

Original Article

The Role of Superoxide Dismutase in the Survival of *Mycobacterium tuberculosis* in Macrophages

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SUMMARY: There is a large amount of information available regarding the chemical structure and biological activity of superoxide dismutase (SOD), which is abundantly generated by *Mycobacterium tuberculosis* in the early stages of growth. SOD is a strong superoxide radical scavenger, which plays a significant role in resisting oxidative stress. On the other hand, SOD mutant strains have been constructed to define the role of this molecule in the immune response to *M. tuberculosis* infection. These studies have suggested that the absence or attenuation of SOD can motivate innate immunity and SOD avoid destruction or growth inhibition of *M. tuberculosis*. For detailed investigation of how SOD proteins aid *M. tuberculosis* survival within macrophages, we cloned 2 SOD genes (SODA and SODC) from the *M. tuberculosis* H37Rv genome, overexpressed, identified, and purified the proteins, and then exposed macrophages to the proteins. Following this, we assessed NO production, the secretion of cytokine interferon- γ (IFN- γ) and intercellular adhesion molecule-1, the expression of IFN- γ receptor and Toll-like receptor 2 on the surface of macrophages, and caspase-3 enzyme activity as well as macrophage apoptosis. Our results showed that both SODA and SODC proteins considerably reduced the production of NO and oxygen radicals and impaired cell immunologic function in early infection.

INTRODUCTION

The control of diseases caused by *Mycobacterium tuberculosis* remains a challenge to humans, as approximately one-third of the world's population is being affected by this pathogen. In addition to causing 1.7 million deaths annually, most *M. tuberculosis* infections exist as life-long latent infections and become sources of larvaceous infections (1).

Physiologically, superoxide dismutase (SOD) is a strong superoxide radical scavenger, which can convert the virulent superoxide radical O_2^- into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), eliminate the toxic effects of O_2^- , and prevent the formation of higher H_2O_2 concentrations via other reactions (2-4). SOD is one of the largest secretory proteins among the bacterial culture filtrate proteins in growing *M. tuberculosis* cultures (5-7), and clinical *M. tuberculosis* isolates from tuberculosis patients are rich in SOD (8). These data indicate that SOD secretion may play a valuable role in *M. tuberculosis* pathogenesis. Two types of SODs are found in *M. tuberculosis*: SODA and SODC. SODA is abundantly released in the culture medium (9,10) and present in comparable amounts in the cytoplasmic fraction and to a relatively lower extent in the cell wall fraction (7,10,11). SODC accounts for only a small proportion of the total SOD. Immunogold electron microscopy analysis has shown that SODC can

adhere to the outer bacterial membrane through lipoprotein binding, thereby forming a protective membrane for *M. tuberculosis* and resisting extracellular superoxide (12-14). Comparatively, SODA seems to be the most effective factor in resisting an "oxidative burst." SODs from various bacteria share similar functions but have different molecular structures, metal ions, and properties such as thermostability and enzyme activity (15-17).

Several studies have investigated the key role of SOD in the long-term survival of *M. tuberculosis* in macrophages and reported that large quantities of 2 toxic molecules, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), are produced after pathogens undergo ample digestive fermentation in endolysosomes of phagocytes, known as the respiratory burst, during which most microbial pathogens are damaged or killed (18-21). However, *M. tuberculosis* survives and replicates in macrophages and consequently causes persistent clinical infections.

In fact, the eradication of the "oxidative burst" alone is insufficient to protect bacteria from being destroyed within macrophages. Other similar functions of SOD have also been investigated. For example, it has been shown that SOD-attenuated *M. tuberculosis* reduces SOD production, and compared with controls, SOD-diminished isolates enhance rapid mononuclear cell infiltration into the lung in addition to apoptosis of infected cells (22). Other research has revealed that inactivation of the *secA2* gene in *M. tuberculosis*, which encodes a component of the virulence-associated protein secretion system (23), can enhance apoptosis of infected macrophages by diminishing secretion of mycobacterial SOD, while the deletion of *secA2* markedly increases priming of antigen-specific CD8⁺ T cells

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in vivo (24). These findings suggest that the absence or attenuation of SOD can enhance immune function; therefore, SOD may be a key immune evasion strategy for *M. tuberculosis* survival.

Although researches in this field have investigated the roles of SOD-null mutants, there is no direct evidence regarding the individual role of SOD. Hence, the present study is aimed to assess the contribution of SOD to the survival of *M. tuberculosis* in macrophages for providing further and direct evidence in this area. We used a different experimental strategy from that of previous research. First, we cloned SODA and SODC genes from the genome of the virulent *M. tuberculosis* strain H37Rv and constructed recombinant expression vectors, PET32a(+)-SODA and PET32a(+)-SODC. Following this, we overexpressed SODA and SODC proteins in *Escherichia coli* and purified the target SOD proteins. Finally, we treated murine peritoneal macrophages (ANA-1 cells) with different concentrations of SODA and SODC to assess NO and cytokine production, immunity receptor expression, macrophage apoptosis, and attendant caspase activity.

Our results showed that these 2 proteins contribute to the survival of *M. tuberculosis* in 3 aspects: (i) they rapidly scavenge ROIs and reduce toxic NO through the inhibition of iNOS activity in the early phase; (ii) they suppress immune responses, e.g., transiently reduce the secretion of the cytokines interferon- γ (IFN- γ) and intercellular adhesion molecule-1 (ICAM-1) and lastingly down-regulate the expression of IFN- γ receptor (IFN- γ R) and Toll-like receptor 2 (TLR2); and (iii) they effectively inhibit macrophage apoptosis by restraining caspase-3 activity.

