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Superoxide dismutase 3 as an inflammatory suppressor in A549 cells infected with *Mycoplasma pneumoniae*

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Herein, we found that serum concentration of superoxide dismutase 3 (SOD3) was significantly reduced in children with mycoplasma pneumonia (MP) infection. To study the roles of SOD3 in inflammatory regulation of MP infection, human A549 type II alveolar epithelial cells were stimulated with 10^7 CCU/ml of MP to build MP infection *in vitro*. Secretion of pro-inflammatory cytokine interleukin (IL)-8 and tumor necrosis factor (TNF)-α were measured via enzyme-linked immunosorbent assay (ELISA) to assess the inflammatory response of A549 cells. Levofloxacin (LVFX) was used as an anti-inflammatory drug while recombinant TNF-α was used as an inflammatory promotor in MP-infected cells. Transcriptional activity of nuclear factor (NF)-κB was assessed by detecting protein levels of nuclear NF-κB and cytoplasm NF-κB using Western blot analysis. Our data suggested that the expression of SOD3 mRNA and protein, as well as content of SOD3 in cultured supernatant, were time-dependently inhibited in MP-infected A549 cells. However, lentiviruses-mediated SOD3 overexpression alleviated inflammatory response of MP-infected A549 cells, and prevented the unclear translocation of NF-κB, as evidenced by obviously reducing the production of IL-8 and TNF-α in cell cultured supernatant, as well as decreasing nuclear NF-κB while increasing cytoplasm NF-κB. Inspiringly, SOD3 overexpression induced anti-inflammatory effect and the inactivation of NF-κB was similar to that of 2 μg/ml of LVFX, but reversed by additional TNF-α treatment. Therefore, we can conclude that transcriptional activity of NF-κB was the underlying mechanism, by which SOD3 regulated inflammatory response in MP infection *in vitro*.

Keywords. A549 cells; mycoplasma pneumonia; NF-κB

1. Introduction

Mycoplasma pneumoniae (MP), a gram-negative microorganism, has lipoproteins or lipopeptides with powerful inflammatory property (Saraya 2017), and is widely considered as the most common cause for community-acquired pneumonia among children over five years (Atkinson and Waites 2014; Kumar 2018). Commonly, after MP infection, there is an incubation period of 2–3 weeks in human body, followed by a series of clinical manifestations, such as pharyngitis, rhinitis, tracheitis, bronchiolitis, and even a severe case of *Mycoplasma pneumoniae* pneumonia (MPP). To date, antibiotics, especially 14/15-memberd ring macrolides, serve as first-line drugs for MP infection (Saraya 2017). Unfortunately, the increasing

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emergence of resistance of MP to macrolides in some areas of the world makes MP infection more difficult to control and treat (Bajantri *et al.* 2018; Pereyre *et al.* 2016). Therefore, increasingly, attention has been focused on exploring novel biomarkers to prevent this disease.

Extracellular superoxide dismutase (EC-SOD/SOD3) is highly localized to lung alveolar type II epithelial cells, rendering those cells play a central role in mediating antioxidant function of SOD3 (Folz *et al.* 1997). As a major antioxidant enzyme outside cells, SOD3 is useful to ameliorate inflammation (Hernandez-Saavedra *et al.* 2005). However, whether SOD3 functioned in inflammatory regulation of MP infection has been reported scarcely.

So far, the role of SOD3 in inflammation is not simply through radical scavenging, but also through affecting signal initiation (Kwon *et al.* 2012). Nuclear factor kappa B (NF-κB), a pleiotropic transcription factor, modulates the processes of inflammation, and can be activated in bacterial infections (Struzik *et al.* 2015). Stimulation of the cells leads to the phosphorylation of inhibitor κB and the subsequent translocation of NF-κB to nucleus, where takes place the transcription of genes processing pro-inflammatory cytokines. Our previous work demonstrated that MP induced inflammatory process with a central role of NF-κB to regulate the expression of pro-inflammatory cytokines. Evidence indicates that SOD3 abrogates NF-κB activation through inhibition of the unclear localization of NF-κB, which makes it unable to bind DNA and affects the followed transcription of inflammatory cytokines in rat ischemic injury (Laurila *et al.* 2009). However, whether and how NF-κB acted in the inflammatory regulation of SOD3 in MP infection remained largely unknown.

