Original Article
Preliminary study about the mechanism of IL-33 acting on neutrophil apoptosis in rates with acinetobacter baumannii pneumonia

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Abstract: IL-33 is a recently discovered member of interleukin-1 (IL-1) family. Binding to the complex of receptor ST2 and the associated signal transduction protein AcP receptor located on the cell surface, IL-33 is able to carry out the intracellular signal transduction and thus lead to a cascade effect. Acinetobacter baumannii, a non-fermentative and gram-negative genus Acinetobacter bacterium, often leads to human pneumonia and sepsis in clinical setting and has become a superbug in hospitals in China. In this study, a rat model with A. Baumannii pneumonia was constructed. Low and high dose of IL-33 were used to treat experimental animals for 24 h. The expression of IL-33 receptor ST2 gene in lungs and peripheral blood was detected by QRT-PCR. Expression of major proteins associated with apoptosis of neutrophils was detected by Western Blot. Cell apoptosis was detected using double staining flow cytometry. The results showed that IL-33 was able to elevate ST3 gene expression in pneumonia lung tissues and peripheral blood in rats infected by A. baumannii, inhibit the expression of caspase-3 and Bax, and in the meantime increase the expression of anti-apoptotic factor Bcl-2.

Keywords: IL-33, acinetobacter baumannii pneumonia, apoptosis

Introduction

Acinetobacter baumannii is a type of opportunistic pathogen widely distributed in nature, with capsule and pilli but lacking flagella and spores. A. baumannii can leads to respiratory infection, sepsis, urinary tract infections, meningitis, peritonitis, among which pneumonia and sepsis are the most common disease [1]. Multidrug-resistant and pandrug-resistant A. baumannii have shown worldwide epidemic, which also become the most important superbug in China [2]. Neutrophils are round shaped with a diameter of 10-12 µm, accounting for 50-70% of total white blood cells. Neutrophils play an important defensive role in the body, with phagocytic ability and active deformation capability. When part of the body is infected by bacteria, neutrophils can release chemotactic chemicals toward bacterial products and infected tissues, penetrate the capillaries by deformation movement, gather at the site infected by bacteria, engulf bacteria and result in the formation of phagosomes, which can fuse with specific granules and lysosome. The bacteria are eventually killed and digested by a variety of hydrolase, oxidase, lysozyme and other bactericidal proteins or polypeptides.

Discovered in 2005, IL-33 is a new member of interleukin-1 family (IL-1), which has been reported as an inflammatory factor in studies of autoimmune disease, allergic disease and heart disease [3, 4]. IL-33 can bind to the complex of receptor ST2 and signal transduction protein AcP receptor located on the surface of cell membrane, activate the intracellular pathway, and recruit the downstream adapter protein myeloid differentiation factor 88 (MyD88). At least two independent pathways are activated, including mitogen-activated protein kinase (MAPK) and phospholipase D (PLD)-sphingosine kinase (SPHK) pathway. After a series of signal transduction, immune responses are regulated via NF-κB and MAPK activation and release of effector protein IL-5 [3, 5-7]. IL-33 may activate effector cells through ST2/AcP receptor on cell surface. The soluble ST2 and caspase-1 can
regulate the activity of IL-33. IL-33 participates in the immune regulation by binding to cell surface receptor ST2, driving the immune response to Th2 type, promoting the release of Th2 type cytokines such as IL-4, IL-5, IL-10 and IL-13, and regulating the activity and functions of epithelial cells, fibroblasts and immune cells such as mass cells and macrophages [8-10]. In addition, IL-33 may act as an intranuclear chromatin binding factor. IL-33 can also function as an endogenous danger signal or alarmin, playing a protective role in infection, cardiovascular disease and Alzheimer’s disease but exerting a negative effect in asthma and skin inflammation. Therefore, IL-33 and its signaling pathway has become a potential target for the treatment of these diseases.