MATERIALS AND METHODS

Gene isolation and constructions of recombinant expression plasmids: Using *M. tuberculosis* H37Rv genomic DNA as a template, a SODA gene fragment (624 bp) and a SODC gene-truncated fragment (625 bp) (25) whose signal peptide had been removed were PCR-amplified using primers P1 (5'-GCGAATTCGTGGCCGAATACACCTTGC-3') and P2 (5'-GCAAGCTTCAGCCGAATATCAACCC-3') and P'1 (5'-GCGAATTCATGGCCTCGTCGCCGACGACGC-3') and P'2 (5'-GCAAGCTTCTAGCCGGAACCAATGACA-3'). The PCR reaction system contained 1 μ l of template genomic DNA, 1 μ l of each primer, 4 μ l of dNTPs (2.5 mM each; TianGen, Beijing, China), 0.5 μ l of Pfu DNA polymerase (TianGen), 5 μ l of Pfu 10 \times buffer (TianGen), and 37.5 μ l of 3dH₂O in a total volume of 50 μ l. The reaction was performed using the following procedure: an initial denaturation step at 96°C for 5 min; followed by 30 cycles of denaturation at 95°C for 50 s, annealing at 62°C for 50 s, and extension at 72°C for 1 min. After purification using the Cycle-Pure Kit (Omega Bio-Tek Inc., Norcross, Ga., USA), the PCR products and expression plasmid PET32a (+) were digested using the restriction enzymes *Eco*R1 and *Hind*III (Fermentas, Waltham, Mass., USA), respectively, at 37°C for 4 h, and linked together by DNA ligase (TaKaRa BIO Inc., Otsu, Japan) at 16°C overnight to obtain the PET-SODA and PET-SODC recombinant plasmids. *E. coli* BL21 (DE3) (TianGen) was

transformed with the 2 recombinant plasmids, and chosen after growth in Luria-Bertani (LB) medium containing 1% ampicillin. Nucleotide sequencing of the recombinant plasmids was performed by Shanghai Invitrogen Biotechnology Co. (Shanghai, China).

Recombinant protein expression and purification: *E. coli* carrying the SODA or SODC recombinant plasmid was induced by adding 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), cultured in LB culture media at 37°C by rotating at 170 rpm for 5 h. Following this, the bacteria were harvested, sonicated, and centrifuged to separate the bacterial extract and sediment. The extract and sediment were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis, respectively. The results demonstrated that the PET-SODA fusion protein predominantly existed in the form of inclusion bodies and that the PET-SODC fusion protein was present as both inclusion bodies and a soluble substance.

The soluble PET-SODC protein was directly purified using a His trap affinity column according to standard procedures, and PET-SODA inclusion bodies were isolated from the bacterial sediment using urea gradient and purified and renatured as reported previously (26). In brief, the inclusion bodies were first cleared, denatured, and then solubilized with solution A (50 mM Tris-Cl, 100 mM NaCl, 0.5% Triton-X-100), solution B (50 mM Tris-Cl, 100 mM NaCl, 2 M urea) and then with solution C (8 M urea), which resulted in soluble PET-SODA recombinant protein. Following this, the soluble protein was purified using a His trap affinity column, and renatured with buffer (50 mM Tris, 50 mM NaCl, 10% glycerol, 1% glycine, 0.05 mM oxidized glutathione, 0.5 mM reduced glutathione, pH 8.0, 0.2 mM ammonium ferric citrate) 4 times, each for 8 h (32 h in total) before being dialyzed with 1 \times PBS for 24 h. Both proteins were stored in 1 \times PBS at -70°C.

His-labeling of proteins and endotoxin removal: PET-SODA and PET-SODC fusion proteins (0.4 mg each) were digested with 1 μ l of enterokinase (Guangdong Zhongda, Zhuhai, China) (27) at 25°C for 8 h. The lysate was filtrated using a His trap affinity column to elute the transudate, which contained the target protein. Following this, the target protein was treated with Detoxi-Gel (Thermo Fisher Scientific Inc., Waltham, Mass., USA) to remove endotoxin. According to the general operation at our laboratory, the endotoxin level was usually maintained at <0.03 endotoxin units (EU) per microgram of protein. Finally, the concentration of purified target proteins was estimated with BCA reagent according to the manufacturer's instruction (28).

Cell culture: ANA-1 cells, which are normally functioning macrophages derived from murine bone marrow and infected with a recombinant retrovirus J2 (V-raf/V-myc), were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin at 37°C in an atmosphere of 5% CO₂. Log-phase ANA-1 cells were seeded on 6-well plates at a density of 1 \times 10⁶ cells/ml and activated by murine recombinant IFN- γ (100 u/ml) (PeproTech, Rocky Hill, N.J., USA) overnight. After replacing the culture medium, the cells were incubated with different concentrations of SODA or SODC protein (10, 50, and

100 $\mu\text{g/ml}$) for different indicated times. The untreated cells acted as blank controls. At each indicated time-point, cell-culture supernatants were collected for analysis of NO, IFN- γ , and ICAM-1 concentrations, and cells were collected for detection of iNOS and GSH, mRNA quantification of IFN- γ R and TLR2 by semi-quantitative RT-PCR, and TLR2 flow cytometry.

To assess caspase-3 activity and apoptosis, after treatment with SOD proteins, the fresh culture medium containing the apoptosis promoter staurosporine (1 mM; Sigma-Aldrich, St. Louis, Mo., USA) was replaced and maintained for 3 h for caspase-3 analysis and for 12 h for apoptosis detection to obtain obvious apoptotic bodies. ANA-1 cells only stimulated by staurosporine in the absence of SOD proteins were established as positive groups.