To study the roles and the mechanisms of SOD3 in inflammatory regulation of MP infection, human A549 type II alveolar epithelial cells were stimulated with 10^7 CCU/ml of MP to build MP infection *in vitro*. Secretion of pro-inflammatory cytokine interleukin (IL)-8 and tumor necrosis factor (TNF)-α were quantified to assess the inflammatory response of A549 cells. Transcriptional activity of NF-κB was assessed by detecting measuring protein levels of nuclear NF-κB and cytoplasm NF-κB. Our data suggested that up-regulation of SOD3 alleviated inflammatory response of MP-infected A549 cells, and inhibiting transcriptional activity of NF-κB was the underlying mechanism.

2. Materials and methods

2.1 *Cells culture and treatment*

A549 cells were maintained in RPMI-1640 (SH30243.01, Hyclone) with 10% (v/v) of fetal bovine serum (6000-044, Gibco, USA), and 1% (v/v) of penicillin (P1400-100, Solarbio) at 37°C under 5% CO_2. A549 cells in logarithmic growth were seeded in a 6-well plate (3×10^5/well), and continued to culture overnight before treatment.

To study the effect of MP on SOD3, IL-8 and TNF-α, cells were infected with 10^7 CCU/ml of MP.

To study the effect of Levofloxacin (LVFX) on SOD3 content in supernatant of A549 cells, cells were stimulated with 10^7 CCU/ml of MP, and then treated with LVFX (100986-85-4, Aladdin) at a dose of 0.5, 1, 2, 4, and 8 μmol/l, respectively.

To study the involvement of NF-κB in inflammatory regulation of SOD3 in MP infection, cells with or without lentiviruse transfection were triggered with 10^7 CCU/ml of MP, and then treated with LVFX (2 μmol/l) or recombinant TNF-α (10 ng/ml).

2.2 *Enzyme-linked immunosorbent assay (ELISA)*

45 serum samples of children with MP infection and 45 serum samples of the corresponding healthy controls were recruited with the written informed agreement from their parents. Our protocol was approved by West China Hospital of Sichuan University Biomedical Research Ethics Committee. Levels of SOD3, IL-8 and TNF-α in serum sample or cell cultured supernatant were assessed using ELISA following a provided protocol (X-Y Biotechnology Co., Ltd. Hangzhou, China).

2.3 *Fluorogenic quantitative (FQ)-RT-PCR*

Serum MP-DNA content from 45 MP-infected children and 45 healthy children was evaluated by FQ-RT-PCR (Cat. #DA-D064, Da An Gene Co., Ltd. of Sun Yat-Sen University) and ABI Prism 7300 SDS Software (Applied Biosystem, USA) following a provided introduction. Cycling conditions was programed as: denaturation at 95°C for 15 s followed by elongation at 60°C for 45 s for 40 cycles and annealing at 95°C for 15 s.

2.4 *Isolation of nuclear and cytoplasmic fractions*

After digestion, A549 cells were washed with phosphate buffer saline (PBS) (JRDUN Biotechnology, Shanghai, China). Total proteins in the cytoplasm and nuclear were extracted by NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents (#78835, Themo) following the manufacturer's instruction.

2.5 *Quantitative real-time (qRT)-PCR*

Trizol regent (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction. First-strand cDNA was harvested using RevertAid First Stand cDNA Synthesis kit (Fermentas, Hanover, Maryland, USA). Quantification of mRNA levels of SOD3 within A549 cells was performed using SYBR Green Mix (Thermo) and ABI Prism 7300 SDS Software. The amplification condition was set as the same mentioned above. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The primer pair of SOD3 was: 5′-GTGTTCCTGCCTGCTCCTG-3′ (forward) and 5′-CTCCGTGACCTTGGCGTAC-3′ (reverse), 118 bps, position of 15-132. The primer pair of GAPDH was: 5′-AATCCCATCACCATCTTC-3′ (forward) and 5′-AGGCTGTTGTCATACTTC-3′ (reverse), 218 bps, position of 436-653.

