some intracellular bacteria, such as Shigella and Listeria, have been shown to directly trigger apoptosis of macrophages to evade the host immune response (37, 38). Recent studies have demonstrated that apoptosis in macrophages is also induced postphagocytosis of E. coli (39–41). Therefore, the extents of apoptosis in peritoneal macrophages from SRC-3−/− and wild-type mice were examined.
The rates of apoptosis in SRC-3 ger, involved in apoptosis, we treated cells with an antioxidant scavenger to investigate whether the intracellular production of ROS was increased macrophages. As Annexin V staining. As shown in Fig. 5A, the sub-G1 population of SRC-3−/− macrophages was significantly larger than that of wild-type macrophages in both the absence and presence of E. coli. The results of Annexin V staining confirmed that SRC-3−/− macrophages had higher apoptotic rates (Fig. 5B, Supplemental Fig. 4). Furthermore, we investigated the percentage of apoptotic cells in peritoneal fluid from mice postinfection with E. coli. The results in Fig. 5C showed that peritoneal cells from E. coli-infected SRC-3−/− mice had higher apoptotic rates compared with that from E. coli-infected wild-type mice.

Several antioxidant enzymes, including catalase, superoxide dismutase, and glutathione peroxidase, are involved in protecting cells against ROS. The levels of catalase, superoxide dismutase 1, and glutathione peroxidase mRNAs in both genotypic macrophages were measured, but only catalase expression in SRC-3−/− macrophages was significantly lower than that of wild-type cells (Fig. 5F, Supplemental Fig. 5). Consistent with reduced catalase expression, the enzymatic activity of catalase was also significantly reduced in SRC-3−/− macrophages compared with wild-type macrophages (Supplemental Fig. 6). To examine whether the intracellular production of ROS was involved in apoptosis, we treated cells with an antioxidant scavenger, N-acetylcysteine (NAC), to abrogate the intracellular ROS. The rates of apoptosis in SRC-3−/− and wild-type macrophages were significantly decreased to similar levels after NAC treatment (Fig. 5E), indicating that ROS is indeed responsible for the increased macrophage apoptosis.

Catalase is an antioxidant enzyme for which expression is transcriptionally regulated by some factors such as SP1, NF-Y, and WT1/Egr-related factor (31, 46). Because the expression of catalase is reduced in SRC-3−/− macrophages, we hypothesize that catalase expression is regulated by SRC-3. To determine whether SRC-3 regulates de novo transcription of the catalase gene, we examined the effects of SRC-3 upregulation and downregulation on the transcriptional activity of the catalase promoter in a transient transfection and luciferase reporter assay. Catalase promoter activity was increased in RAW264.7 cells cotransfected with SRC-3 expression vector and decreased in RAW264.7 cells cotransfected with SRC-3 specific siRNA. After transfection of control siRNA, Western blot analysis revealed a downregulated of SRC-3 protein by SRC-3 siRNA but not control siRNA. Data are presented as the means ± SD (n = 3).

Several lines of evidence demonstrate that ROS can suppress the expression of antiapoptotic gene Bcl-2 (44, 45). Consistently, we found that the catalase-specific inhibitor 3-AT, which was shown to be able to increase ROS accumulation (Fig. 5H), effectively suppressed the expression of Bcl-2 in macrophages (Fig. 5K). Because ROS accumulation was increased in SRC-3−/− macrophages, we examined whether the expression of Bcl-2 is decreased in these cells. As anticipated, deficiency of SRC-3 led to a significant decrease of Bcl-2 expression (Fig. 5L).

SRC-3 regulates the expression of catalase in macrophages

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with SRC-3–specific siRNA (Fig. 6A, 6B), suggesting that SRC-3 is required for transcriptional activation of catalase. Furthermore, the results of the ChIP assay demonstrated that SRC-3 was recruited to the catalase promoter (Fig. 6C). These data suggest that during E. coli–induced peritonitis, SRC-3 prevents macrophage apoptosis and enhances bacterial clearance by directly upregulating the expression of catalase in macrophages (Fig. 7).

