Protective Role of Peroxiredoxin I in Heat-Killed *Staphylococcus Aureus*-infected Mice

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Abstract. *Background/Aim: Staphylococcus aureus (S. aureus) is a major gram-positive pathogen, which can cause toxic and immunogenic injuries both in nosocomial and community-acquired infections. Peroxiredoxin (Prx) I plays crucial roles in cellular apoptosis, proliferation, and signal transduction as well as in immunoregulation. The present study aimed to investigate whether Prx I protects mice from death caused by the heat-killed Staphylococcus aureus. Materials and Methods: In the present study, we challenged the wild-type and Prx I-deficient mice with heat-killed S. aureus (HKSA). The effects of Prx I were evaluated by a series of in vitro and in vivo experiments including western blot, Haematoxylin and Eosin staining, splenocyte analysis and cytokines analysis. Results: Intra-peritoneal (ip) inoculation of HKSA resulted in increased mortality of Prx I-knockout (KO) mice with severe liver damage and highly populated spleens with lymphocytes. Furthermore, HKSA infections also bursted the production of both pro-inflammatory and anti-inflammatory serum cytokines in Prx I KO compared to wild-type mice. Conclusion: Enhanced mortality of S. aureus-infected mice with Prx I deficiency suggested that Prx I may protect against the infection-associated lethality of mice.*

Peroxiredoxin (Prx) I, an antioxidant enzyme, belongs to 2-cysteine Prxs (1). Recent evidence shows that Prx I participates in several cellular signaling pathways by interacting with diver proteins to regulate cell differentiation, apoptosis and proliferation (2-4). Accumulation of reactive oxygen species (ROS) in cells can cause oxidation of DNA, membrane lipids and proteins, resulting in increased cellular damage (5). Prx I plays an important role in scavenging ROS in cells (6). In the last decade, the regulatory role of Prx I in tumorigenesis has been well defined both *in vivo* and *in vitro*. Depletion of Prx I can spontaneously induce the development of several malignant cancers and severe haemolytic anaemia, as well as oxidative DNA damage and decreased cell proliferation (7). Furthermore, a previous study by our group has also shown that Prx I suppresses the K-ras-induced lung carcinogenesis *via* the ROS/ERK/cyclin D1 signaling pathway (8), suggesting that Prx I may be a tumor suppressor that could prevent carcinogenesis.

Apart from its anti-cancer properties, the immune regulatory roles of Prx I was recently considered.by Riddell *et al.*, who reported that recombinant Prx I stimulates the secretion of pro-inflammatory cytokines, such as TNF-α and IL-6 in macrophages, by binding to toll like receptor 4 (TLR-4) (9). Furthermore, knockdown of Prx I modulates the balance of IL-10, IL-1β and TNF-α secretion in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells (10). In a previous study we have also shown that mouse deficiency of Prx I attenuates the phagocytic activity of macrophages in clearing damaged red blood cells (11). These findings suggest that Prx I possibly plays key roles as an immunoregulator both *in vivo* and *in vitro*.

Staphylococcus aureus (*S. aureus*) is a major gram-positive pathogen present in both nosocomial and community-acquired infections (12). There is a great number of secreted proteins and cell-wall associated factors in *S. aureus* that can cause toxic and immunogenic responses (13, 14). Furthermore, it has also been reported that heat-killed *S. aureus* (HKSA) infection can cause septic shock in mice, through the activation of the TLR2- signalling pathway (12, 15). Thus, HKSA is often used as a sepsis mouse model in experimental studies.

In the present study, we investigated the role of Prx I in initiating an immune response and cytokine production, as well as in the viability of *S. aureus* infected mice. Intra-peritoneal (ip) inoculation of HKSA resulted in increased death of Prx I knockout (KO) mice with severe liver damage, immune reactions, and increased production of cytokines and apoptosis in the liver. This study aimed to investigate the protective role of PRX I against *S. aureus*-induced lethality in mice .