2.6 *Western blot analysis*

After homogenization in RIPA buffer (JRDUN, Shanghai, China), total protein levels in A549 supernatant was quantified using BCA protein assay kit (Thermo), 25 μg of which was separated by 15% SDS-PAGE. Proteins of SOD3, nuclear NF-κB and cytoplasmic NF-κB in the electrophoretic pure were transferred onto PVDF membranes (Millipore, USA) and incubated with primary antibodies: anti-SOD3 (ab83108, abcam), anti-NF-κB (ab16502, abcam), anti-GAPDH (#5174, Cell Signaling Technology) and H3 antibody (#4499S, CST) at 4°C overnight followed by a second antibody (Beyotime, Shanghai, China) for 1 h at 25°C. ECL system (GE Healthcare/Amersham Biosciences) was used for analysis. GAPDH normalized SOD3 and cytoplasmic NF-κB while H3 normalized NF-κB.

2.7 *SOD3 overexpression vectors*

The primers of human SOD3 gene (NCBI NM_018643.4) were designed as follows: 5′-CGGAATTCATGCTGGCGCTACTGTGTTC-3′ (forward) and 5′-CGGGATCCTCAGGCGGCCTTG-CACTC-3′ (reverse), which were inserted into pLVX-Puro vector (Clontech). Plasmids (pLVX-Puro-SOD3, psPAX2 and pMD2G) were exerted using Endo-free Plasmid Mini Kit I (OMGEA, E.Z.NA®), and co-transfected into 293T cells (ATCC) using Lipofectamine^{TM} 2000 according to supplier's protocol (Invitrogen). 1.5 μg of high-titer recombinant lentiviruses with SOD3 expression was transfected into A549 cells using Lipofectamine 2000 reagent. Meanwhile, cells transfected with pLVX-Puro without SOD3 expression were thought as the corresponding control. After transfection for 72 hours, A549 cells in a 96-well (3000 cells/well) were cultured overnight, and then stimulated with MP (10^7 CCU/ml) or recombinant TNF-α (10 ng/ml) prior to the following experiments.

2.8 *Statistics and data analysis*

Standard error of the mean (SEM) was measured by three independent parallels. Date was described as mean ±SEM. One-Way ANOVA analysis with the post hoc test was used for comparisons between groups, and $P < 0.05$ was statistical significance.

3. Results

3.1 *Levels of SOD3, IL-8, TNF-α and MP-DNA in children with MP infection*

Concentrations of SOD3, IL-8 and TNF-α, as well as expression levels of MP-DNA in serum samples from 45 healthy and 45 MP-infected children were detected, by ELISA and FQ-PCR, respectively. Our data suggested that SOD3 was down-regulated while IL-8,

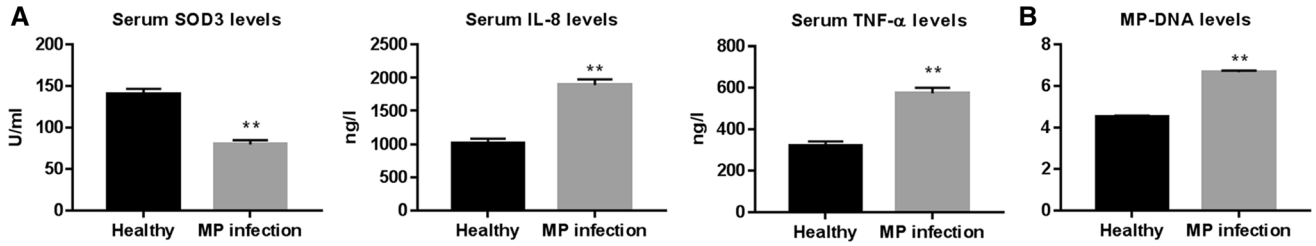

Figure 1. Serum levels of SOD3, IL-8, TNF-α and MP-DNA in children with MP infection. (**A**) Concentrations of SOD3, IL-8 and TNF-α, as well as (**B**) MP-DNA content in 45 pairs of serum samples from healthy and MP-infected children were measured by ELISA and FQ-PCR method, respectively. **P < 0.01 vs. Healthy group.

