Research Paper

Toll-Like Receptor 9/Myeloid Differentiation Factor 88 Signal Pathway Mediates Immune Regulation of Neonatal Acute Lung Injury

YING TANG, LING CHEN, XIAO FENG AND FANGLIN YANG*

Department of Pediatrics, Zhongshan Hospital, Xiamen University, Xiamen, Fujian 361003, China

Tang *et al.*: Molecular Mechanism of Neonatal Acute Lung Injury

To study the molecular mechanism of immune regulation of toll-like receptor 9/myeloid differentiation factor 88 signal pathway in neonatal acute lung injury. Fifty-one newborn mice were divided into control group (n=17), model group (n=17) and experimental group (n=17). The model of acute lung injury in neonatal mice was established by intraperitoneal injection of kanamycin. The mice in the control group and model group were fed and treated normally, while the mice in the experimental group were treated with toll-like receptor 9 inhibitor chloroquine to inhibit toll-like receptor 9/myeloid differentiation factor 88 signal pathway. Compared with the model group, the degree of lung injury in the experimental group was alleviated after administration of toll-like receptor 9 inhibitor and the pathological score of lung tissue in the experimental group was lower than the model group, but higher than the control group. Compared with the model group, the toll-like receptor 9 and myeloid differentiation factor 88 messenger ribonucleic acid in the experimental group decreased. The toll-like receptor 9 and myeloid differentiation factor 88 in the model group model group was up-regulated after acute lung injury, while toll-like receptor 9 and myeloid differentiation factor 88 in the experimental group was decreased. Compared with the model group, the expression of toll-like receptor 9 and myeloid differentiation factor 88 protein in the experimental group decreased. Compared with the model group, the content of superoxide dismutase in the experimental group increased and the content of malondialdehyde decreased, the difference was significant. Compared with the model group, the levels of tumor necrosis factor-alpha and interleukin-6 in the lung tissue of the experimental group were down-regulated. The results show that toll-like receptor 9/myeloid differentiation factor 88 signaling pathway is a potential therapeutic target for inflammatory response in neonates with acute lung injury.

Key words: Toll-like receptor 9/myeloid differentiation factor 88 signaling pathway, neonatal acute lung injury, immune regulation, molecular mechanism

Neonatal Acute Lung Injury (NALI) is a common critical disease with high incidence and mortality, usually characterized by severe symptoms such as dyspnea, hypoxemia, pneumonia and inflammation[1]. It is one of the main causes of neonatal hemorrhagic shock, multiple organ dysfunction and death. The pathogenesis of NALI is not completely clear, nonetheless, immune control is regarded as one of the key contributors to its pathogenesis[2]. The mechanism of Acute Lung Injury (ALI) involves many factors, including infection, inflammation, oxidative stress and so on. Immune control is important for the incidence and progression of NALI, but its specific molecular mechanism is not clear. Toll-Like Receptor 9 (TLR9) is an important immunosensor receptor, which plays a key role in immune response. TLR9 participates in the regulation of various inflammatory responses through Myeloid Differentiation Factor 88 (MyD88)-mediated signaling pathways[3]. TLR9/MyD88 signaling pathway is an important regulatory pathway of inflammatory and immune response, which participates in the recognition

***Address for correspondence**
E-mail: kongjiaozhan050544@163.com

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms

Accepted 15 November 2023
Revised 10 July 2023
Received 19 January 2023
Indian J Pharm Sci 2023;85(6):1713-1718

and immune response of bacteria and viruses. Recent research revealed that the TLR9/MyD88 signaling pathway is crucial in the development of pediatric illnesses[4], but its specific regulatory mechanism in the occurrence of NALI has not been deeply studied. Therefore, in-depth study of the molecular mechanism of immune regulation is of great significance to understand the pathogenesis of NALI and find treatment strategies. The goal of this investigation is to explore the role of TLR9/MyD88 signal pathway in newborn NALI and its related molecular mechanism.

