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Immunomodulatory effect and molecular mechanism of deoxyribonucleic acid receptor toll-like receptor 9 signaling pathway on newborn babies with acute lung injury

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ABSTRACT

This work was to demonstrate the immunomodulatory effect of toll-like receptor 9 (TLR9) signaling on newborn babies with acute lung injury (ALI) and the mechanism of TLR9 in vivo, so as to provide experimental basis for clinical treatment of newborn babies with ALI. Firstly, the expression of TLR9 in peripheral blood mononuclear cell (PBMC) was compared among ALI and healthy newborn babies. Then, PBMCs of newborn babies with ALI were extracted and divided into three groups. They were added with non-methylated cytosine purine-guanine dinucleotide sequence oligodeoxyribonucleotide (CpG ODN), ODN without non-methylated CpG, and blank nutrient solution, respectively, so as to determine the proliferation changes of PBMC. The immunohistochemistry (IHC) method was applied to detect the protein expression of TLR9-myeloid differentiation factor 88 (MyD88) signaling in lung tissue, and the number of T cell subsets ($CD3^+$, $CD4^+$, and $CD8^+$) was detected by flow cytometry. Besides, enzyme-linked immunosorbent assay (ELISA) was employed to determine the concentration of interferon-α (INF-α) and INF-γ. The results revealed a neglectable difference in TLR9 expression in PBMCs among ALI and healthy newborn babies ($P>0.05$). Additionally, the proliferation number of PBMC cultured in CpG ODN group was greatly superior to the number of ODN and blank groups ($P<0.05$), and the INF-α and INF-γ of CpG ODN group increased obviously versus those of blank and ODN groups ($P<0.05$). In conclusion, TLR9 in PBMCs was present in both ALI and healthy newborn babies. CpG ODN could specifically recognize the TLR9 signaling, so as to activate the immune function of T lymphocyte subsets in newborn babies with ALI and promote the release of inflammatory factors from the neonatal patient's immune cells, thereby mediating the immune response of neonatal patients.

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Introduction

Acute lung injury (ALI) is the damage of alveolar epithelial cells and capillary endothelial cells caused by various factors that lead to diffuse pulmonary interstitial and pulmonary edema, causing acute positive respiratory insufficiency (1). It is manifested as progressive hypoxemia and respiratory distress, and pulmonary imaging shows uneven permeability lesions, which can develop into acute respiratory distress in severe cases (2). According to statistics, the annual incidence of ALI can reach 0.08%. Its incidence resulted in different causes is also very different. In severe infection, its incidence can reach 25-50%, and multiple traumas can lead to its incidence up to 25%. When ALI is severe to a certain degree, it can develop into acute respiratory distress syndrome and has a higher mortality rate (3). At present, there is relatively few clinical research on neonatal ALI, and there is no uniform standard at home and abroad, and its diagnosis and treatment still adopt adult standards. As a natural pattern recognition receptor on the surface of important mammalian cells, TLRs can be expressed on neutrophils, macrophages, epithelial cells, endothelial cells, and other immune cells, and act in acquired immunity and natural anti-infection immunity. TLR9, as a DNA sensor, is a member of the TLRs family and is located in intracellular vesicles. It is mainly involved in the signal transduction of immune-stimulating sequences to activate immune cells. By participating in the recognition of the nucleic acid part of microorganisms, it can recognize CpG-ODN, cause T helper 1 (Th1)-like inflammatory response, activate B cells, promote the release of immune cells, and produce cytokines, so as to mediate immune function (4,5). T lymphocyte subsets are mainly responsible for cell immune function and have the effect of regulating the function of the immune system. Its function depends on the total value of T lymphocytes ($CD3^+$) and the relative composition of its subgroups ($CD4^+$ and $CD8^+$). $CD3^+$ is a mature T lymphocyte, which represents the body immune function status. $CD4^+$ is a vital hub for regulating the immune response, in which $CD8^+$ directly kills cells (6).

Therefore, there was an investigation of the mechanism of deoxyribonucleic acid (DNA) receptor TLR9 signaling on neonatal acute pneumonia tissue and its effect on immune cell function, which could provide a basis for clinical treatment of neonatal ALI.