The A. baumannii pneumonia rat model was established by endotracheal intubation in this study. Using this model, this study investigated the role and mechanism of IL-33 in treating pneumonia in rats infected by A. baumannii.

Materials and methods

Experiment animals

All SD male rats were purchased from Beijing HFK Biotechnology Co. LTD, without skin deformity, injuries or skin infections, weighing (200±10) g. Rats were fed with the standard granule food provided by Tengxin Bio, and raised at a temperature of 20-25°C and relative humidity 55%-70%. The rats were divided into four groups with five in each group: control group (A), A. baumannii pneumonia rats (B), A. baumannii pneumonia rats treated with low dose of IL-33 at 24 h after infection (C), and A. baumannii pneumonia rats treated with high dose of IL-33 at 24 h after infection (D).

Establishment of A. baumannii pneumonia rat model

Based on previous experiences, A. baumannii pneumonia rat model was established by endotracheal administration. A pilot study was conducted to determine the appropriate amount of A. baumannii to inoculate rats. SD rats were randomly divided into control group and immunodeficiency group. Prior to infection, the immunodeficiency group was injected with cyclophosphamide and dexamethasone to induce immunodeficiency. During the experiment, for the infected immunodeficiency group 12 ul activated A. baumannii (1.2*10^9 CFU/ml) was injected into trachea, while for the control group 12 ul saline was injected.

ST2 gene detection by QRT-PCR

Rat lung RNA was extracted by Trizol kit and reversely transcribed to cDNA using random

Table 1. ST2 gene expression in lung and peripheral blood

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<th>Ct3</th>
<th>Mean of Ct value</th>
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IL-33 and Acinetobacter baumannii pneumonia

A

Graphs showing data analysis of IL-33 and Acinetobacter baumannii pneumonia.
Figure 1. QRT-PCR Results of ST2 and internal standard. A: ST2. B: Internal Standard. Note: 1. Lung tissues of normal rat group; 2. Lung tissues of low dosage IL-33 treated pneumonia rat group; 3. Lung tissues of high dosage IL-33 treated pneumonia rat group.
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primers. ST2 specific primers were designed and used with sybr green I fluorescent dye for quantitative fluorescence PCR. SYBR Green I was a type of fluorescence dye which can bind to double strand DNA. The fluorescence was greatly enhanced after binding with double stranded DNA. With increased amplification cycles, the fluorescent signal was accumulated. Thus, the strength of the signal of SYBR Green I was correlated with the number of double stranded DNA, the number of which in the PCR system can be detected by fluorescence signals. The maximum absorption wavelength of SYBR Green I was 497 nm and the emission wavelength was 520 nm.

**Extraction and culture of neutrophils**

The peripheral blood of the experiment animals was collected using anti-clotting tubes. The neutrophils were isolated and extracted using peripheral blood neutrophil isolation kit, following the instructions. Cells were cultured as description previously.

**Neutrophil protein detection by western blot**

Western blot employed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to detect proteins, using antibodies as probes and secondary antibodies for development. The protein samples separated by SDS-PAGE were transferred to a solid phase (such as NC membrane or PVDF membrane), which can absorb the proteins in a non-covalent

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**Figure 2.** Caspase-3, Bax and Bcl-2 protein expression in Neutrophils. Note: 1 and 2, neutrophils in the peripheral blood of normal rat group; 3 and 4, neutrophils in the peripheral blood of pneumonia rat model group; 5 and 6, neutrophils in the peripheral blood in low dose IL-33 treated pneumonia rat model group; 7 and 8, neutrophils in the peripheral blood in high dose IL-33 treated pneumonia rat model group.