TNF-α and MP-DNA were up-regulated in MP-infected children when compared with the corresponding healthy group (all P < 0.01; figure 1).

3.2 *MP reduced SOD3 while enhanced IL-8 and TNF-α in A549 cells*

A549 cells were trigged using 10^7 CCU/ml of MP, and then at 0, 12, 24 and 48 h, respectively, the expression of SOD3 mRNA and protein was assessed using qRT-PCR and Western blot, whereas, the concentrations of SOD3, IL-8 and TNF-α in cell cultured supernatant were measured via ELISA. As shown in figure 2, MP time-dependently suppressed SOD3 while increased IL-8 and TNF-α, suggesting an A549 cells inflammation induced by MP stimulation.

3.3 *LVFX increased SOD3 in MP-infected cells*

Normal or MP infected A549 cells were treated with LVFX at a dose of 0.5, 1, 2, 4 and 8 μg/ml, and then concentrations of SOD3 in cell cultured supernatant were evaluated. Our data suggested that MP decreased SOD3 production when compared with the normal control. However, the secretion of SOD3 could be enhanced by LVFX in a dose-depended manner in MP-infected cells (figure 3).

3.4 *Anti-inflammatory regulation of SOD3 overexpression via NF-κB activation in MP-infected cells*

To substantiate the roles of SOD3 in inflammation of MP-infected A549 cells, lentiviruses-mediated SOD3