Materials and Methods

Research materials

A total of 32 neonatal patients with ALI admitted to Southern University of Science and Technology Hospi-

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tal from January 2018 to May 2020 were recruited. The criteria for inclusion were defined to include neonatal patients who had clinical manifestations such as bruising and dyspnea, had chest X-rays with diffuse infiltrates of both lungs, were full of rales in both lungs and had PaO_2 with less than 6.67kPa and $PaCO_2$ with more than 6.67kPa. The criteria for exclusion were defined to include neonatal patients who suffered from congenital respiratory disease, cardiac insufficiency, myocardial injury, or other genetic diseases.

There were 18 male neonatal patients and 14 female neonatal patients, and 32 healthy newborn babies were selected as controls (16 males and 16 females). The age and gender differed slightly among neonatal patients with ALI from the two groups (P>0.05). The informed consent form had been signed by the families of all newborn patients, and the experiment had been approved by the Ethics Committee of Southern University of Science and Technology Hospital.

Detection of peripheral blood mononuclear cells

The peripheral anticoagulant blood was diluted with phosphate buffer. Under a microscope, the cells were counted, and the concentration was diluted to 10^6 cells/mL. Afterward, the lung injury cells were separated, the cells were counted and their concentration was adjusted, and the culture concentration of PBMC was adjusted to 2*106 cells/mL. Interleukin-2 (IL-2) was added into the culture medium to promote the proliferation of B cells and secretion of antibodies. The three mediums were added with CpG ODN, ODN, and blank nutrient solution in turn, and cultured for 2 days. The cells and supernatant were saved for later use. Then, Roswell Park Memorial Institute(RPMI)-1640culture solution was added with IL-2, and the separated PBMCs were placed in the culture solution. Next, the mixed solution was added with 10mg/L CpG ODN, placed in a 37°C incubator, and cultured for 3 days. The hydrogen3-thymine deoxyriboside (3H-TdR) incorporation method was adopted to detect the proliferation of PBMCs.

Detection of expression of TLR9-MyD88 signaling in lung tissue through Immunohistochemistry method

The lung tissues from neonatal sputum were collected and fixed with 10% paraformaldehyde solution for 24 hours. Then, they were embedded with paraffin and sectioned. The xylene and gradient ethanol were employed to dewax, and sodium citrate was used for antigen retrieval. The primary antibody was added according to the instructions of the ready-to-use immunohistochemical hypersensitivity kit, diaminobenzidine (DAB) was used for coloring, hematoxylin was adopted to counterstain, and heavy gum was used for sealing sections. In addition, the immunohistochemical staining score (IRS) = staining intensity (SI) × percentage of positive cells (PP), and there were observations from 5 fields of view under a light microscope for scoring.

Detection of immune cells and inflammatory factors

The inflammatory factors of neonatal patients were determined by ELISA. 2mL of peripheral venous blood was taken from each patient and added with heparin for anticoagulation. Then, it was placed at room temperature. Within 6 hours, the inflammatory factors, including INF-α and INF-γ, were detected based on the kit instructions. Flow cytometry was applied to measure T cell subsets, and fluorescein isothiocyanate (FITC)-labeled $CD3^+$, isophycocyanin-labeled $CD8^+$, and peridinin chlorophyll protein complex (PerCP)-labeled $CD4^+$ were added to PBMC to detect the number of T cell subsets ($CD3^+$, $CD4^+$, and $CD8^+$).

Statistical analysis

Using SPSS21.0, measurement data were indicated as $\bar{x}\pm s$, and *the* test was adopted for pairwise comparison. What's more, count data were denoted as percentages (%), which were tested by the χ^2 test. P<0.05 meant a statistically substantial difference.

Results and discussion

Chest X-rays of neonatal patients in the two groups

Figure 1 shows a chest X-ray of a normal newborn baby with symmetrical thoracic structures, normal lung field penetration, clear texture, and no abnormal density increase. Besides, Figure 2 indicated a chest X-ray of a neonatal patient with ALI, and its image presented patchy shadows in both lungs.