NO, iNOS, and GSH detection: The analyses of NO, iNOS, and GSH were performed according to the manufacturer's instructions (NJCBio, Nanjing City, China). SOD protein (10 $\mu\text{g/ml}$) was added into three reaction systems separately. NO can be changed to NO_3^- which is translated to NO_2^- via catalysis by nitrate reductase. Chromogenic NO_2^- absorbs light waves at 550 nm, and its OD was read by an ELISA reader. For the measurement of iNOS and GSH content, ANA-1 cells were lysed by repeated freeze/thaw cycles, and the lysate supernatants were used for analysis. Chromogenic products of iNOS and GSH absorb light waves at 530 nm and 420 nm, respectively, and their ODs were read by an ELISA reader. NO, iNOS, and GSH concentrations were calculated according to the formula listed in the instructions.

Cytokine ELISA: Activated cells were incubated with various concentrations of SOD proteins (10, 50, and 100 $\mu\text{g/ml}$) for 6, 12, 18, and 24 h. Untreated cells served as blank controls. At each indicated incubation time, cell-free culture supernatants were harvested, aliquoted, and frozen at -70°C . INF- γ and ICAM-1 levels in the supernatant were measured in duplicate using the commercially available mouse IFN- γ ELISA Kit (NeoBioscience, Shenzhen, China) and Soluble mouse ICAM-1 ELISA Kit (Boster, WuHan, China) according to the manufacturer's instructions.

Caspase-3 activity assay and cell apoptosis detection: Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin at 37°C and 5% CO_2 . After co-culture with various concentrations of SOD proteins for 6, 12, 18 and 24 h, for the caspase-3 activity assay, apoptosis was induced in a group of cells using staurosporine (1 mM; Sigma) for 3 h. Another group was exposed to the same conditions for 12 h for apoptosis detection to obtain obvious apoptotic bodies. The cells stimulated with staurosporine in the absence of SOD proteins were considered as positive controls, and those untreated with either staurosporine or SOD proteins were considered as negative controls. At 3 h post-treatment, caspase-3 activity in the cell lysate was analyzed using the Caspase-3 Activity Assay Kit (BestBio, Shanghai, China). Luminescence was measured at 405 nm, and caspase-3 activity was expressed in luminescence units. For detection of cell apoptosis, at 12 h, the cells were collected, stained with Hoechst 33258 at room temperature for 5 min in the dark according to the manufacturer's instructions, and then uniformly spread

on glass slides. Fluorescence intensity was measured using a fluorescence microscope (Leica DM2500 system; Leica, Wetzlar, Germany) with an excitation wave at 350 nm and an emission wave at 460 nm. Apoptotic bodies were smaller and white or slightly bluish-white, while the nuclei of normal cells were larger and dark blue. Each experiment was performed in duplicate.

Receptor expression assay by semi quantitative RT-PCR: After pre-treatment with SOD proteins for different indicated times, total RNA was extracted from the ANA-1 cells using the Total RNA Extraction Kit (BioFlux, Beijing, China) according to the manufacturer's protocol. The quality of the RNA samples was assessed by agarose gel electrophoresis. Three microliters of total RNA was reverse transcribed to cDNA using the RevertAidTM First Strand cDNA Synthesis kit (Fermentas). These cDNA were used as templates for semi-quantitative RT-PCR of IFN- γ R (743 bp) and TLR2 (410 bp). The primers P1 (sense: 5'-TGGAGCTTTGACGAGCACTAGG-3') and P2 (antisense: 5'-CCAGGATAACTACTGTAAAGACGGT-3') were used to amplify 743 bp of IFN- γ R mRNA, and TLR2 was amplified using the sense (5'-TTTGCTCCTGCGAACTCC-3') and antisense (5'-CAGCTTAAAGGCGGGTC-3') primers; length of the PCR product was 410 bp. The primers for inner contrast β -actin were p1 (sense: 5'-GAGACCTTCAACACCCCAGC-3') and p2 (antisense: 5'-ATGTCACGCACGATTTCCC-3'), and a 263 bp product was obtained. Quantity One analysis software (Bio-Rad Laboratories, Hercules, Calif., USA) was used for semi-quantitative analysis of the PCR products with the aid of 1% agarose gel electrophoresis.

Flow cytometry analysis: The cells were incubated with two concentrations of SOD proteins (10 and 100 $\mu\text{g/ml}$) for 6, 18, 24 and 48 h at 37°C , in an atmosphere of 5% CO_2 . At each specified time point, the treated cells were washed and then incubated with 1.25 $\mu\text{g}/100\ \mu\text{l}$ of PE-conjugated monoclonal anti-mouse CD282 (TLR2) (eBioscience, San Diego, Calif., USA) for 30 min at 4°C in the dark. After washing with $1 \times$ PBS twice, the cells were resuspended in 1% paraformaldehyde/PBS at a range of 10^5 to 10^8 cells/test. Finally, analysis was performed using a FACScan flow cytometer (Becton, Dickinson and Co., Franklin Lakes, N.J., USA). Results were analyzed using Flowjo 7.6 software version. Each experiment was performed in duplicate.

Nucleotide sequence accession numbers: The sequences obtained in this study were deposited in GenBank under accession numbers "SODA": NC_000962 REGION: 4320704 . . . 4321327 and "SODC": NC_000962 REGION: 519600 . . . 520322.

Statistical analysis: Statistical analysis was performed using SPSS 11.5 for Windows software. Results are expressed as mean \pm SD of the number of samples evaluated. Differences between groups were evaluated by 1-way ANOVA. A P value of <0.05 was considered statistically significant.