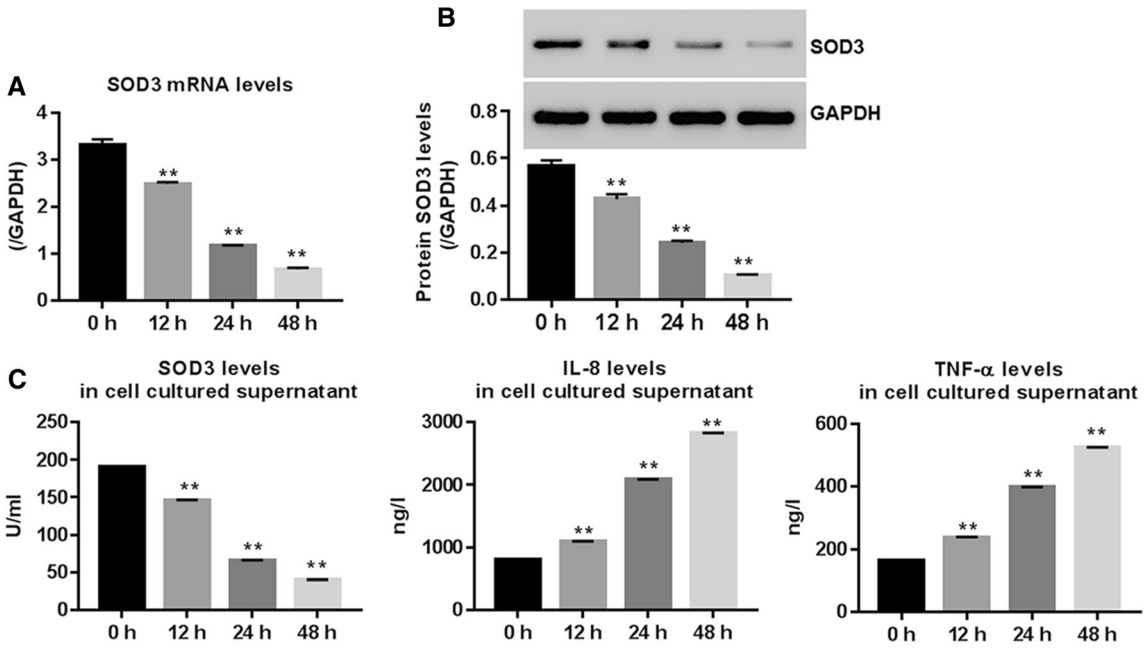

Figure 2. MP-induced inflammation in A549 cells. A549 cells were treated with 10^7 CCU/ml of MP. (**A**) mRNA levels of SOD3, assessed by qRT-PCR at 0, 12, 24, and 48 h; (**B**) protein levels of SOD3, determined by Western blot at 0, 12, 24, and 48 h; and (**C**) concentrations of SOD3, IL-8 and TNF-α, assessed by ELISA at 0, 12, 24, and 48 h. **P < 0.01 vs. 0 h.

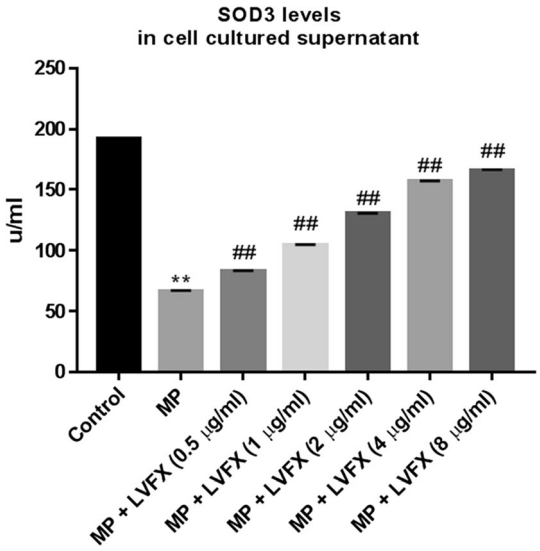

Figure 3. Effect of LVFX on the secretion of SOD3 in MP-infected cells. A549 cells stimulated with 10^7 CCU/ml of MP were treated with 0.5, 1, 2, 4 and 8 μg/ml of LVFX, and then concentrations of SOD3 in cell cultured supernatant were evaluated. **P < 0.01 vs. Control; ##P < 0.01 vs. MP group.

overexpression were transfected to A549 cells after MP stimulation, and then contents of SOD3, IL-8 and TNF-α in cell cultured supernatant were assessed. Figure 4A indicates that the production of SOD3 were significantly elevated in MP + SOD3 group when compared with MP, demonstrating a successful establishment of overexpression of SOD3 within MP-infected cells. Besides, up-regulation of SOD3 suppressed the production of IL-8 and TNF-α in MP-infected cell, and the inhibitory effect of which was similar to that of LVFX treatment (2 μg/ml), but reversed with additional recombinant TNF-α treatment (10 ng/ml), suggesting that promoting SOD3 was effective to alleviate MP-induced inflammation of A549 cells.

Furthermore, to substantiate the mechanism, by which SOD3 overexpression delivered anti-inflammatory actions, protein levels of NF-κB in A549 cell cytoplasm and nuclear were measured. As shown in figure 4B, MP promoted the expression of nuclear NF-κB while reduced cytoplasm NF-κB when compared with the normal control, suggesting the activation of NF-κB in MP-induced inflammation in A549 cells. On the contrary, both LVFX and SOD3 overexpression resulted in the opposite way, significantly decreasing nuclear NF-κB while promoting cytoplasm NF-κB. However, nuclear import of NF-κB in SOD3 overexpression group was strongly augmented by additional recombinant TNF-α treatment. Above all, these data suggested that SOD3 regulated inflammation of MP-infected A549 cells via modulating transcriptional activity of NF-κB.

4. Discussion

Diagnosis of MP infection is challenging, rendering the prevalence of this disease wide underestimation (Bajantri *et al.* 2018). In addition to FQ-PCR detection of MP-DNA content (Wang *et al.* 2009), serum content of IL-8 and TNF-α can serve as reference indexes to predict the severity of MP infection (Mei *et al.* 2015; Song *et al.* 2013). In our present study, 45 serum samples from MP-infected and healthy children were harvested, respectively. We confirmed the statistically significantly increased IL-8, TNF-α and MP-DNA in MP-infected children when compared with the corresponding healthy (figure 1). Meanwhile, our data firstly suggested that SOD3 was significantly reduced in MP infection (figure 1), which provided experimental basis for MP diagnosis.