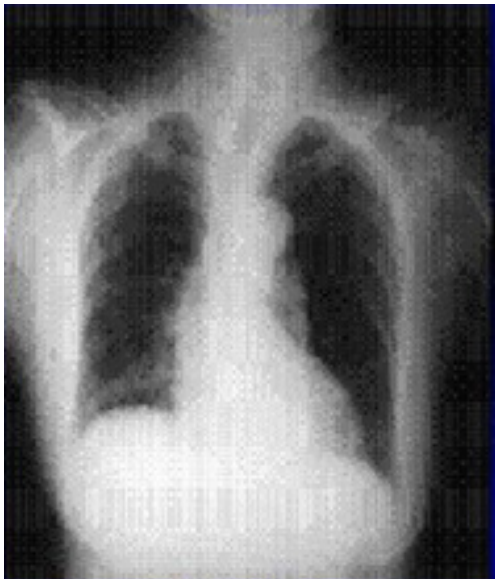

Figure 1. Chest X-ray image of one healthy newborn baby.

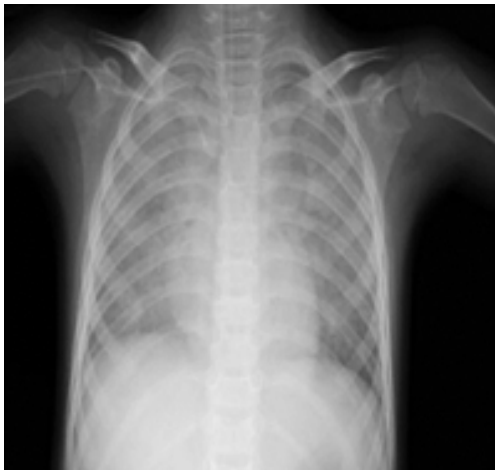

Figure 2. Chest X-ray image of one neonatal patient with ALI.

Results of TLR9 expression in peripheral blood mononuclear cells among neonatal patients

In Figure 3, the peripheral blood monitoring results of 32 neonatal patients and 32 healthy infants revealed that PBMC all expressed TLR9. Relative to experimental and blank groups, there was no obvious difference (P>0.05). It indicated that PBMC existed in both neonatal patients with ALI and healthy infants to express TLR9. Although the expression intensity was slightly different (P>0.05).

Effects of TLR9 on the number and proliferation of monocytes

The number of PBMC proliferation in the subjects from the three groups is shown in Figure 4 below. The number of PBMC proliferation in neonatal patients from the CpG ODN group was obviously superior to that of ODN and blank groups (P<0.05). Although there were slight differences in the number of PBMC proliferation in the subjects from ODN and blank groups (P>0.05). It suggested that CpG ODN was a specific nucleotide sequence with non-methylated CpG as the core, which could specifically bind to TLR9 and induce the proliferation of PBMC in neonatal patients with ALI.

Table 1 disclosed that the ratio of $CD69^+$ to $CD3^+$ in neonatal patients from the CpG ODN group was dramatically superior to the ratio of ODN and blank groups (P<0.05). However, no marked differences were indicated in the ratio of $CD69^+$ to $CD3^+$ of the subjects from ODN and blank groups (P>0.05). It showed that CpG ODN could specifically activate the function of PBMC and promote the expression of $CD69^+$ on the surface of $CD3^+$ molecules.