RESULTS

SOD proteins scavenge reactive oxygen species and reduce NO release by inhibiting iNOS activity: In this study, NO was assessed as a representative of ROIs, and

GSH, which is one type of reactive oxygen species scavenger and exhibits an oxidation-reduction state, could be considered as a marker of reactive oxygen species in macrophage. Fig. 1 shows the effect of SOD pro-

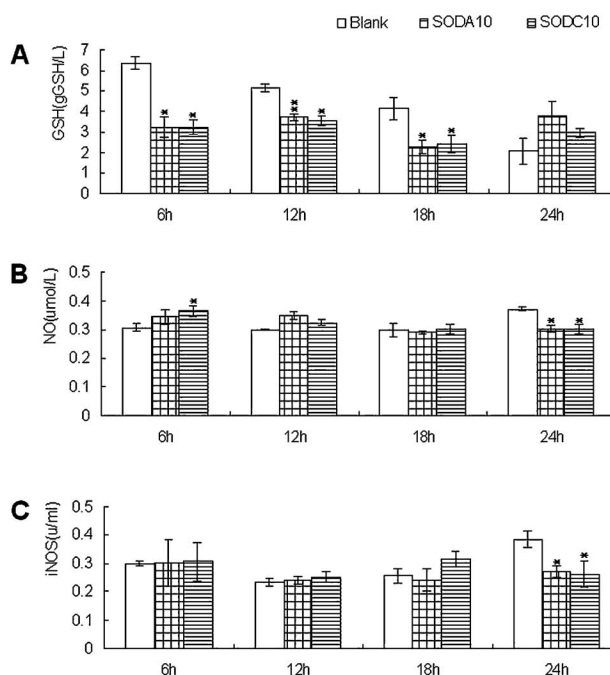


Fig. 1. SOD proteins scavenge reactive oxygen species, reduce NO release by inhibiting iNOS activity. Activated ANA-1 cells were treated with SODA/SODC proteins (10 $\mu\text{g}/\text{ml}$) for 6, 12, 18 and 24 h respectively. GSH contents, NO release and iNOS activity were detected at indicated time points. The data were expressed as the mean ($\pm\text{SD}$) of 3 separate donors. $**P < 0.01$, $*P < 0.05$, versus blank control. (A) GSH contents sharply reduced during 6–18 h, recovered to normal or even slightly higher level at 24 h. (B) The release of NO displayed a going-down tendency, and fell significance at 24 h time point in contrast with blank control. (C) The activity of iNOS enzyme was inhibited obviously at 24 h time point.

teins on reactive oxygen species, NO generation, and iNOS activity. As expected, the SOD proteins were regarded as foreign bodies and subsequently endocytosed by ANA-1 cells; an oxidative burst rapidly occurred during the early stage, particularly in activated cells, and GSH concentration was sharply reduced during 6–18 h post-treatment, but, recovered or even reached a slightly higher level at 24 h post-treatment (Fig. 1A). These results revealed that reactive oxygen species ROS were already cleared by SOD proteins at that time. In contrast, the generation of NO in culture supernatant increased at 6 h, but dropped sharply at 24 h (Fig. 1B). iNOS activity was found to be coincident with the change in NO, and at 24 h, it was obviously inhibited (Fig. 1C), which suggested that the decrease in NO was directly associated with the reduction in iNOS activity. Similar results were obtained with the resting ANA-1 group, with the exception that the oxidative burst was not stronger or longer than that of the activated ANA-1 group (data not shown). Therefore, our data supported the proposition that SODA and SODC proteins not only scavenge reactive oxygen species but also reduce the release of NO by inhibiting iNOS activity.

SOD proteins transiently inhibit cytokine secretion in the early stages of infection: The data shown in Fig. 2 clarify the impact of SOD proteins on cytokine secretion. Both SODA and SODC proteins inhibited IFN- γ secretion at 6 h post-treatment (data not shown, except those at a concentration of 100 $\mu\text{g}/\text{ml}$) and the effect of only the highest level of SODA (100 $\mu\text{g}/\text{ml}$) lasted to 12 h (Fig. 2A). However, the SODA protein had no impact on ICAM-1 secretion (data not shown), and the secretion of ICAM-1 was only found to be significantly reduced at mid and high levels of the SODC protein at 6 h post-treatment (Fig. 2B). These findings suggested that the inhibition of SOD proteins on cytokine secretion was transiently limited to early phases of infection.

SOD proteins down-regulate IFN- γ R expression on the cell surface: The amplified products of the IFN- γ R gene fragment in the cDNA genome, which was reverse

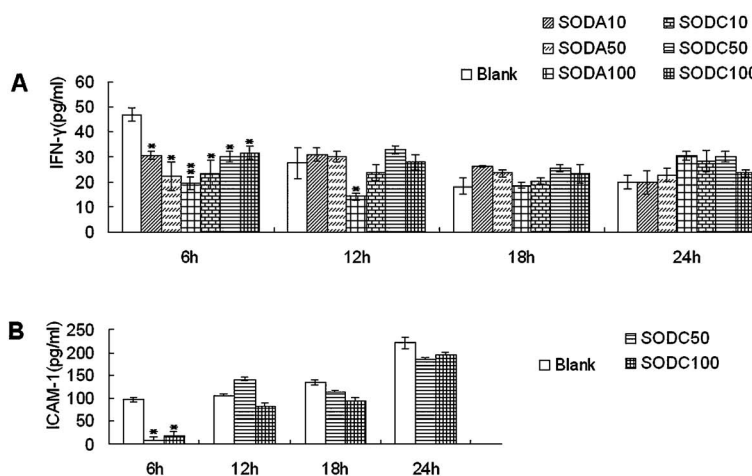


Fig. 2. SOD proteins inhibit cytokine IFN- γ and ICAM-1 secretion in the early stages. The release of IFN- γ and soluble ICAM-1 after ANA-1 cells were treated with different concentration (10, 50, and 100 $\mu\text{g}/\text{ml}$) of SOD proteins, and tested at 6, 12, 18 and 24 h respectively. Results are expressed as the mean ($\pm\text{SD}$) of 3 separate donors. $**P < 0.01$, $*P < 0.05$, versus blank group. (A) IFN- γ secretion was affected by SOD proteins, different concentrations with a range of 10–100 $\mu\text{g}/\text{ml}$. It was inhibited in early phase (6 h), and high level SODA did not yet relieve inhibition until 12 h. (B) ICAM-1 release was influenced by medium and high level SODC proteins at 6 h, recovery to normal after 12 h.