MATERIALS AND METHODS

Experimental animal:

Fifty-one newborn mice, male or female, were selected and purchased from the Institute of Model Animals, Nanjing University. The mice were reared in separate cages, the temperature range of the feeding environment was 20°~25°, the humidity range was 45°~65°, and the light was 12 h per day for 24 h to ensure the ventilation and heating conditions.

Construction and grouping of animal models:

Fifty-one newborn mice were divided into Control Group (COG) (n=17), Model Group (MOG) (n=17) and Experimental Group (EXG) (n=17). The ALI model of newborn mice was established by intraperitoneal injection of kanamycin in model group and experimental group. After intraperitoneal injection, the mice were placed back into the cage to allow free movement and intake of water and feed. The clinical symptoms, such as dyspnea, were observed within 24 h after injection. Then the mice in the COG and MOG were fed and treated normally, and the mice in the experimental group were treated with TLR9 inhibitor Chloroquine (CQ) to inhibit TLR9/MyD88 signal pathway.

Histopathological observation of lung:

Mice were injected with euthanasia to make them lose consciousness and stop breathing. Quickly open the mouse chest, lavage the chest with normal saline, empty the blood, and rinse the lung tissue with normal saline to remove blood and foreign bodies. Carefully remove the entire lung and place it in a formalin solution containing 10 % buffer formalin to soak it completely. The fixed lung tissue is removed from formalin, washed and dehydrated. The lung tissue was cut by a rotary slicer to prepare tissue sections with a thickness of 4-5 microns. Place the tissue section on the slide and dye it. To determine the severity of lung damage, tissue sections were examined under a microscope, and a semi-quantitative analysis was carried out using the Smith score. The score is mostly determined by the amount of atelectasis, inflammatory cell infiltration, alveolar bleeding, pulmonary interstitial edema, and alveolar edema, respectively. The criteria, normal is 0; lesion range is <25 %; score 1 and score 2 between 25 % and 50 %; 3 points between 50 % and 75 %; and 4 points when >75 %.

Real-time quantitative Polymer Chain Reaction (RT-qPCR):

The mouse lung tissue was taken out and put into the Ribonuclease (RNase)-free centrifuge tube, and the lung tissue samples were added to the cell fragmentation buffer, and the tissue cells were completely broken by cell breaker or ultrasonic crusher. According to the instructions of the kit, total Ribonucleic Acid (RNA) was extracted from broken lung tissue using phenol/chloroform or column RNA extraction kits. The total RNA extracted was reverse transcribed with reverse transcriptase and random primers to obtain complimentary Deoxyribonucleic Acid (cDNA). The cDNA produced by reverse transcription was mixed with primers TLR9, MyD88 and SYBR™ Green Master Mix to prepare the mixture of PCR reaction. The mixture of PCR reaction was added to the RT-qPCR instrument to carry out PCR reaction. The Ct value of each gene (the number of threshold periods) was calculated by real-time quantitative fluorescence signal curve recorded by PCR instrument. Using $2^{-\Delta\Delta Ct}$ method, the Ct value of each gene was normalized relative to the reference gene, and the relative gene expression level was calculated. The primers seen are shown in Table 1. The experimental results were statistically analyzed, and the differences of TLR9 and MyD88 gene expression levels in different groups were compared.

Immunofluorescence:

The fixed lung tissue was dehydrated, cleaned and impregnated, embedded in paraffin and made into thin sections. The slices were removed and added to distilled water for antigen repair. 5 %

Bovine Serum Albumin (BSA) was used to block the section, block the non-specific binding site, and incubate for 1 h. The first antibody against TLR9 or MyD88 was added to the sample and the incubation temperature was 4° overnight. The sections were washed with buffer or Phosphate-Buffered Saline (PBS), and the fluorescence-labeled second antibody was added to the section to bind specifically with the first antibody. Wash the slices with buffer or PBS to remove the unbound secondary antibodies. The nuclei were stained with fluorescent labeled DNA dye and the sections were sealed with anti-fading tablets. The sealed slices were observed under a fluorescence microscope or a confocal microscope, and the corresponding fluorescence images were taken at appropriate wavelengths.