Materials and Methods

Mice and genotypic analysis. The Prx I KO mice used in the experiments were generated by heterozygous mice, and the genotype was confirmed by PCR and western blotting (16) (Figure 1A). C57BL/6J (n=12) and Prx I KO mice (n=12) pathogen-free mice used for the heat-killed *Staphylococcus aureus* (HKSA) challenge were at 8-10 weeks of age. Their survival was carefully observed for 4 days. All animals were housed in microisolator cages with laminar air flow under ambient light. *S. aureus* strain Wood 46 (ATCC 10832) was kindly provided by the laboratory of Professor Yu-Dong Cui (College of Life Science & Technology, Heilongjiang Bayi Agricultural University). HKSA was diluted in sterile PBS [approximately 2.6×10^{11} colony-formation units (CFU)/ml] and was injected intra-peritoneally (ip) to the animals. The primers used for the mouse genotype analysis are as follows: i) for the Neo gene: forward primer 5'-gcttgggtggagaggctattcg-3'; reverse primer 5'-gtaaagcacgaggaagcggtcagcc-3' and ii) for the Prx I gene: forward primer 5'-ctggaaacctggcagtgata-3'; reverse primer 5'-ctgtgactgata gaagattggt-3'. The Institutional Animal Care and Use Committee approved the both animal care and experiments.

Cytokines analysis. Serum and tissue (liver and lungs) were collected from HKSA treated wild type and Prx I KO mice at the following times: i) 0 hours, ii) 36 hours, iii) 48 hours and iv) 60 h, Tissues were minced and lysed in a protein lysis buffer (150 mM NaCl, 1% Nonidet p-40, 50 mM Tris, pH 8.0) containing protein inhibitor (cOmplete™, Mini, EDTA-free, Merck, NJ, USA). The lysis mixtures were then centrifuged and the supernatants were extracted. The levels of cytokines (IL-6 and IL-10) were detected using ELISA (R&D Systems, Minneapolis, USA), according to manufacturer's instructions. The absorbance at 540 nm was measured using a UV MAX kinetic microplate reader (Molecular Devices, Menlo Park, CA).

Haematoxylin and eosin staining. To understand the pathological changes following HKSA infection, the livers and lungs were collected from wild type and Prx I KO mice at the indicated times following HKSA injections. Tissues were harvested after removing the blood by heart perfusion and fixation with perfused heparinized saline containing 3.7% formaldehyde through the left ventricle. The tissue sections (4 μM in thickness) were stained with haematoxylin and eosin (H&E) and were observed under a microscope (Olympus IX70 Fluorescence Microscope, Olympus, Japan). The experiments were performed with 10 wild type and 10 Prx I KO mice.

Splenocyte isolation and analysis. To assess the effect of HKSA infection on mice on lymphocyte distribution, spleens from wild type and Prx I KO mice were collected following HKSA infection at indicated times. The splenocytes were obtained using the spleen lymphocyte cell separation medium kit (Solarbio Life Sciences, P.R. China), according to the manufacturer's instructions. Subsequently, cells were stained with anti-CD4-FITC, anti-CD8-PE, anti-CD25-PE, anti-CD11b-PE and anti-NK1.1-PE antibodies (Santa Cruz, USA) for 30 minutes at 4˚C in cold FACs buffer (1X PBS and 1% FBS). The cytotoxic T cells (CD8 positive), regulatory T cells (CD4/CD25 positive), monocytes (CD11b positive) and NK (NK1.1 positive) were analysed using flow cytometry (BD FACS Calibur, CA, USA) and the numbers of the cells were presented on a histogram.

Western blot analysis. Tissue protein lysates obtained from wild type and Prx I KO mice following HKSA injection were separated in 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and were transferred onto nitrocellulose (NC) membranes (Millipore, Bedford, MA, USA). The membranes were blotted with primary antibodies against Prx I, cleaved caspase-3, Bcl-2 (Santa Cruz, USA), and β-actin (Sigma-Aldrich, St. Louis, USA) at 4˚C overnight. Following washing of the membranes with tris-buffered saline (TBS) [10 mM Tris-HCl (pH 7.5), 150 mM NaCl] and 0.2% Tween20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich) or anti-mouse IgG (Merck) for 1 hour at room temperature (RT). The specific binding was detected using a chemiluminescence detection system (Amersham, Berkshire, UK), according to the manufacturer's instructions.