**Figure 3.** ST2 protein in Neutrophils. Note: 1, Lung tissue in normal control group; 2, Lung tissue in A. baumannii pneumonia; 3, Lung tissue in high dosage IL-33 treated A. baumannii pneumonia rats; A, Peripheral blood in normal control group; B, Peripheral blood in A. baumannii pneumonia; C, Peripheral blood in high dosage IL-33 treated A. baumannii pneumonia.
way while keeping the polypeptide nature separated by electrophoresis and biological activities. The proteins or polypeptide on the solid phases would work as antigens and induced immune response with the corresponding antibodies, which then recognized the enzyme or isotope labeled secondary antibody. The proteins expressed from specific target gene and separated by electrophoresis can be detected by substrate colorimetric detection or autoradiography. The whole proteins were extracted from cultured neutrophils and detected by Western Blot using corresponding antibodies.

Detection of Neutrophil apoptosis by double staining flow cytometry

Apoptosis is one of the primary characteristics of cells, playing an important role in embryogenesis, tissue repair and the maintenance of homeostasis. Annexin V, a Ca\(^{2+}\) dependent phospholipid binding protein with a molecular weight of 35-36 kDa, can bind to the phosphatidylserine (PS) which flips to the extracellular space during apoptosis, with a high specificity. Propidium iodide, a nuclear dye impermeable to intact cell membrane, can penetrate the membrane of dead cells or cells during mid-late stage apoptosis, and stain the nucleus with red color. Apoptosis can be detected by flow cytometry or fluorescence microscopy using FITC labeled Annexin V and propidium iodide as the fluorescent probes.

Detection of Neutrophil ROS by flow cytometry

Reactive oxygen species (ROS) including superoxide radicals, hydrogen peroxide as well as the downstream peroxide and hydroxide products, will damage cell and gene structure, and interfere with multiple physiological and pathological procedures such as cell proliferation, differentiation, senescence and apoptosis [14]. DCFH-DA (2, 7-dichlorofluorescin diacetate) is by far the most commonly used and most sensitive intracellular ROS detection probe. Without fluorescence, DCFH-DA is hydrolyzed into dichlorofluorescin (DCFH) by esterase after entering the cells. In the presence of ROS, DCFH is oxidized to a membrane impermeable substance (DCF) with strong fluorescent green color. The fluorescence of DCF exhibits the maximum peak at an excitation wavelength of 503 nm and emission wavelength of 530 nm. The strength of fluorescence is positively correlated with the cellular ROS level. This ROS detection system has a low background, high sensitivity, as well as good reproducibility, which is easy to operate.

Results

**ST2 Gene detected by quantitative fluorescent real time PCR (QRT-PCR)**

24 h after the treatment with IL-33, the lung tissues of experiment animals in each group were separated and cut into small pieces. 1 ml RIPA lysis buffer was added to 100 mg tissue. The tissues were homogenized manually using glass homogenizer up and down for 20 times,
transferred to a 1.5 ml centrifuge tube, and centrifuged at 12000 g, 4°C for 5 min. The supernatant was collected and stored. Peripheral blood was collected and RNA was extracted from the cells using Trizol kit. RNA was reversely transcribed into cDNA using random primers (100 pmol/μL). Specific primers were designed (rST2F: GGCATGATTTGCTTACCCTCAC, ST2R: TTGTTTCCTTCTCAGTCTTACGG). Specific primers and SYBR Green I fluorescence dye were used in quantitative fluorescence PCR to detect ST2 gene expression in lung tissues and peripheral blood in experiment animals from each group. Results are shown in Table 1 and Figure 1.

As shown in quantitative fluorescence PCR, the number of ST2 gene copies in IL-33 high dosage treated pneumonia rat group was similar to that of IL-33 low dosage treated pneumonia rat group, both higher than that in control group. In peripheral blood, the number of ST2 gene copies in IL-33 high dosage treated pneumonia rat group was significantly higher than that in IL-33 low dosage treated pneumonia rat group, and also higher than control group. This indicated that IL-33 treatment may promote the amplification of ST2 gene expression, which was higher in peripheral blood than in lung tissue.