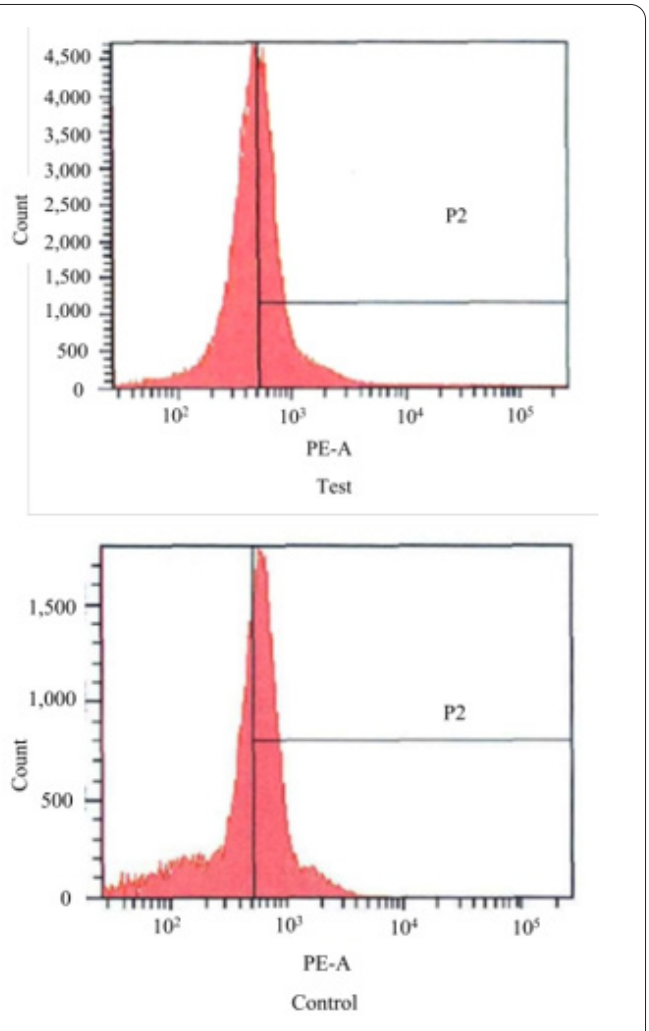

Figure 3. The expression intensity of TLR9 in PBMCs of the subjects from the two groups.

The proteinsofTLR9-MyD88 signaling

TLR9 and MyD88protein expression in neonatal patients from the CpG ODN group was dramatically superior to that of blank and ODN groups (P<0.05). It revealed that CpG ODN could activate the TLR9-MyD88 signaling transduction pathway, and TLR9 could trigger MyD88-dependent pathways, activate NF-κB, and induce the secretion of inflammatory factors. Moreover, TLR9 could specifically bind to non-methylated CpG-DNA and activate the signaling transduction pathway, and then, activate downstream molecules to promote the secretion of inflammatory factors, which was in line with the results of Muxel et al. (7) (Table 2).

Measurement results of interferon level

There was a comparison of the interferon concentration among the subjects from the three groups, as shown in Figure 5. It was found that INF-α and INF-γ in neonatal patients from the CpG ODN group increased markedly in contrast to those of blank and ODN groups, suggesting considerable difference (P<0.05). This was in accord with the findings of Seliga et al. (8).

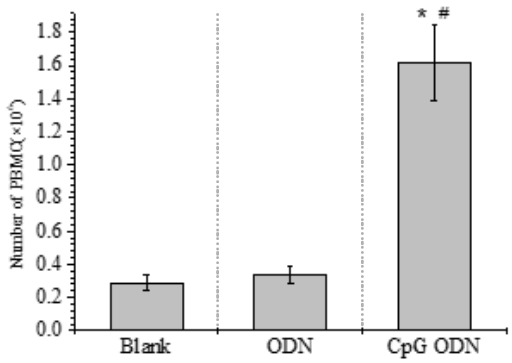

Figure 4. The number of monocyte proliferation in the subjects from the three groups. (Note: #P<0.05 vs. blank group; *P<0.05vs.ODN group.)

Table 1. PBMC function activation scale of the subjects from the three groups.

Group	$CD69^+$/ $CD3^+$
Blank group	8.9±0.9
ODN group	9.1±0.8
CpG ODN group	38.5±8.8*#
t	—
P	<0.05

Note: # P<0.05 vs. blank group; * P<0.05 vs. ODN group.

Table 2. IRS scores of ALI tissue in the subjects from the three groups.

Group	MyD88	TLR9
Blank group	1.81	1.21
ODN group	1.93	1.46
CpG ODN group	8.57*#	8.13*#
t	—	—
P	<0.05	<0.05

Note: # P<0.05 vs. blank group; * P<0.05 vs. ODN group.