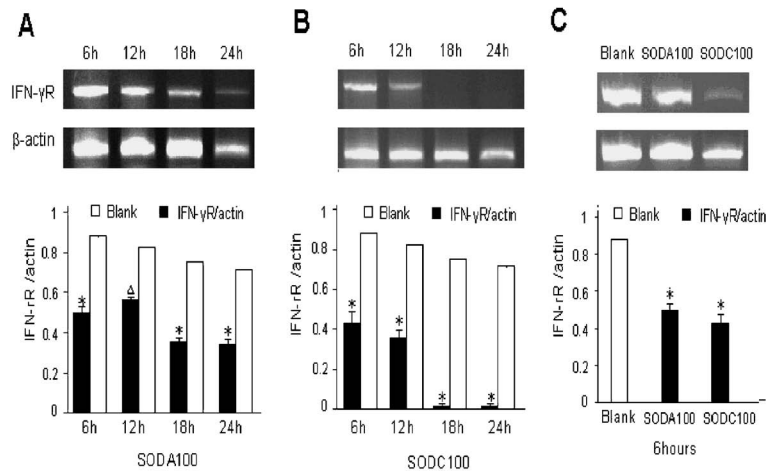


Fig. 3. SOD proteins down-regulate the expression of IFN- γ R on surface of cells. Activated ANA-1 cells were incubated with SODA and SODC proteins (100 μ g/ml) for 6, 12, 18 and 24 h respectively. Total RNA was extracted at the indicated times and translated into cDNA. Untreated time-matched ANA-1 cells were treated at the same time as blank group. PCR products of IFN- γ R were assayed by semi-quantitative RT-PCR. Black bar diagram represents treated cells. White bar diagram represents untreated blank cells. Data were expressed as the mean (\pm SD) of 3 separate donors. ** P < 0.01, * P < 0.05, versus blank group. (A) The expression of IFN- γ R elicited from SODA100 (100 μ g/ml) at indicated times, displayed a decreasing tendency in a time-dependent manner. (B) The expression of IFN- γ R originated from SODC100 (100 μ g/ml) at the indicated times, displayed a decreasing tendency in time-dependent manner. (C) SOD proteins (100 μ g/ml) obviously down-regulated the expression of IFN- γ R at 6 h.

transcribed from the total RNA, could partially represent the expression of IFN- γ R on the surface of ANA-1 cells. IFN- γ R expression was obviously down-regulated in a time-dependent manner from 6 to 24 h by both SOD proteins (100 μ g/ml) (Fig. 3A-C). Comparatively, the inhibition of SODC was stronger than that of SODA (Fig. 3B and C) because IFN- γ R expression was barely detectable after 18 h of exposure to the SODC protein. The effect of SOD proteins at concentrations under 50 μ g/ml (data not shown) was similar to that under 100 μ g/ml, as mentioned above. Collectively, these results suggested that the effect of SOD proteins on IFN- γ R expression was potentially persistent and effective.

SOD proteins inhibit cell apoptosis by restraining caspase-3 activity: We previously noted that caspase-dependence was mainly involved in the apoptosis of macrophages. Therefore, apoptosis was confirmed by the assessment of caspase-3 activity and the amount of apoptotic bodies produced. Compared with the positive control, caspase-3 activity was significantly decreased in cells pre-treated with various concentrations of SOD proteins (Fig. 4). Furthermore, the inhibition of caspase-3 activity in activated ANA-1 cells was much stronger than that in resting cells in a time-dependent manner. Meanwhile, the number of apoptotic bodies was counted. Pyknotic bodies, a category of apoptotic bodies, could be extensively observed in the positive controls. Comparatively, the number of apoptotic bodies induced by staurosporine was significantly lessened following pre-incubation with different concentrations of SOD proteins (Fig. 5). These findings suggested that the SOD proteins aided the cells to be remarkably resistant to apoptosis induced by the apoptosis promoter staurosporine. Furthermore, apoptotic bodies in the activated cells were less than those in the resting cells under the same conditions (data not shown), which