Western blot analysis of apoptosis associated protein in neutrophils

Peripheral blood was collected in anti-clotted tubes from each experiment model group after treatment. Neutrophils were isolated and extracted using peripheral neutrophil isolation kit following the instructions. Neutrophils were then cultured. The detailed procedures were as follows. 1 ml fresh anti-clotted blood was mixed 1:1 with whole blood and tissue diluted buffer and centrifuged at 500 g (approximately 1800 rpm) for 25 min (with horizontal rotor diameter of 15 cm). Cells were separated into four lays in the centrifuge tube: 1st lay was plasma; 2nd layer was mononuclear cells in round shape milk color; 3rd layer was the slightly turbid isolation buffer rich in neutrophils; the 4th layer was red blood cells. The 1st layer plasma and the 2nd layer mononuclear cells were discarded. The 3rd layer isolation buffer and 4th lay RBC were collected in 10 ml test tube containing cell wash buffer. After thorough mixing, cells were centrifuged at 500 g (approximately 1800 rpm) for 30 min. The precipitants were washed once and the red blood cells were lysed using RBC lysis buffer. After another three times of washing, the debris of red blood cells was removed and the neutrophils in the precipitants were collected and cultured.
As shown in Figure 2, the expression of cell apoptosis factor caspase-3 and Bax in the neutrophils of peripheral blood was significantly higher in the rat pneumonia model group than that in other experiment groups; the expression of anti-apoptosis factor Bcl-2 was significantly lower than other experiment group, which meant rats with immunodeficiency experienced cell apoptosis after infected by A. baumannii. The expression of anti-apoptosis factor Bcl-2 in neutrophils in peripheral blood in IL-33 high dosage treated pneumonia rat group was higher than that in IL-33 low dosage treated pneumonia rat group, indicating that IL-33 treatment may have an inhibitory effect toward apoptosis. The expression of apoptosis factor caspase-3 and Bax was comparable in the two IL-33 treated groups, both higher than that in control group.

As shown in Figures 3 and 4, the expression level of ST2, NF-κB and TLR4 in lung tissue, alveolar lavage fluid and peripheral blood of IL-33 high dose treated A. baumannii pneumonia rats, was more close to that in control group rats, compared to A. baumannii pneumonia rats. This demonstrated that the treatment with IL-33 may provide longer survival time for A. baumannii pneumonia rats.

Apoptosis of neutrophils detected by annexin V-FITC and PI double staining

Neutrophils isolated from each experiment animal group were double stained with Annexin V-FITC and PI to differentiate the early stage apoptotic cells, late stage apoptotic cells and dead cells. Cells were detected immediately after labeling. Results are shown in Figure 5. The results of flow cytometry showed that the degree of apoptosis of A. baumannii pneumonia rats 24 h group was significantly higher than that in other experiment groups. The degree of apoptosis of IL-33 high dosage treatment 24 h group (D) was lower than that in IL-33 low dosage treatment 24 h group (C). This indicated that IL-33 might inhibit neutrophil apoptosis in A. baumannii pneumonia rats. The results were consistent with the Western Blot results of caspase-3, Bax and Bcl-2 expression in neutrophils.

ROS in neutrophils detected by flow cytometry

Twenty four hours after IL-33 treatment, animals were euthanized. The alveolar lavage fluid

Figure 6. ROS in Neutrophils in Alveolar Lavage Fluid. Note: A-C represent rat experiment groups.
IL-33 and acinetobacter baumannii pneumonia

Three rat experiment groups detected by flow cytometry showed that, A. baumannii pneumonia rats without IL-33 treatment exhibited the highest ROS level in neutrophils, indicating cell senescence and apoptosis. After treated with high dose of IL-33, the ROS level in neutrophils of A. baumannii pneumonia rats was greatly improved, which was less than half of that in group B. This indicated that IL-33 treatment can significantly relieve neutrophil senescence and apoptosis in A. baumannii pneumonia rats and beneficial for survival.

