

Original Article

Role of Interleukin-17 in defense against pseudomonas aeruginosa infection in lungs

Xilin Xu¹, Bing Shao², Ran Wang³, Sijing Zhou⁴, Zhongzhi Tang¹, Weihua Lu¹, Shengdao Xiong⁵

¹Department of Emergency Medicine, Wuhan General Hospital of Guangzhou Military Command, Wuhan 430070, China; ²Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; ³Department of Respiratory Medicine, First Affiliated Hospital of Anhui Medical University, Hefei 230001, China; ⁴Department of Occupational Medicine, Hefei Third People's Hospital, Hefei 230001, China; ⁵Department of Respiratory Medicine, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

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Abstract: Background: *Pseudomonas aeruginosa* may cause severe or even fatal infection in hosts with immunodeficiency. Interleukin-17 (IL-17) is a newly discovered pro-inflammatory cytokine, which promotes the recruitment and activation of neutrophils in the respiratory tract by inducing release of chemokine C-X-C. Objective: This study was conducted to explore the role of IL-17 in host defense against acute *pseudomonas aeruginosa* infection in lungs. Methods: The expression of IL-17 and its downstream effectors (IL-1 β , MIP-2 and G-CSF) were detected in mouse lungs with acute *pseudomonas aeruginosa* infection; 48 h after intratracheal administration of justice plasmid, mice were infected with *pseudomonas aeruginosa* again, and the bacterial clearance rate and the expression of downstream effectors of IL-17, as well as the mice death rate, were determined 6 h later. Results: The expression of IL-17 and its downstream effectors (IL-1 β , MIP-2 and G-CSF) significantly increased in mouse lungs with acute *pseudomonas aeruginosa* infection. After intratracheal administration of justice plasmid expressing IL-17, the expression of IL-17 and its downstream effectors significantly increased, accompanied by increase in neutrophil count. The justice plasmid expressing IL-17 was intratracheally administered before acute *pseudomonas aeruginosa* lung infection, which significantly increased the expression of IL-17 and its downstream effectors (IL-1 β , MIP-2 and G-CSF) in the respiratory tract, leading to increasing clearance rate of bacteria and survival rate. Conclusion: IL-17 may recruit neutrophil to the infected areas in the early phase of *pseudomonas aeruginosa* lung infection.

Keywords: IL-17, *pseudomonas aeruginosa*, pneumonia infection, gene transfection, host defense

Introduction

Pseudomonas aeruginosa (PA), a conditioned pathogen, hardly causes disease in people with normal immunity, but severe and even fatal infection may be caused in hosts with immunodeficient conditions such as HIV, organ transplant and tumors [1-4]. The extensive application of antibiotics in recent years increases the tolerance of PA, making it one of the most important bacteria causing refractory infection [5, 6]. Interleukin-17 (IL-17) is a newly discovered cytokine and named because it is generated by Th17 cells. IL-17 is a powerful pro-inflammatory cytokine that can promote the recruitment and activation of neutrophils in the respiratory tract by inducing the release of

C-X-C chemokines [7-10]. A variety of studies have shown that IL-17 plays important roles in some inflammatory diseases of the airways like asthma and chronic obstructive pulmonary diseases [9, 11-15]. This study was undertaken to investigate the role of IL-17 in acute PA lung infection, and explore whether the high IL-17 expression can increase the local expression of vital immune defense factors like interleukin-1 β (IL-1 β), macrophage inflammatory protein-2 (MIP-2) and granulocyte colony-stimulating factor G (G-CSF) in the lung, and whether IL-17 benefits the attenuation of acute PA lung infection and the survival of PA infected mice. Our findings may provide theoretical and practical evidence for the treatment of PA lung infection.

Materials and methods

Construction and transfection of justice plasmids expressing IL-17

Mouse IL-17 sequence was obtained from NCBI GeneBank, and restriction analysis and primer design were carried out. Total RNA was extracted from the lungs and then subjected to RT-PCR for amplification. The products were spliced to plasmid pcDNA3.1 (+) with gene clone; bacteria transformation and enzyme electrophoresis were conducted to identify and check order. All were designed and synthesized in the Invitrogen. 60 µg of justice plasmid expressing IL-17 was intratracheally administered to mice, and the lungs were collected 48 h later and processed for sectioning. The airway epithelial cells of these sections were observed under a fluorescence microscope.