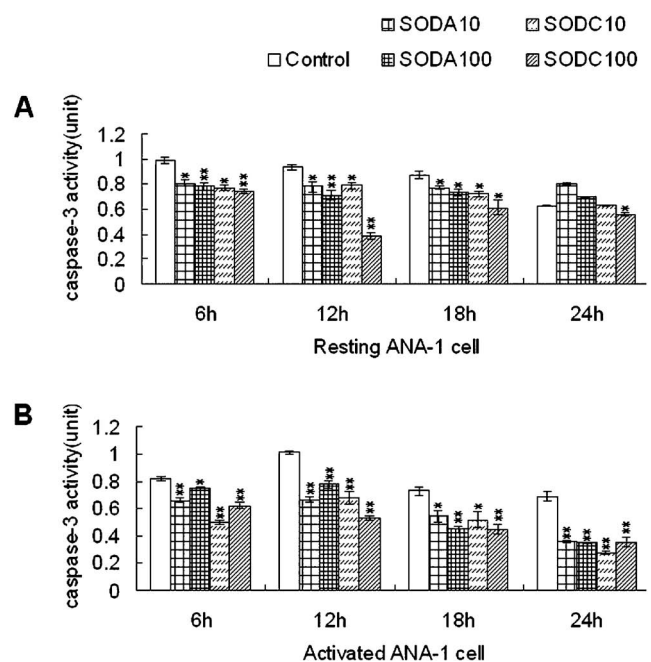


Fig. 4. SOD proteins inhibit the activation of caspase 3. Resting and activated ANA-1 cells were treated with two concentrations (10 and 100 μ g/ml) SOD proteins for indicated time respectively. After inducing them for 3 h with apoptosis promoter staurosporine, we tested caspase-3 activity. Data were expressed as the mean (\pm SD) of 3 separate donors. ** P < 0.01, * P < 0.05, versus the positive control group. Results showed that any concentration of SOD proteins could inhibit caspase-3 activity in both resting (A) and activated cells group (B) at 6, 12, 18, and 24 h. Caspase-3 activity inhibition was stronger in activated cells than in resting cells at 24 h, which suggested that SOD proteins protected activated cells from apoptosis more than resting cells.

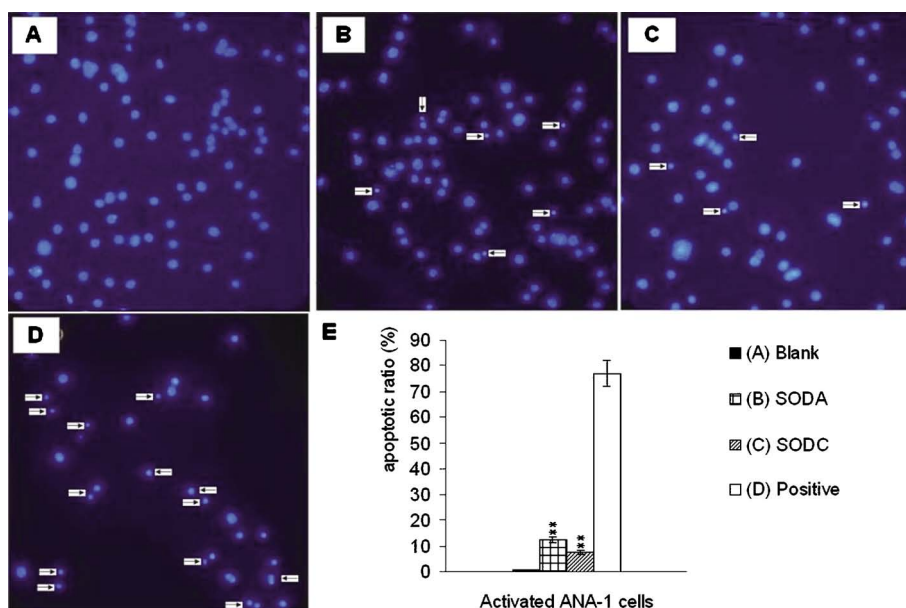


Fig. 5. (Color online) SOD proteins inhibit cells apoptosis. Activated ANA-1 cells were incubated with apoptosis promoter staurosporine for 12 h post-treated with SOD proteins (SODA and SODC, 100 $\mu\text{g}/\text{ml}$) for 24 h, and then stained with Hoechst fluorescence. Apoptotic bodies appeared to be smaller and slightly whiter than normal nuclei by fluorescence microscope. (A) Untreated cells were considered as blank group and few apoptotic bodies could be seen in this picture. (B) Cells treated with SODA protein, staurosporine, and Hoechst were considered as SODC group and at least 6 apoptotic bodies were shown in this picture. (C) Cells treated with SODC protein, staurosporine and Hoechst were considered as SODC group and at least 5 apoptotic bodies were seen in this picture. (D) Cells treated with staurosporine and Hoechst, but with an absence of SOD proteins were considered as positive control and at least 20 apoptosis bodies could be seen in this picture. (E) Comparison of apoptosis rate of various groups, the white bar diagram represented apoptosis rate of positive control, grid bar diagram represented apoptosis rate of SODA protein, solidus bar diagram represented apoptosis rate of SODC protein, a black bar represented apoptosis rate of blank group which was considered as zero.

suggested that *M. tuberculosis*-infected activated cells escaped from apoptosis more easily than resting cells.

SOD proteins down-regulate TLR2 expression by interfering with TLR2 mRNA transcription: Expression of the cell surface receptor TLR2 was determined by flow cytometric analysis. To investigate whether SOD proteins were involved in innate immunity and inflammation, we detected TLR2 expression by flow cytometry and transcription of TLR2 mRNA. The results showed that the intensity of TLR2 expression in cells following pre-treatment with SOD proteins was significantly decreased after 24 h (Fig. 6A–F). Following this, TLR2 mRNA transcription was observed to be interfered from 18 to 24 h post-treatment (Fig. 7); however, we observed that this inhibition was temporary. Therefore, we presumed that down-regulation of the TLR2 may have occurred via unknown pathways other than the weakening of TLR2 mRNA transcription in macrophages.

DISCUSSION

M. tuberculosis is a typical intra-cellular pathogenic bacterium, which can resist degradation by macrophages of the host immune system during early invasion and remain latent for as long as 10 years, thereby resulting in a series of problems, such as latent infection, relapse, need for long-term therapy, and drug resistance. However, thus far, the pathogenic mechanism of *M. tuberculosis* remains unclear. It is well known that as a dependable vaccine, BCG belongs to an aviru-

lent strain; compared with the virulent strain H37Rv, it loses the RD1 domain (29), but the loss of the virulent domain does not influence its invasion, survival, and long-term stimulation of the host immune system (30). Both the virulent strain H37Rv and the nonpathogenic mycobacterium secrete large amounts of SOD proteins (6). Related research also found that treatment with diethyldithiocarbamate, a potent inhibitor of SOD, increased *Mycobacterium lepraemurium* survival in murine splenic macrophages (31), notably suggesting that SOD proteins probably contribute to the long-term survival of mycobacterium in vivo.