Statistical analysis. The data are depicted as a mean±SEM. Student's *t*-tests were performed using the GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA), and $p < 0.05$ was considered as indicative of significant difference.
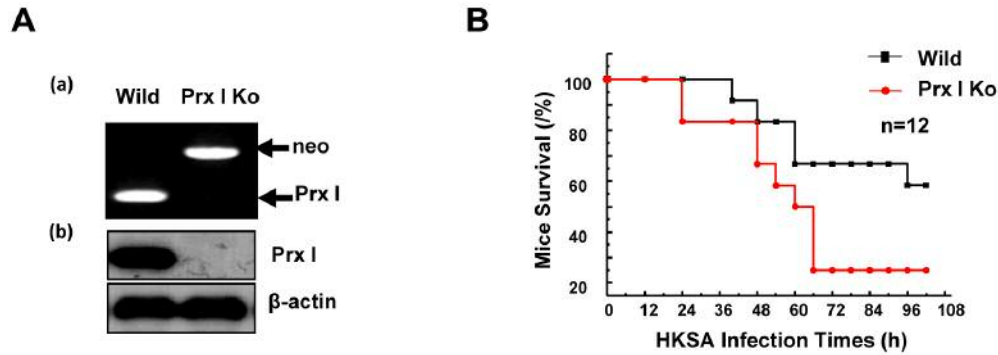
Figure 1. *Prx I deficiency increases the HKSA induced mortality in mice. (A) (Upper Panel) PCR analysis of genomic DNA isolated from wild-type (lane 1) and Prx I homozygous knockout mice (lane 2). The 700 bp (Neo) and 250 bp (Prx I) arrows indicate the PCR products of Prx I KO and wild-type mice, respectively. (Lower Panel) Western blot analysis of Prx I expression in the liver from wild and Prx I KO mice. (B) The wild (n=12) and Prx I KO (n=12) mice were ip injected with (approximately 2.85×10^{10} CFU/mouse) HKSA, and mortality was observed at the indicated times. No additional mice died 96 h following the injection. HSKA: Heat-killed Staphylococcus aureus; PRX I KO: peroxiredoxin I knockout; h: hours.*
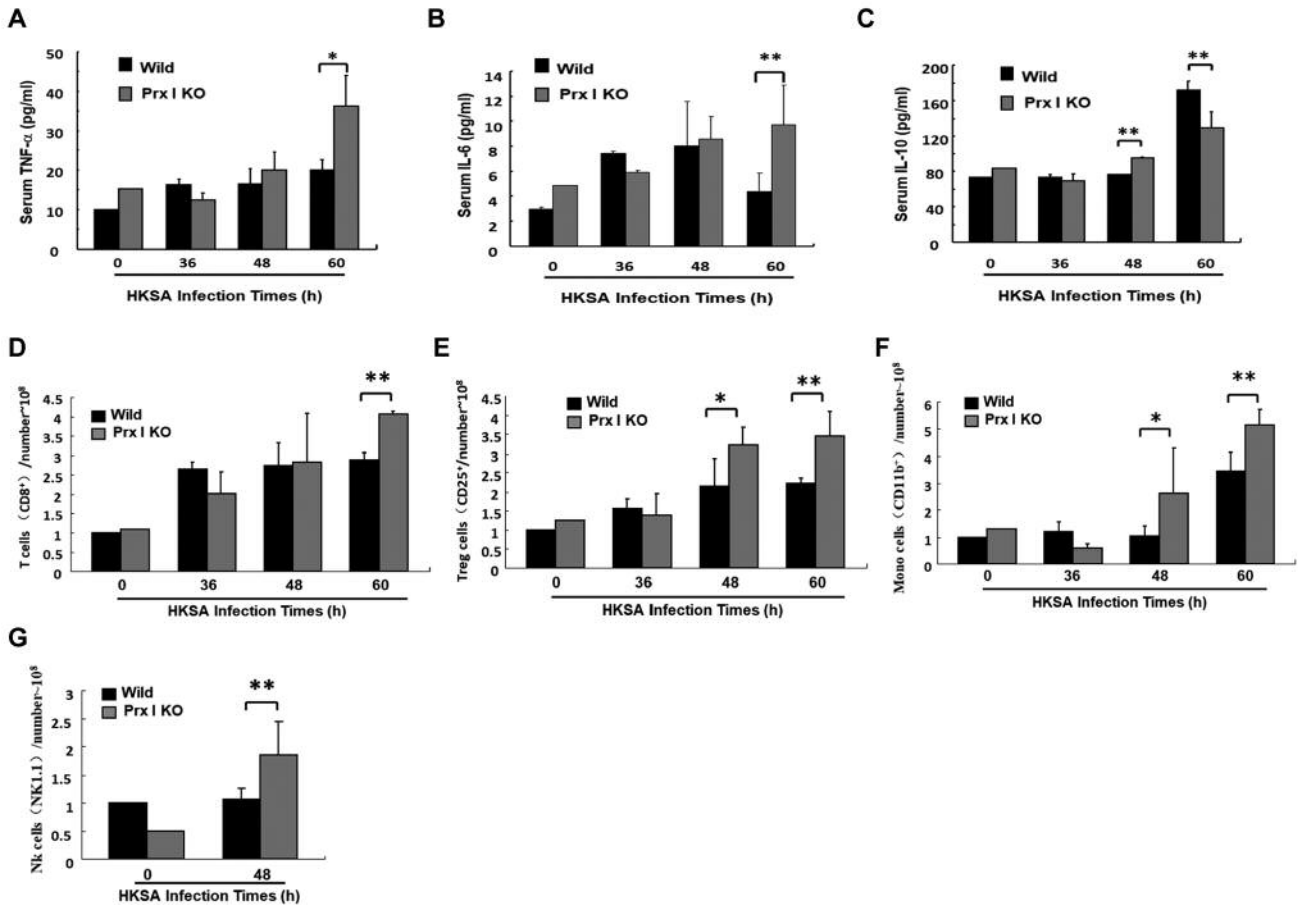