Animals

Specific pathogen free C57BL/6 mice (male, 6-8 weeks, weighing 18-20 g) were purchased from the Experimental Animal Center of Wuhan University and housed independently in the Experimental Animal Center of Tongji Hospital of Huazhong University of Science and Technology. Animals were given ad libitum access to food and water. PA strain PAO1 was kindly provided by the Inspection Division of Microbial Room of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology.

Modeling of lung infection

Intraperitoneal anesthesia with chloral hydrate was conducted in C57BL/6 mice, which were then fixed on a sterilized operation table. After a midline incision was made at the neck, the trachea was exposed, and 50 µL of PA suspension or control suspension was slowly injected into the trachea with a 1-ml syringe. The amount of bacteria instilled was 2.5×10^6 CFU per mouse. On day 1, 2 and 3, 8 mice were sacrificed, respectively, and 0.7 ml of blood was collected from the heart into an anticoagulant syringe followed by centrifugation at 1200 r/min for 10 min at 4°C. The supernatant was collected and stored at -80°C. The lungs were collected as a whole, and then the one of leaf lobe of the right lung was ligated, and lavaged with PBS solution twice (0.7 mL/time). The bron-

choalveolar lavage fluid (BALF) after the first lavaging was centrifugated at 1200 rpm/min for 10 min at 4°C; and the supernatant was collected and stored at -80°C for the detection of cytokines. The cells of BALF were mixed with BALF after the second lavaging, and then the total number of cells and differential cells was determined. Left lung tissue was harvested and homogenized in 1 ml of cold sterile saline. The homogenate was 10-fold serially diluted, and then 10 µL of homogenate at different concentrations were inoculated to LB agar coated plate. Incubation was done for 24 h at 37°C and the colonies were counted. The right lung was embedded in paraffin, and the rest leaf lobes were stored at -80°C for RNA extraction and subsequent real-time fluorescence quantitative PCR [16, 17].

To induce PA lung infection, 72 C57BL/6 mice were randomly assigned into normal group, PBS group and PA group. On days 1, 2^d and 3 after PA infection, the IL-17 mRNA expression in the lung was detected with RT-PCR, IL-17 content of the BALF and serum was measured with ELISA, and the neutrophils of BALF were counted. To over-express IL-17 in the lung, 72 C57BL/6 mice were divided into normal group, pcDNA3.1 (+) blank plasmid group and IL-17 plasmid group. After intratracheal administration of PBS/plasmid, IL-17 mRNA expressions in the lung and IL-17 content of the BALF as well as content of IL-17 downstream effectors (IL-1β, MIP-2 and G-CSF) and the number of neutrophils in BALF were determined on days 1, 2 and 3. To investigate the effect of IL-17 on early infection, 64 mice were divided to IL-17 justice plasmid group and blank plasmid group in which mice were intratracheally treated with IL-17 at 48 h before PA treatment. At 6 h after PA treatment, the level of IL-17 and its downstream effectors (IL-1β, MIP-2 and G-CSF), and the amount of bacteria in the lung homogenate were determined. In addition, 36 mice were randomly divided into PA group, IL-17 justice plasmid group and blank plasmid group. Mice were intratracheally treated with IL-17 plasmid, pcDNA3.1 (+) plasmid in the later two groups, respectively. At 48 h after treatment, mice were infected with PA and then observed once every 24 h and the survival rate was determined [18].

ELISA detection

IL-17 ELISA kit was purchased from the eBioscience, while the ELISA kits for IL-1β, MIP-2 and

IL-7 against pseudomonas aeruginosa

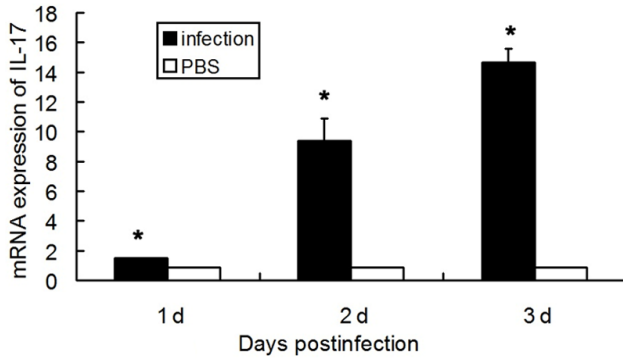


Figure 1. IL-17 mRNA expression in the lung homogenate after PA infection (n=8, * $p < 0.05$).