Since 1969, when McCord and Fridovich first time found the enzymatic activity of SOD, which catalyzes the dismutation of superoxide free radicals (32), a large number of researches investigated SOD protein structure, activity, distribution, secretion, and its function as an antioxidant against free radicals. However, before 2001 and 2007 when Edwards et al. (22) and Hinchey et al. (24), respectively, constructed SOD mutant strains, the function of SOD proteins on immune responses was not introduced to the world.

In the present study, the SOD proteins were researched, mainly focusing on the host immune regulation. The concentrations of SODA and SODC proteins used for the cell studies were 10, 50, and 100 $\mu\text{g}/\text{ml}$, which were determined according to XTT test (data not shown). Only one set of data of one concentration was used as a representative to explain. Our results that are discussed below:

GSH parameters represented levels of oxidative stress

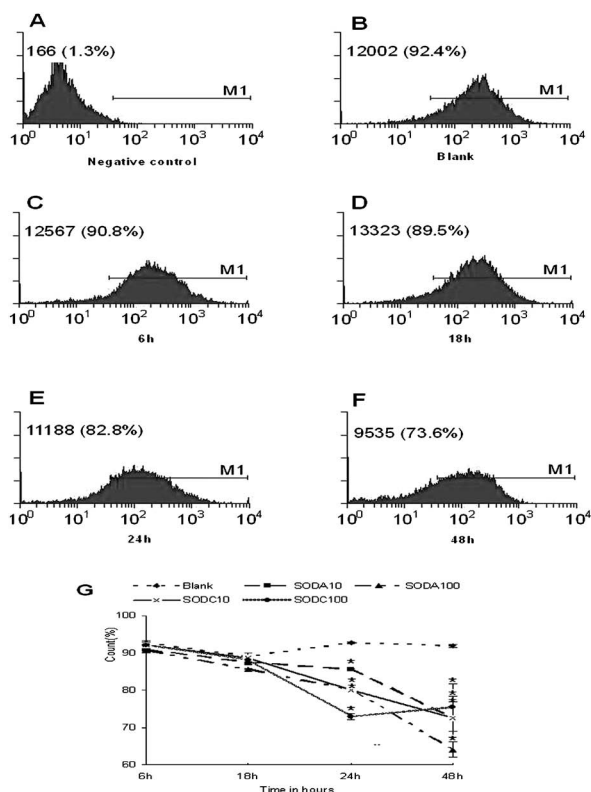


Fig. 6. SOD proteins down-regulate TLR2. Flow cytometry analysis showed proportion of TLR2 expression. Panels A and B are representative histograms of unlabeled anti-TLR2 ANA-1 cell control group and labeled anti-TLR2 untreated blank cell respectively. Panels C, D, E, and F showed representative histograms of labeled anti-TLR2 positive cell at 6, 18, 24, and 48 h time points, respectively, post-incubated with SODA (100 $\mu\text{g/ml}$). G represent percent counts of TLR2 expression at all indicated time points and explain further above data. Data were expressed as the mean (\pm SD) of 3 separate donors. * $P < 0.01$, versus blank group. It suggested that SOD proteins down-regulated TLR2 expression after 24 h.

in ANA-1 cells. The results showed that GSH levels sharply decreased during 6–18 h and then recovered by 24 h, which inferred a double function of the SOD proteins. As foreign bodies, SOD proteins were endocytosed by the macrophages and elicited cells to generate a violent respiratory burst, and GSH was initially exhausted. Thereafter, the enzyme activity of the SOD proteins scavenged oxygen radicals, thus leading to GSH recovery.

NO is a toxic RNI, which can kill invading pathogenic bacteria. A related study (33) reported that, as a potential novel class of neuronal NOS inhibitors, the SODC protein can block NOS enzyme activity in the brain, and the SODA protein also appeared to hinder NO_2^- production in activated murine macrophages (18). Our research supported this view that SODA and SODC proteins can inhibit iNOS activity in murine macrophages and further inhibit NO production. The following conclusions can thus be drawn from the data: (i) a respiratory burst probably occurred at 6–18 h. Once SOD proteins were endocytosed, it was triggered immediately. However, this process was affected by many factors, such as the state of the phagocytes (our data showed that the release of oxygen radicals in activated ANA-1 cells was much longer and stronger than that in