Figure 2. *Impaired production of cytokines and immune cells in HKSA-infected Prx I KO mice. (A) Wild type (n=10) and Prx I KO mice (n=10) were challenged with HKSA (approximately 2.85×10^{10} CFU/mouse) at indicated times. The serum TNF-α, (B) IL-6 and (C) IL-10 were detected using ELISA. (D) The wild-type and Prx I KO mice were challenged with HKSA (approximately 2.85×10^{10} CFU/mouse) at indicated times and the splenocytes were collected for analysis of immune cell distribution. The number of CD8-positive T cells, CD4/CD25 positive, (E) Treg, (F) CD11b positive monocytes and (G) NK1.1 positive NK cells were analyzed using flow cytometry. n=6, *p<0.05, **p<0.01. HSKA: Heat-killed Staphylococcus aureus; PRX I KO: peroxiredoxin I knockout; h: hours.*
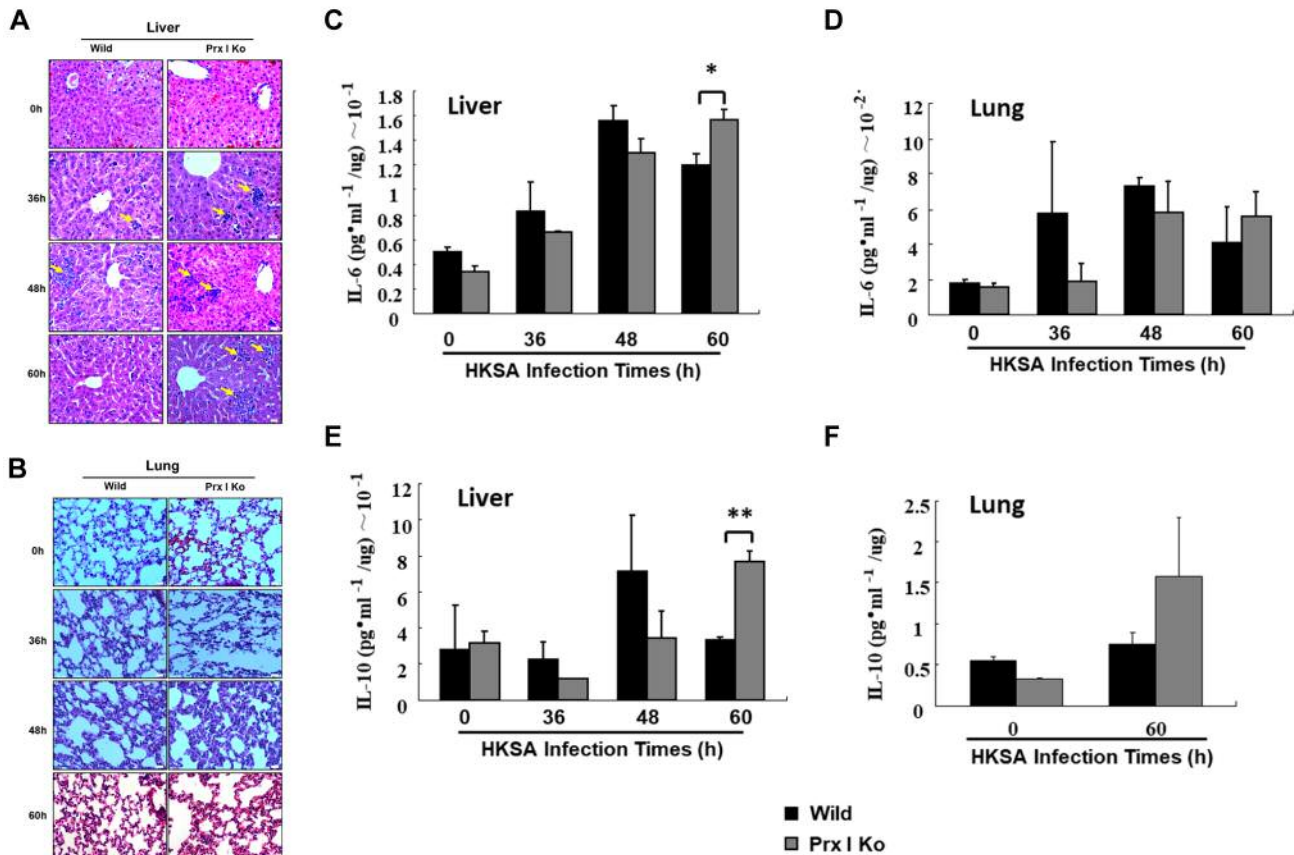
Figure 3. *Severe liver inflammation and increased tissue cytokines in Prx I KO mice in response to HKSA infection. (A, B) The H&E-stained sections from wild type and Prx I KO mice at the indicated times (0, 36, 48 and 60 h) following the HKSA infection, microscope images at 400× show signs of immune cell infiltration in the liver (A) (yellow arrow, immune cell infiltration) and (B) lungs. n=6 (C-F) The cytokine levels IL-6 and IL-10 were analysed in the liver and lung of wild type and Prx I KO mice at the indicated times following the HKSA infection. The produced levels of cytokines are represented as pg/mg of total protein, (n=6 for each experiment) *p<0.05, **p<0.01.*