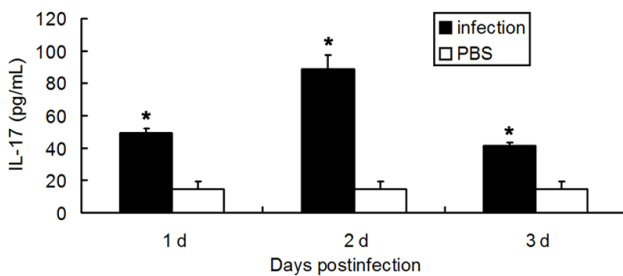


Figure 2. IL-17 content of the BALF after PA infection. (n=8, * $p < 0.05$).

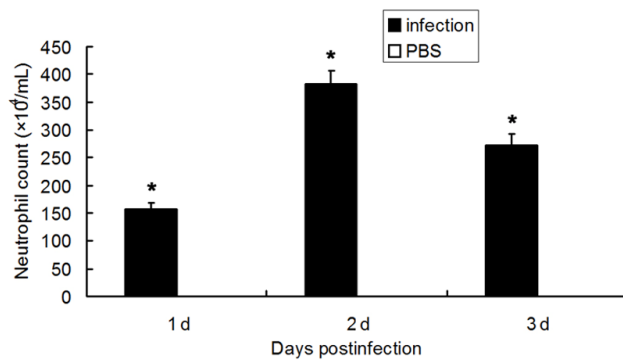


Figure 3. Neutrophil count of the BALF after PA infection (n=8, * $p < 0.05$).

G-CSF were from Raybio. In brief, specimens and standards at different concentrations were added into wells followed by incubation for 2.5 h at room temperature. After washing, the biotinylated antibody was added followed by incubation for 60 min. The plate was washed again and avidin - peroxidase complex (Streptavidin) was added followed by incubation for 45 min. The plate was washed, and disclosing solution was added followed by incubation in dark for

30 min. The reaction was stopped and the absorbance was measured at 450 nm with an automatic microplate reader Elx800. The standard curve was delineated and the content of samples was determined according to this standard curve.

Real time PCR

Primers were synthesized by the Sangon Engineering Company, and Real time PCR and reverse transcription kit were bought from TAKARA. Total RNA was extracted from the lung, followed by reverse transcription and Real Time-PCR according to manufacturer's instructions. The Ct value was used to calculate relative expression with $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were expressed as mean \pm standard deviation. One way analysis of variance was employed for comparisons of means among groups, t-test between two groups, and survival rate was compared with time sequence log-rank. A value of $P < 0.05$ was considered statistically significant.

Results

After PA infection, mice had a significantly higher IL-17 content in BALF and higher IL-17 mRNA expression in the lung than PBS treated mice in 3 consecutive days ($P < 0.05$), while no IL-17 was detected in the uninfected mice. In the uninfected mice, PBS treated mice and PA infected mice, IL-17 was undetectable in the serum. Data are listed in **Figures 1** and **2**. Moreover, the infected mice had more neutrophils than PBS treated mice within 3 days ($P < 0.05$), and the number of neutrophils peaked on the second day. The neutrophil count in normal group was $0.05 \times 10^4/\text{mL}$, as shown in **Figure 3**.

After intratracheal administration of justice plasmid expressing IL-17, the IL-17 mRNA expression in the lung on day 3 in IL-17 plasmid group was significantly higher than that in blank plasmid group ($P < 0.05$). The BALF level of IL-17 and its downstream effectors (IL-1 β , MIP-2 and G-CSF) was markedly higher than that in blank