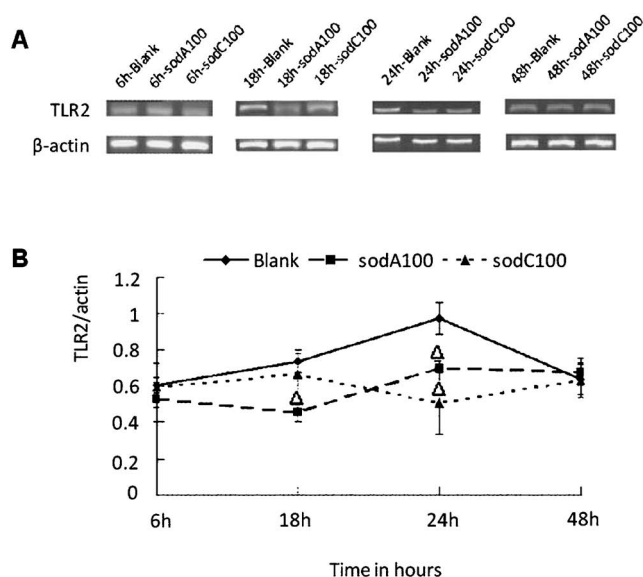


Fig. 7. SOD proteins restrain TLR2 mRNA transcription. ANA-1 cells were incubated with SODA or SODC proteins (100 $\mu\text{g/ml}$) for 6, 18, 24, and 48 h and total RNA was isolated at indicated time point and reversed transcription into cDNA. Untreated time-matched ANA-1 cells were considered as blank group. (A) PCR products (TLR2 and internal control β -actin) were exhibited on a piece of Sepharose and assayed by semi-quantitative RT-PCR. (B) Bar diagram represents TLR2/actin ratio of each group. SODA protein decreased TLR2/actin ratio in period of 18–24 h, SODC protein reduced TLR2/actin ratio in 24 h time point. Data were expressed as the mean (\pm SD) of 3 separate donors. $\triangle P < 0.05$, versus blank group.

resting ANA-1 cells), bacterial strains, and enzyme species and activity (34). (ii) Enzyme activity of the SODC protein was not less than that of the SODA protein in terms of inhibiting the generation of ROIs and scavenging of oxygen radicals. This is a subject that has not been discussed before. (iii) The SOD proteins reduced the generation of toxic NO by inhibiting iNOS activity.

On the other hand, acquired immunity response is generally accepted as the primary mechanism of killing and resistance to *M. tuberculosis*. Our results indicated that the inhibition of SOD proteins on cytokine secretion was transitory, slight, and complex. In fact, in our study, SOD proteins bidirectionally regulated cytokine $\text{IFN-}\gamma$ secretion, it was inhibited in the early stage of infection, recovered quickly, and then slightly increased after 24 h. A previous research regarding the antigenicity of mycobacterial SOD (35) showed that SOD stimulated mice to produce various cytokines, including $\text{IFN-}\gamma$, which coincided with our research. ICAM-1 was implicated in mononuclear cell infiltration, and the decrease in ICAM-1 secretion predicted that the local inflammatory response would be attenuated in the early stage of infection. Cytokine ICAM-1 was detected and found to be clearly inhibited by SODC at 6 h. In addition, the SODA proteins did not influence the release of ICAM-1.

However, the effect of SOD on cytokine receptor expression was strong and persistent. $\text{IFN-}\gamma$ is an important cytokine produced by T-lymphocytes and macrophages, which can regulate immunity. $\text{IFN-}\gamma$ interacts with cells through the cell surface receptor $\text{IFN-}\gamma\text{R}$, which then activates various specific genes and elicits

macrophage activation and subsequent antigen presentation, phagocytosis, and killing (36). IFN- γ R is a glycoprotein specific to IFN- γ , which can penetrate through the cell membrane. Hence, its quantity, affinity, and subsequent intra-cellular reactions will affect the biological activities and individual physicochemical features of IFN- γ . The loss of IFN- γ R can lead to disruption of IFN- γ function (37). Our results showed that SOD proteins inhibited IFN- γ R expression on the surface of ANA-1 cells in both concentration- and time-dependent manners. In addition, IFN- γ R expression was hardly detected after 18 h post-induction of SODC proteins. These findings suggested that the SODC protein was more effective in inhibiting IFN- γ R expression than SODA protein. Therefore, SOD proteins can impair acquired immune responses primarily by down-regulating IFN- γ R expression and the efficacy of SOD proteins influence on receptor expression more than cytokine secretion.

Innate immunity is the first line of defense against infection, and inhibition of innate immunity can cause recurrence of infection and deterioration of superinfections. TLRs have been found to be involved in the innate immune response of fruit flies and other insects (38–40), and several similar proteins have been discovered in a variety of vertebrates including humans (41–43); for example, TLR2 was reported to be closely associated with mycobacterium infections (44). In our study, SOD proteins down-regulated TLR2 expression after 24 h. Prior to this, from 18 h to 24 h, TLR2 mRNA transcription had already decreased, which suggested that SOD proteins probably depressed TLR2 expression by disturbing TLR2 mRNA transcription. However, according to our data, the time gap between the two processes (transcription and expression) was very short; although, in theory, this should not be the case. It is therefore considered that there are probably factors other than SOD proteins that affect these two processes; however, further evidence is needed to demonstrate the details of these mechanisms.

Apoptosis inhibition is an immune evasion strategy usually used by intra-cellular bacterial parasites to interfere physiological deletion of infected cells, which causes immortalized infection. Previous researches have indicated that apoptosis of host cell is inhibited because of increasing SOD enzyme activity (45–48). In this study, apoptosis of ANA-1 cells was evaluated by caspase-3 activity and development of apoptotic bodies by Hoechst fluorescence staining. Compared with positive control, SODA and SODC proteins could significantly inhibit caspase-3 activity and ANA-1 cell apoptosis induced by the apoptosis promoter staurosporine. However, apoptosis of activated cells was much less than that of resting cells. Therefore, the SOD protein secretion could remarkably prevent apoptosis of *M. tuberculosis*-infected host cells.

Here, we presented evidence that SOD proteins scavenge oxygen radicals, reduce the release of toxic NO by inhibiting iNOS activity, impair acquired immunity through down-regulation of IFN- γ and IFN- γ R, resist caspase-dependent apoptosis of phagocytes, and impair innate immunity by down-regulating TLR2 expression and TLR2 mRNA transcription. These data demonstrated that SOD proteins can assist mycobacterium to

survive within macrophages. However, the mechanisms like intra-nuclear regulation and signal transduction pathway mediated by SOD proteins have not yet been discussed. Therefore, future studies should focus on identifying target points of SOD, which will have implications for clinical therapy.

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Conflict of interest None to declare.

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