Western blot:

The mouse lung tissue samples were transferred to the centrifuge tube, and the tissue was broken evenly with tissue fragmentation liquid Radio-Immunoprecipitation Assay (RIPA) buffer. The tissue breakage fluid was centrifuged by a centrifuge. The protein in the supernatant was diluted with protein loading buffer and the reductant beta-mercaptoethanol was added. Electrophoresis using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate the boiled sample, and then the protein was transferred to the polyacrylamide gel Polyvinylidene Difluoride (PVDF) membrane. Seal the PVDF membrane with 5 % skimmed milk powder or 1 % BSA solution. Specific antibodies to TLR9 and MyD88 were used to incubate the membrane and wash the membrane. Add mitoxin horseradish peroxidase which binds to specific antibody and wash membrane. Observed and recorded by X-ray film or imaging system.

Enzyme-Linked Immunosorbent Assay (ELISA):

The mouse lung tissue sample homogenize was put into the cell lysis buffer to destroy the cell structure and release protein. Centrifuge it and collect the supernatant. Prepare and mark the sample diluent and standard according to the instructions of the ELISA kit. Remove the orifice lath from the enzyme plate and add the diluted standard or sample. The detection antibody and enzyme-labeled secondary antibody were added. Put the orifice plate in the shaker or incubator for incubation. Pour out the liquid from the orifice plate and wash the orifice plate with washing buffer to remove non-specific reactants. Repeat this step 3-4 times. Add substrate solution and incubate for a period of time. The substrate combines with the enzyme-labeled secondary antibody to form a color product. Add the stop liquid to stop the color development process. The absorbance value was read by enzyme labeling instrument.

Statistical method:

Using Statistical Package for the Social Sciences (SPSS) 21.0 analysis, the data are expressed as ($\bar{x}\pm s$), t test is used for comparison between the two groups, variance analysis or rank sum test is used for comparison among the three groups, Chi-square (χ^2) test is used for comparison of counting data, and the difference is statistically significant.

RESULTS AND DISCUSSION

The lung tissue of the COG was intact, the alveolar structure was clear, and there was no injury, while the lung tissue of the MOG showed obvious characteristics of ALI. The degree of lung tissue injury and alveolar destruction were significantly improved in the EXG.

According to the semi-quantitative analysis of Smith score, it was found that the score of the MOG was higher than the COG, and the pathological score of lung tissue of the EXG was lower than the MOG, but higher than the COG as shown in Table 2.

Compared with the COG, the expression level of TLR9 and MyD88 mRNA in the MOG was up-regulated; compared with the MOG, the expression level of TLR9 and MyD88 mRNA in the EXG was lower, but higher than the COG as shown in Table 3.

There was only a small amount of expression of TLR9 and MyD88 in the lung tissue of neonatal mice in the COG, while the TLR9 and MyD88 in the MOG was up-regulated after ALI, while the TLR9 and MyD88 in the EXG was less than the EXG.

The TLR9 and MyD88 protein in the MOG was higher than the COG, while the TLR9 and MyD88 protein in the EXG was lower than the COG, but higher than the COG, as shown in Table 4.

TABLE 1: PRIMER SEQUENCES FOR qPCR

Gene		Sequence
TLR9	Forward	TGTCACCAGCCTTTCCTTGT
	Reverse	GCAGTTCCACTTGAGGTTGAGA
MyD88	Forward	CGCCTGTCTCTGTTCTTGAA
	Reverse	TCCGCTTGTCTCCAGTT

TABLE 2: COMPARISON OF PATHOLOGICAL SCORES OF LUNG TISSUE IN THREE GROUPS OF NEWBORN MICE

Group	n	Smith score
Control	17	0.32±0.14
Model	17	1.65±0.17
Experimental	17	1.04±0.16
F		305.06
p		<0.001

TABLE 3: COMPARISON OF TLR9 AND MYD88 mRNA IN THREE GROUPS OF NEWBORN MICE ($\bar{x}$±s)