IL-7 against pseudomonas aeruginosa

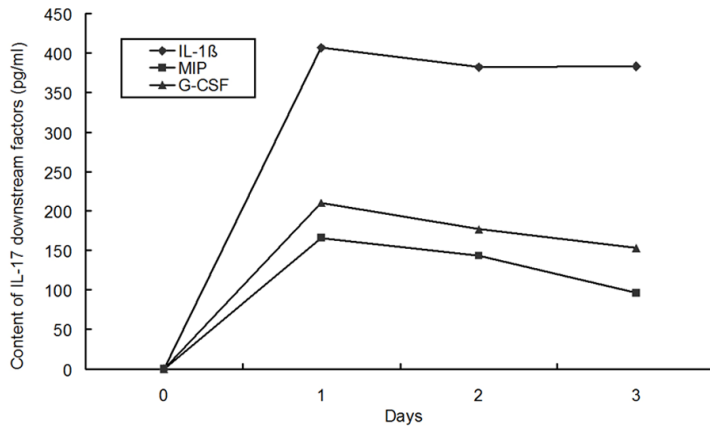


Figure 4. Contents of IL-1 β , MIP-2 and G-CSF in the BALF after intratracheal administration of pcDNA3.1-IL-17 (n=8).

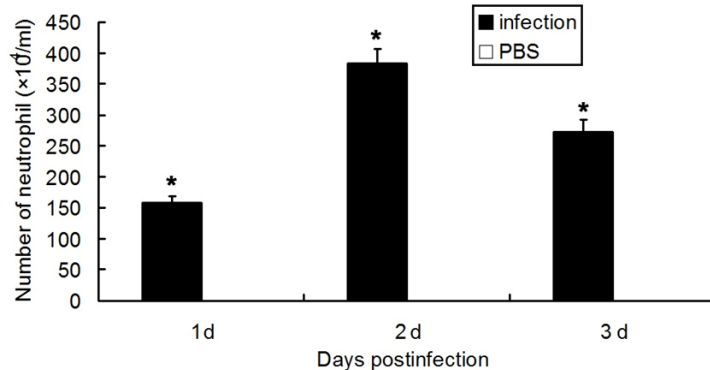


Figure 5. Neutrophil count of the BALF after intratracheal administration of pcDNA3.1-IL-17 (n=8, *P<0.05).

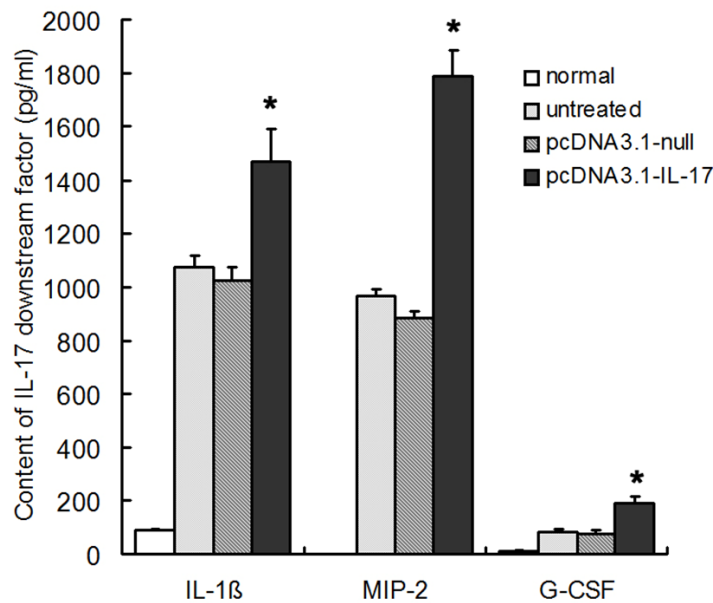


Figure 6. PcDNA3.1-IL-17 pretreatment increased the contents of IL-1 β , MIP-2 and G-CSF after PA challenge (n=8, *P<0.05).

plasmid group (P<0.05). The IL-17 and its downstream effectors were not detected in normal group, nor was IL-17 expression in the serum. Data are listed in **Figure 4**. On day 3, the neutrophil count in IL-17 plasmid group was significantly higher than that in blank plasmid group (P<0.05), and peaked on the second day, as shown in **Figure 5**.