Discussion

IL-33 is a recently discovered inflammatory factor. IL-33 can bind to the complex of ST2 receptor and signal transduction protein AcP receptor located on the surface of cell membrane, pass the signal into cells and lead to a cascade effect [24, 25]. Bcl-2 protein is located in the mitochondria, endoplasmic reticulum and continuous perinuclear membrane, extensively expressed in embryonic tissues. Bcl-2 can prevent the release of cytochrome c from mitochondria into cytoplasm, inhibit transmembrane flow of calcium ions, and act as an ion channel protein and anchoring protein to inhibit cell apoptosis. Bcl2 and Bax can each form homodimers, which may also bind to each other and form heterodimers. The ratio of Bcl-2 and Bax in the heterodimers may determine the life or death of cells [19, 20]. NF-κB is an early stage transcription factor, reacting in response to the harmful stimulation to the cell. LPS located on the surface of gram-negative bacteria can activate downstream NF-κB signal pathway through TLR4 and regulate gene expression [23]. Reactive oxygen species (ROS) is a type of byproduct during biological aerobic metabo-

The results of ROS in alveolar lavage fluid and peripheral blood neutrophils from A, B and D inhibit transmembrane flow of calcium ions, and act as an ion channel protein and anchoring protein to inhibit cell apoptosis. Bcl2 and Bax can each form homodimers, which may also bind to each other and form heterodimers. The ratio of Bcl-2 and Bax in the heterodimers may determine the life or death of cells [19, 20]. NF-κB is an early stage transcription factor, reacting in response to the harmful stimulation to the cell. LPS located on the surface of gram-negative bacteria can activate downstream NF-κB signal pathway through TLR4 and regulate gene expression [23]. Reactive oxygen species (ROS) is a type of byproduct during biological aerobic metabo-

was collected and peripheral blood was collected. Neutrophils were isolated, cultured and labeled with fluorescence. 10 µM DCFH-DA was added to cell media and cells were cultured at 37 °C for 2 h. Cells were then collected and centrifuged at 1500 g for 6 min. Cells were washed once with PBS and centrifuged again at 1500 g for 6 min. Cell precipitants were collected and resuspended using PBS. Cellular ROS level was detected through fluorescence using flow cytometry with the optimal excitation wavelength of 500 nm and emission wavelength of 525 nm. Results are shown in Figures 6 and 7.

The results of ROS in alveolar lavage fluid and peripheral blood neutrophils from A, B and D
lism, including $O_2^-$, $H_2O_2$, $HO_2^-$, $OH$, etc. In cell biology studies, high concentration of ROS induces apoptosis by cellular oxidative stress and may even leads to necrosis. Studies of free radical biology discovered that ROS can regulate both of the apoptosis and proliferation of tumor cells, associated with cell signal transduction, and that low concentration of free radicals may interfere with a series of signal transduction pathway [14]. Free radicals in the body control the balance between cell life and cell death through the concentration. Studies of tissue regeneration have demonstrated that ROS is critical in activation of Wnt signal and plays an essential role in initiating and maintaining regeneration reactions.

In this study, Acinetobacter baumannii infected pneumonia rat model was established. Experiment animals were treated with low and high dose of IL-33 for 24 h. The expression of IL-33 receptor st2 gene in the lungs and peripheral blood was detected by QRT-PCR. Protein expression associated with apoptosis in the neutrophils was detected by Western blot. Cell apoptosis was detected by double staining flow cytometry. The results showed that IL-33 was able to elevate st2 gene expression in lung tissues and peripheral blood to some extent in the A. baumannii infected pneumonia rats, inhibit the expression apoptotic factor caspase-3 and Bax and in the mean time increase anti-apoptosis factor Bcl-2 expression. Accordingly, IL-33 extended the survival of neutrophils in A. baumannii infected pneumonia rats and made rats live better. However, further research is needed to investigate how IL-33 increases st2 gene copy number in A. baumannii infected pneumonia rats, how IL-33 passes the signal to caspase-3, Bax and Bcl-2 after binding to ST2 receptor, and the effect of IL-33 treatment on other organs.

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Disclosure of conflict of interest

None.

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