Results

Prx I deficiency increased the HKSA-induced mortality in mice. To examine the possible protective role of Prx I against the *S. aureus* infection, wild type (n=12) and Prx I KO mice (n=12) were challenged with HKSA injections and their survival was observed for 4 days. The results show that deficiency of Prx I increased their susceptibility to succumb to HKSA infection compared with wild type mice (Figure 1B).

Impaired production of cytokines and immune cells in HKSA-infected Prx I KO mice. To examine the effect of *S. aureus* infection on cytokine responses, wild type (n=10) and Prx I KO mice (n=10) were challenged with HKSA injections as previously at indicated times. Interestingly, we observed that the production of the pro-inflammatory cytokines, such as IL-6 increased in Prx I KO mice while the anti-inflammatory

cytokine IL-10 compared to wild type mice infected with HKSA (Figure 2A-C).

The results from our splenocytes' analysis of immune cells' distribution showed that the CD8^{+} T cells, CD11b^{+} monocytes, NK1.1^{+} NK cells and CD4^{+}/CD25^{+} regulatory T cells were significantly increased in Prx I KO mice compared to wild type mice infected with HKSA (Figure 2D-G).

Severe liver inflammations in Prx I KO mice as a response to HKSA infection. To investigate the histopathological changes following *S. aureus* infection, the HKSA-challenged liver and lung were collected both from wild type and Prx I KO mice for analysis. Our results show that *S. aureus* increased the invasion of immune cells in liver and lung tissues in both infected wild type and Prx I KO mice. Although there was no difference between wild type and Prx I KO concerning the
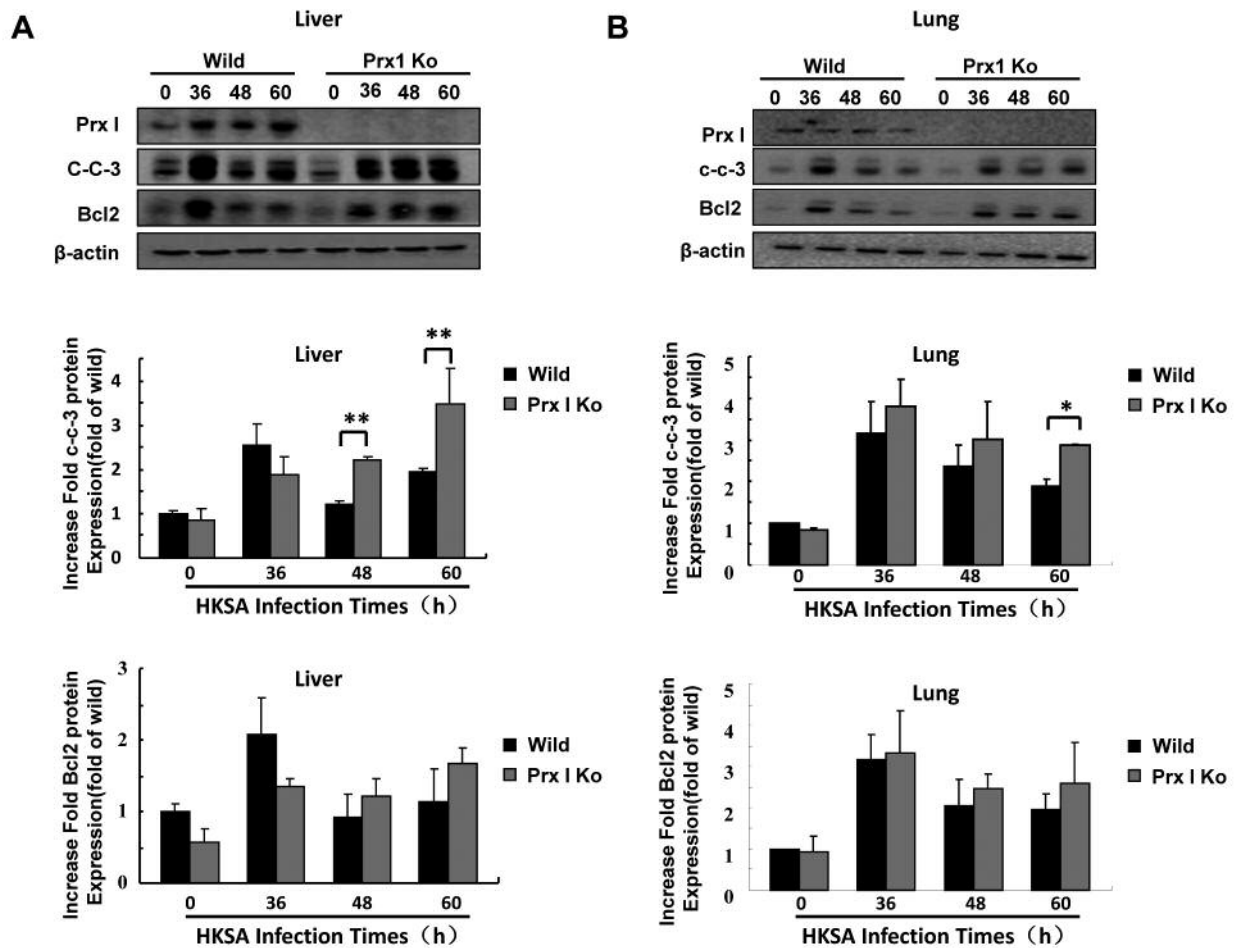
Figure 4. *Increased apoptosis in the liver of Prx I KO mice in response to HKSA infection. The protein expression levels of cleaved caspase-3 and Bcl-2 in both liver (A) and lungs (B) of wild type and Prx I KO mice following HKSA infection. The protein expression levels are presented as means±SD (C-F). (n=6 for each experiment) *p<0.05 and **p<0.01.*

lungs, a severe inflammatory response was observed in the liver of Prx I KO mice compared to the liver of wild type mice (Figure 3A and B). Furthermore, IL-6 and IL-10 production was also significantly higher in the liver of Prx I KO mice compared to wild type mice (Figure 3C-F), but not in the lungs, suggesting that the liver of Prx I KO mice were the ones most affected by the *S. aureus* infection.