6 h after IL-17 plasmid's intervening PA lung infection, the contents of IL-17 downstream effectors (IL-1 β , MIP-2 and G-CSF) in IL-17 plasmid treated mice were significantly higher than those in PA infected mice which were not treated and treated with blank plasmid (P<0.05), as shown in **Figure 6**. In IL-17 plasmid group, the bacteria count (4.7 log₁₀ CFU) was significantly lower than that in infection group (6.22 log₁₀ CFU) and blank plasmid group (6.26 log₁₀ CFU) (P<0.05), as shown in **Figure 7**. In IL-17 plasmid group, the 3-day survival rate was 67%, while that was 41% in infection group and blank plasmid group, significant difference was noted between IL-17 plasmid group and infection group/blank plasmid group (P<0.05), as shown in **Figure 8**.

Discussion

In both developing and developed countries, bacterial pneumonia is the major cause of death and has a high mortality, especially in host with immunodeficiency such as HIV, organ transplant and cancers. PA is a conditioned pathogen and may cause severe or even fatal infection. Although great progress has been achieved in the antibacterial treatment, the wide application of broad-spectrum antibiotics increases the drug tolerance of bacteria [19, 20]. Therefore, a better treatment is required to increase the immunity against bacterial infection.

IL-7 against pseudomonas aeruginosa

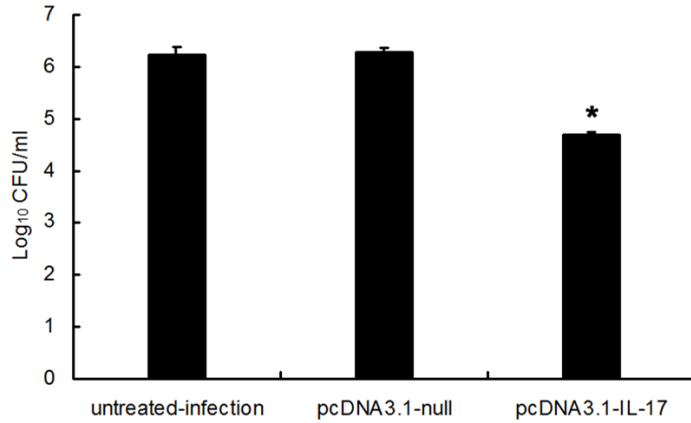


Figure 7. PcDNA3.1-IL-17 pretreatment augmented the clearance of PA in the lung (n=8, *P<0.05).

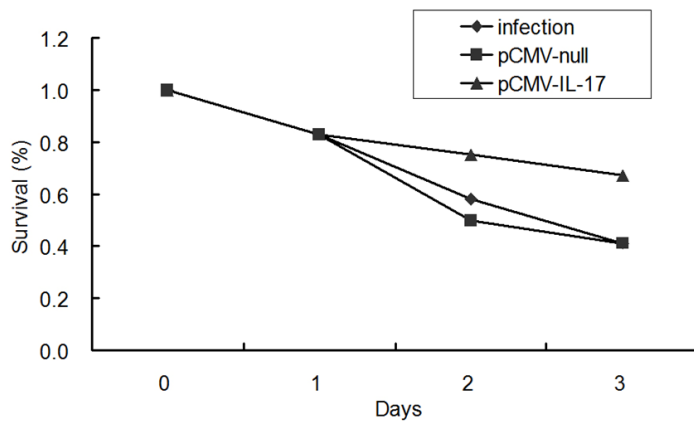


Figure 8. PcDNA3.1-IL-17 pretreatment increased early survival rate after PA challenge (n=12).

IL-17 is a newly discovered cytokine, being a homodimeric glycoprotein consisting of 155 amino acids with 35 kD in molecular weight. Besides IL-17A, IL-17 includes IL-17B, IL-17C, IL-17D and IL-17F [21-24]. It is produced by not only Th17 cells but also other cells like thymocytes, epithelial cell and vascular endothelial cells. IL-17 functions via binding to IL-17 receptor (IL-17R) on cells *in vivo*. IL-17 receptor is distributed widely and expressed in almost every kind of tissues and cells [25]. IL-17 is a powerful pro-inflammatory cytokine and promotes the recruitment and activation of neutrophils in the respiratory tract via inducing the release of C-X-C chemokines, by which it increases the clearance of pathogens, such as mycoplasma and gram-negative bacteria [8-10, 26-30]. These results also indicate the important role of IL-17 in the host defense [10, 31-34].