INF is a type of glycoprotein, which is an inflammatory factor. It has a high degree of species specificity and can resist viruses, inhibit cell proliferation, and regulate immunity and anti-tumor effects. INF-α is an effective inducer of major histocompatibility complex class 1 (MHC-1) protein exposure. It regulates IL-1, IL-2, and tumor necrosis factor through the cytokine network, so as to promote the proliferation of cytotoxic T lymphocytes and activate immunocompetent cells. Besides, INF-γ is a vital immune regulatory factor in the body. It can improve the expression of MHC on the cell surface to convert constitutive proteasomes into immune proteasomes, make MHC-1 molecules better bind to $CD8^+$, and make MHC-2 molecules better combine with $CD4^+$, so as to enhance the sensitivity of T lymphocytes.

Measurement results of TLR9 for cellular immune function

As shown in Table 3 below, $CD4^+$ and $CD4^+/CD8^+$ of neonatal patients from the CpG ODN group rose dramatically but its $CD8^+$ dropped sharply in contrast to those of ODN and blank groups (P<0.05). Nevertheless, no obvious difference in $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ was suggested among the subjects from the blank and ODN groups, which was not statistically great (P>0.05). Blackwell et al. (9) noted the similar results.

$CD4^+$ is mainly responsible for inducing and helping T cells, and the increase of $CD4^+$ reflects that there is an immune response in the body. $CD8^+$ is mainly in charge of inhibiting T cells and killing toxic T cells. A decrease in $CD8^+$ means that a strong immune response has occurred. The ratio of $CD4^+/CD8^+$ can judge the sensitivity of clinical diagnosis of immune system disorders. When $CD4^+/CD8^+$ increases, it indicates that the cellular immune function is in an active state.

The expression of TLR9 on PBMCs was compared between neonatal patients with ALI and healthy infants. The lung tissues of neonatal patients with ALI were separated and the lung tissue cells CpG ODN were mediated, so as to detect the lung tissue TLR9-MyD88 pathway protein expression scores, levels of inflammatory factors INF-α and INF-γ, and changes of $CD4^+$ and $CD8^+$ cellular immune function. The results revealed no marked difference in the expression of TLR9 in PBMCs between neonatal patients with ALI and healthy infants. TNR9 could promote NF-κB to induce B cells and plasmacytoid dendritic cells through the MyD88-dependent pathway. The plasmacytoid cells were specifically combined with CpG ODN to promote the activation and proliferation of PBMC, increase the ratio of $CD4^+$ T cells in PBMCs, improve the production of INF-α, and enhance $CD8^+$ T cells to secrete INF-γ. Thereby, it would start a variety of signaling transduction pathways, activate downstream molecules, and regulate the immune cells. To sum up, the DNA receptor TLR9 could mediate the cellular immune function of neonatal patients with ALI, so as to regulate the immune response of patients by promoting the secretion of inflammatory factors.

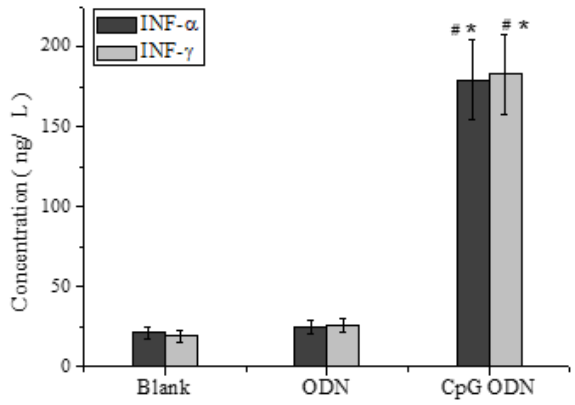

Figure 5. Comparison of INF of subjects. (Note: # P<0.05 vs. blank group; * P<0.05 vs. ODN group.)

Table 3. Comparison results on the immune function of lung tissue in the subjects from the three groups.

Group	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Blank group	42.46±5.37	12.48±3.24	3.39±0.22
ODN group	13.61±3.25	8.53±3.12	1.59±0.19
CpG ODN group	12.42±2.97	7.96±3.21	1.56±0.20

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