Increased liver apoptosis in Prx I KO mice as a response to HKSA infection. To assess whether tissue inflammation and increased cytokine production led to increased hepatocyte or lung epithelial apoptosis we assessed the expression of certain apoptosis-related proteins in these tissues. Our results show that cleaved caspase 3 protein expression was upregulated both in the liver and lungs of Prx I KO mice compared to wild type mice, however, there was no

significant difference in Bcl2 protein expression levels between wild type and Prx I KO mice following HKSA infection (Figure 4A and B).

Discussion

In the present study, we analyzed the regulatory function of Prx I on both lethal shock reaction and host immune response to *S. aureus* infection in mice. Several studies have previously shown that *S. aureus* promotes a host immune response from minor skin infections to severe systemic inflammations, including septic lethal shock (17, 18). TLR2 is known as a key molecule to contribute to host immune response against *S. aureus* infections (19, 20), and deletion of TLR2 increases the mortality of mice during *S. aureus* infection (15, 21-23) combined with impaired cytokines

production and severe inflammatory burden. Similarly, in this study, Prx I KO mice showed an enhanced susceptibility to *S. aureus* infection within four days post-infection as well as impaired production of cytokines in plasma serum, suggesting a possible protective role of Prx I against *S. aureus* infection. Our previous study has shown that deficiency of Prx I results in increasing lipopolysaccharide (LPS) induced lethal shock in mice (24), which ismainly stimulated through TLR4 dependent signaling. Furthermore, stimulation of RAW264.7 mouse macrophages with LPS could significantly induce Prx I mRNA as well as protein expression *in vitro via* the Src/PI3k/JNK signaling pathway (25). Moreover, the regulatory function of Prx I on LPS-induced macrophages through TLR4 was also reported recently *in vitro* (9, 10). These findings demonstrate that Prx I plays an essential role in a TLR4-induced host immune response, but the regulatory function of Prx I in gram-positive bacterial infection is not yet understood.

Previous studies have shown that the TNF-α and IL-6 are considered early cytokines that are induced by *S. aureus* infection (26-28), both in immune and non-immune cells *in vivo*. In addition, the anti-inflammatory cytokine, IL-10, has been shown to be up-regulated by LR2 signaling initiated by *S. aureus* infection (29, 30). Similarly, in our experiments both pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory (IL-10) production were higher in the serum of Prx I KO mice compared to wild-type mice following *S. aureus* infection. At the same time, the executive immune cells, such as CD8 positive T cell, monocytes, NK cells and regulatory T cells were also increased in the spleen of Prx I KO compared to wild type mice. Our results revealed that Prx I deficiency could promote both an innate and an adaptive immune response to *S. aureus* infection, but the possible mechanisms should be further studied.

Liver is a large organ that offers immune surveillance against bacterial infections and can help to clear the entrance of bacteria into bloodstream (15, 31). Herein, our results showed an increased invasion of inflammatory cells and higher accumulation of IL-6 and IL-10 in the liver of Prx I KO mice compared to wild type, which led to increased apoptosis through the up-regulation of cleaved caspase 3. These results suggest that deletion of Prx I increase the susceptibility to *S. aureus* infection in mice and may influence the host liver defense functions against this particular bacterial infection. In conclusion, our findings demonstrate that Prx I deficiency enhances the mortality of mice caused by *S. aureus* infection, and increases the circulating cytokines production as well as the spleen lymphocytes distribution, which lead to severe inflammatory damage in these mice's liver. Additional future studies should provide more clinical data concerning the role of Prx I on *S. aureus* infection so as to provide strategies for its treatment.

Authors' Contributions

HNS, YL, TK, and YHH. designed and wrote the whole manuscript, HNS, YL, TK, and YHH performed the experiments, CW, RL, LZK, XZ, NC, YDC, SUK, DSL, DYY, JSK, and DKJ contributed in the revision of the manuscript. All authors read and approved the final manuscript.

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