Miyamoto et al [30] found that, 6 h after endotoxin's partial airway administration, the IL-17 content of BALF significantly increased and the anti-IL-17 antibody completely inhibited the recruitment of neutrophils into respiratory tract. Shellito et al found when the mice treated with alcohol developed klebsiella pneumonia lung infection, the IL-17 was inhibited in lung tissues, the recruitment of neutrophils was compromised and the death rate increased [10]. Mice with IL-17R deficiency had shortened survival time after Klebsiella pneumonia infection, and increased lung bacteria, and the infection was easy to spread, which was related to the delayed recruitment of neutrophils and reduced neutrophils in the lungs, as well as to the down-regulated expression of G-CSF and MIP-2. At 48 h after Klebsiella pneumonia infection, mice with IL-17R deficiency had a mortality of 100%, but the mortality in wild-type mice was 40% [31]. Increasing evidence shows that the early recruitment of neutrophils is essential for the resistance to bacterial infection. Therefore, the increased early recruitment of neutrophils may benefit the treatment of PA lung infection [29]. In this study, the role of IL-17 in the immune

response was investigated in mice with acute PA lung infection. Our results indicated that IL-17 mRNA expression was significantly up-regulated after acute PA lung infection, and the IL-17 content of BALF was significantly higher than that in control group (P<0.05). With the increase in IL-17 content, the neutrophil count of BALF increased correspondingly, but IL-17 expression was undetectable in the serum of infected mice. Therefore, the up-regulated IL-17 may function on local immune response against acute PA lung infection.

IL-17 induces the recruitment of neutrophils primarily by promoting the release of C-X-C chemokines (especially IL-8). Laan et al [28] found that the intratracheal administration of IL-17 in mice could lead to the recruitment of neutrophils into the lung, which could last at least 8 h.

Thus, we speculated that intratracheal administration of justice plasmid expressing IL-17 could also cause a high IL-17 expression in the lung, and thus increase the production of IL-17's downstream effectors (G-CSF, IL-1 β and MIP-2), which may lead to the recruitment of neutrophils into the lung, increasing the clearance of bacteria. Consequently, the justice plasmid expressing IL-17 was administered to the airway, and the IL-17 plasmid was used to transfect the airway epithelial cells. Results showed the IL-17 mRNA expression in the lung of mice treated with IL-17 plasmid was higher than that in blank plasmid group ($P < 0.05$), and the contents of IL-17 and its downstream effectors (G-CSF, IL-1 β and MIP-2) in the BALF also increased markedly. Meanwhile, the BALF neutrophil count correspondingly increased. When compared with blank plasmid group, statistically significant difference was observed ($P < 0.05$). As a result, the intratracheal transfection of IL-17 plasmid was successful in mice.

Neutrophil functions dually in bacterial pneumonia. Early neutrophil recruitment is important in counting bacterial infection. In later phase, it will generate a great deal of cytokines/chemokines, reactive oxygen species and proteases, thus causing damage to tissues [26, 35]. Peng et al found that the intratracheal administration of IL-17 recombinant adenovirus (AdIL-17) into the lungs of mice before Klebsiella pneumonia infection to induce the over-expression of IL-17, leading to the production of TNF- α , IL-1 β , MIP-2 and G-CSF in some parts, recruitment of neutrophil aggregation, increased clearance of Klebsiella pneumonia and the elevated defense against infection demonstrated by increased survival rate [36]. In our study, at 6 h after infection, the IL-17 content in IL-17 plasmid group was higher than that in both infected group and blank plasmid group. The bacterial count in IL-17 plasmid group was significantly lower than that in the infected group and blank plasmid group. In addition, the 3-day survival rate in IL-17 plasmid group (67%) was markedly higher than that in the infected group and blank plasmid group (41%). Hence, we postulate that the increased early neutrophil recruitment may contribute to the treatment of acute PA lung infection.

In summary, our results demonstrate that the up-regulation of IL-17 may increase the bacterial clearance and survival rate through increas-

ing neutrophil recruitment via IL-17's downstream effectors, and playing protective effect in early phase of acute PA lung infection in mice. If similar mechanisms exist in humans, it seems that IL-17 may be a potential target for the treatment of acute PA lung infection, especially for those with resistant bacterial infection.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shengdao Xiong, Department of Respiratory Medicine, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. Tel: +8627-688781111; E-mail: xiongshengtao00@163.com

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