Besides, our data suggested that MP (10^7 CCU/ml) time-dependently increased IL-8 and TNF-α in cell cultured supernatant of A549 cells (figure 2C), suggesting a successful establishment of *in vitro* model of MP infection, which accompanied severe inflammation in this process. Interestingly, inhibited effect on SOD3 production was also observed as time went by (figure 2A, B, C), demonstrating the potential involvement of SOD3 in inflammatory response in MP-infected A549 cells.

Levofloxacin (LVFX) was an antibiotic frequently used in influenza-virus infection in the lung, due to its anti-oxidative and anti-inflammatory properties (Enoki *et al.* 2015; File 1999). LVFX suppressed SOD activity in the brain (Rawi *et al.* 2011). In our present study, we studied the effect of LVFX on SOD3 in MP-infected A549 cells, and we found that LVFX dose-dependently enhanced the expression of SOD3 (figure 3), suggesting the possible involvement of SOD3 in the therapeutic effect of LVFX in MP infection *in vitro*.

TNF-α accelerates inflammation and oxidative stress in A549 cells via promoting NF-κB nuclear translocation (Wei-Jing *et al.* 2012). However, NF-κB p65 silencing obviously decreased inflammatory cytokines (IL-1β, IL-4 and IL-6) and dramatically increased SOD concentrations in A549 cells injured with 10 ng/ml of TNF-α (Wei-Jing *et al.* 2012). To substantiate the roles of SOD3 and the involvement of transcriptional activity of NF-κB in MP infection, MP-infected cells were treated with LVFX (2 μg/ml), SOD3 overexpression transfection, or SOD3 overexpression plus recombinant