Group	n	TLR9	MyD88
Control	17	1.03±0.04	1.02±0.05
Model group	17	6.17±0.76	5.97±0.43
Experimental	17	4.15±0.38	4.38±0.62
F		472.63	569.64
p		<0.001	<0.001

TABLE 4: COMPARISON OF PROTEIN EXPRESSION OF TLR9 AND MYD88 IN THREE GROUPS OF NEWBORN MICE ($\bar{x}$±s)

Group	n	TLR9	MyD88
Control	17	0.15±0.47	0.28±0.45
Model	17	0.68±0.73	0.75±0.52
Experimental	17	0.51±0.12	0.57±0.24
F		4.86	5.41
p		0.012	0.008

Compared with the COG, the content of Superoxide Dismutase (SOD) in the MOG decreased, while the content of propanediol (Malondialdehyde (MDA)) increased, compared with the MOG, the content of SOD in the EXG increased, but lower than the COG, and the content of MDA decreased but higher than the COG as shown in Table 5.

The expression of inflammatory factors Tumor Necrosis Factor-Alpha (TNF-α) and Interleukin-6 (IL-6) in the lung tissue of the MOG was higher than the COG, and the TNF-α and IL-6 in the lung tissue of the EXG was lower than the MOG, but higher than the COG as shown in Table 6.

NALI is a kind of lung disease that occurs after birth, which is common in premature infants or low birth weight infants. Pharmacologically, respiratory support, oxygen therapy and drug therapy are mainly used in the treatment of NALI[5]. Drug therapy can include supportive therapy, antibiotic therapy, diuretics, lung protective agents and immunomodulatory, etc. Supportive treatment includes correcting acid-base balance, maintaining

water-electrolyte balance, maintaining normal body temperature, etc.,[6]. Antibiotic therapy is mainly used to prevent and treat secondary infection. Diuretics can reduce pulmonary edema, and lung protective agents can protect lung tissue from further damage. The use of immunomodulatory may help to regulate inflammatory response and reduce lung injury[7]. Pathologically, the typical pathological features of NALI are alveolar wall thickening, inflammatory cell infiltration and emphysema. Injury can lead to alveolar wall injury and alveolar edema. The infiltration of inflammatory cells aggravates the injury of lung tissue and leads to the decline of lung function. Complications of NALI include pneumothorax, pneumonia, airway obstruction, asthma, circulatory failure, etc.,[8]. These complications will further aggravate lung injury and pose a great threat to the life and health of children. TLR9 and MyD88 are two key signaling molecules, which play an important role in immune regulation. TLR9 is a member of the TLR9 family. TLR9 can recognize specific structures in bacterial and viral DNA, such as CpG oligonucleotides[9]. When infection occurs, TLR9 binds to these oligonucleotides and activates downstream signaling pathways. In NALI, the activation of TLR9 will lead to the production of inflammatory factors and the activation of immune cells, resulting in inflammatory response and tissue injury[10]. MyD88 is an aptamer, which binds to TLR9 and activates downstream signal transduction molecules, such as Nuclear Factor Kappa B (NF-κB). The activation of MyD88 can initiate the inflammatory signal pathway and promote the production of inflammatory factors[11]. In NALI, the abnormal activation of MyD88 is related to the excessive release of inflammatory response. By regulating the expression and activity of MyD88, it can inhibit the production of inflammatory factors and reduce lung injury[12].