Discussion

We have previously shown that SRC-3−/− mice are highly susceptible to LPS-induced lethality, and SRC-3 is a negative regulator of inflammatory response (22). In the current study, we demonstrate that SRC-3−/− mice are markedly susceptible to the lethality caused by E. coli infection–induced peritonitis. Therefore, the enhanced inflammation as indicated by increased production of proinflammatory cytokines should be at least in part responsible for these phenomena observed in SRC-3−/− mice. An uncontrolled systemic inflammation is harmful to the host, but inflammation is beneficial to the effective clearance of bacteria (3). For example, lack of IL-10, an anti-inflammatory cytokine, increases systemic inflammatory response and causes multiple organ failures and death, whereas it enhances bacterial clearance in mice during E. coli peritonitis (47). We therefore initially hypothesize that enhanced inflammatory responses in SRC-3−/− mice are better for clearing bacteria during E. coli peritonitis. Surprisingly, SRC-3−/− mice display more bacterial loads and systemic dissemination despite the increased inflammatory responses. It indicates that SRC-3−/− mice have defects in controlling both bacterial outgrowth and inflammatory responses. Intensively increased bacterial loads, which provide a more potent inflammatory stimulus, may be able to further enhance inflammation and increase mortality rates of SRC-3−/− mice. In contrast, the exaggerated bacterial loads alone are able to cause the increased mortality. Therefore, the impaired capacity of SRC-3−/− mice to clear a bacterial infection and the overproduction of host proinflammatory cytokines are both responsible for the increased mortality observed.

Macrophages are required for the innate immune response to bacterial infection. Our results demonstrate that SRC-3 deficiency impaired phagocytosis of E. coli by macrophage through reducing the expression of SR-A, an important receptor for FcγR-independent phagocytosis (48). The apoptotic response of macrophages plays a vital role in the pathogenesis of sepsis (40, 49). For example, liver X receptors-null macrophages undergo accelerated apoptosis upon infection and exhibit defective bacterial clearance in vivo (38). Therefore, macrophage survival intimately concerns the host defense against infectious pathogens. Our data demonstrate that macrophages from SRC-3−/− mice are more sensitive to E. coli–induced apoptosis compared with the wild-type macrophages. In addition, increased apoptosis of SRC-3−/− macrophages may explain the slightly decreased peritoneal macrophage count in SRC-3−/− mice (Table I), despite that the levels of MCP-1, MIP-2, and keratinocyte chemoattractant, three major mediators of leukocyte recruitment (50), are significantly increased in the peritoneal cavity at 20 h postinfection (Supplemental Fig. 7).

ROS and the resulting oxidative stress play a pivotal role in apoptosis (42, 51), and a state of severe oxidative stress is encountered in sepsis, with host endogenous antioxidant defenses overcome (52). Alterations in the redox status of host by various antioxidants, such as NAC, block apoptosis in cells (42) and blunt the inflammatory response to sepsis (53). Our data are consistent with this notion that SRC-3−/− macrophages exhibit higher levels of ROS, and the apoptosis induced by E. coli can be repressed by NAC. Catalase, an antioxidant enzyme, has a direct antioxidant activity through catalyzing the conversion of hydrogen peroxide to water. We find that SRC-3 is involved in the regulation of catalase transcription. Therefore, the decreased expression of catalase underlies a higher level of ROS and the more apoptotic rate observed in SRC-3−/− macrophages. Overexpression of catalase increases the Bcl-2 expression (44), which prevents cells from apoptosis by an antioxidative mechanism (42). In this study, a decrease in catalase expression and a concomitant decrease in Bcl-2 expression as well as an increase in apoptosis are observed in SRC-3−/− macrophages. Thus, downregulation of the Bcl-2 expression further sensitizes SRC-3−/− macrophages to E. coli–induced apoptosis.

We have previously shown that in response to LPS stimulation, SRC-3 interacts with translational repressors, T cell intracellular Ag-1, and T cell intracellular Ag-1–related protein to suppress the protein synthesis of proinflammatory cytokines in the cytoplasm, which limits excessive inflammatory response, reduces systemic organ damage, and improves survival (22). In this study, we further demonstrate that during E. coli–induced peritonitis, macrophage SRC-3 not only suppresses proinflammatory cytokine synthesis to limit an inflammatory response, but also enhances the bacterial clearance by macrophages through acting as a transcriptional coactivator in the nucleus to upregulate the expression of SR-A and catalase, leading to an enhanced phagocytosis of bacteria and a reduced macrophage apoptosis. Therefore, the highly coordinated cytoplasmic and nuclear functions of SRC-3 effectively prevent animals from sepsis-induced lethality (Fig. 7). Taken together, these findings clearly demonstrate that SRC-3 is a master regulator of sepsis and plays an essential role in protecting the host against bacterial infection.

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Disclosures

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References