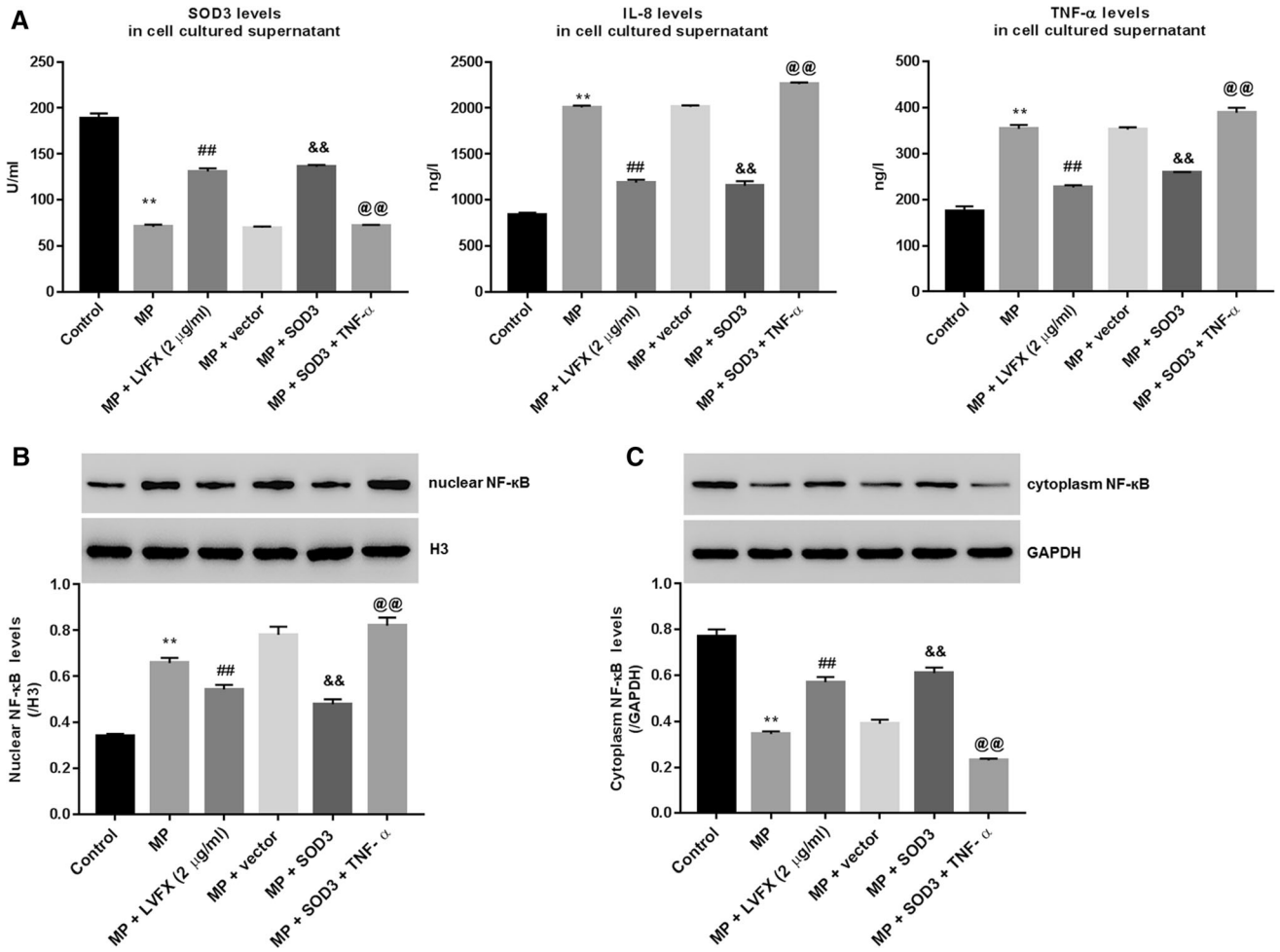

Figure 4. SOD3 blocked the activation of NF-κB to attenuate MP-induced inflammation in A549 cells. A549 cells stimulated with 10^7 CCU/ml of MP were treated with LVFX (2 μg/ml), lentivirus-mediated SOD3 overexpression, or lentivirus plus recombinant TNF-α (10 ng/ml). (**A**) Concentrations of SOD3, IL-8 and TNF-α in cell cultured supernatant, (**B**) nuclear NF-κB and (**C**) cytoplasmic NF-κB within A549 cells, determined by Western blot. **P < 0.01 vs. normal; ##P < 0.01 vs. MP; &&P < 0.01 vs. MP + vector; @@P < 0.01 vs. MP + SOD3.

TNF-α (10 ng/ml). Our data suggested that SOD3 overexpression obviously reduced the production of IL-8 and TNF-α, and inhibited NF-κB nuclear translocation (figure 4), demonstrating the anti-inflammatory effect of this enzyme, and the blockade of NF-κB in this process. Exhilaratingly, the efficacy of anti-inflammatory effect of SOD3 and its suppressed effect on NF-κB activation was comparable to existing LVFX, but worsened by additional TNF-α treatment. Therefore, we can conclude that SOD3 regulated inflammation of MP-infected A549 cells via prevention of transcriptional activity of NF-κB activity. TREM1 is an amplifier of inflammatory response via the NF-κB pathway in MP-infected cells (Liu *et al.* 2018). Evidence suggests that the deficiency of TREM1 enhances anti-oxidants, including SOD1 in injured spinal cord, and suppression of HO-1 expression, may be the underlying mechanism (Li *et al.* 2019). However, whether and how TREM1 regulates SOD3 in MP infection is unclear now and is under further investigation. These results will be reported later.

To sum up, we found that serum SOD3 was dramatically down-regulated in children with MP infection. Enhancing the production of SOD3 ameliorated the inflammatory response in MP infection *in vitro*, and inhibiting NF-κB nuclear translocation was the underlying mechanism. Targeting SOD3 expression may be a promising strategy for MP diagnosis and treatment.

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