The findings demonstrate that TLR9 and MyD88 are crucial in the etiology of NALI and influence the severity of lung damage by controlling inflammatory cytokine and oxidative stress levels. Through histological analysis and pathological score of lung tissue, it was found that the degree of lung injury in the MOG was higher than the COG, while the degree of lung injury in the EXG was reduced after administration of TLR9 inhibitor. This suggests that inhibition of TLR9/MyD88 signal pathway can reduce the degree of NALI. The results of RT-qPCR showed that the TLR9 and MyD88 genes in the MOG were up-regulated, while those in the EXG were lower. The results of immunofluorescence and Western blot confirmed that the TLR9 and MyD88 proteins in the MOG were also up-regulated, while two proteins in the EXG was lower than the MOG. This indicates that TLR9/MyD88 signal pathway is important in the development of NALI, and the degree of lung injury can be reduced by inhibiting this signal pathway. Comparing the contents of SOD and MDA among the three groups, it was found that the SOD content of the MOG was lower, while the MDA content was higher, while the SOD content of the EXG was higher, while the MDA content was lower. This suggests that the inhibition of TLR9/MyD88 signal pathway can improve the oxidative stress response and reduce the lung injury caused by oxidative stress. ELISA results showed that the inflammatory cytokines TNF-α and IL-6 in the MOG were higher than the COG, while the inflammatory cytokines in the EXG were lower. This further confirms the importance of TLR9/MyD88 signaling pathway in the regulation of inflammatory cytokines. By inhibiting TLR9 signal pathway, the degree of lung injury can be reduced and the level of inflammatory factors can be reduced. In addition, the inhibition of this signal pathway can regulate oxidative stress response[13-15].

In summary, TLR9/MyD88 signaling pathway can affect the degree of lung injury by regulating the levels of inflammatory cytokines and oxidative stress. Therefore, TLR9/MyD88 signaling pathway may be a possible therapeutic target for inflammatory response in neonates with ALI.

TABLE 5: COMPARISON OF THE CONTENTS OF SOD AND MDA IN LUNG TISSUE OF THREE GROUPS

Group	n	SOD	MDA
Control	17	1.58±0.29	3.04±0.18
Model	17	1.02±0.15	3.63±0.32
Experimental	17	1.24±0.18	3.19±0.21
F		29.21	26.81
p		<0.001	<0.001

TABLE 6: COMPARISON OF INFLAMMATORY CYTOKINES TNF-α AND IL-6 IN THREE GROUPS OF NEONATAL MICE (x̄±s)

Group	n	TNF-α (ng/ml)	IL-6 (ng/ml)
Control	17	75.51±14.06	27.52±3.26
Model	17	194.72±12.95	65.78±6.83
Experimental	17	106.47±11.23	40.35±6.67
F		397.03	190.03
p		<0.001	<0.001

Conflict of interests:

The authors declared no conflict of interests.

REFERENCES

1. Ren Y, Chen M, Liu X, Feng B. Research progress of extracellular vesicle microRNA in acute lung injury. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 2021;33(5):633-7.
2. Upadhya S, Rehman J, Malik AB, Chen S. Mechanisms of lung injury induced by SARS-CoV-2 infection. Physiology 2022;37(2):88-100.
3. Rizzello C, Cancila V, Sangaletti S, Botti L, Ratti C, Milani M, *et al.* Intracellular osteopontin protects from autoimmunity-driven lymphoma development inhibiting TLR9-MYD88-STAT3 signaling. Mol Cancer 2022;21(1):215.
4. Kim KH, Jo MS, Suh DS, Yoon MS, Shin DH, Lee JH, *et al.* Expression and significance of the TLR4/MyD88 signaling pathway in ovarian epithelial cancers. World J Surg Oncol 2012;10(1):193.
5. Li J, Li X, Huang X, Zhang Z. Noninvasive high-frequency oscillatory ventilation as respiratory support in preterm infants: A meta-analysis of randomized controlled trials. Respir Res 2019;20(1):58.
6. Xie L, Huang W, Li J, Chen G, Xiao Q, Zhang Y, *et al.* The protective effects and mechanisms of modified Lvdou Gancao decoction on acute alcohol intoxication in mice. J Ethnopharmacol 2022;282:114593.
7. Fatima S, Bhaskar A, Dwivedi VP. Repurposing immunomodulatory drugs to combat tuberculosis. Front Immuno1 2021;12:645485.
8. Liu F, Peng W, Chen J, Xu Z, Jiang R, Shao Q, *et al.* Exosomes derived from alveolar epithelial cells promote alveolar macrophage activation mediated by miR-92a-3p in sepsis-induced acute lung injury. Front Cell Infect Microbiol 2021;11:646546.
9. Biswas SK, Tergaonkar V. Myeloid differentiation factor 88-independent Toll-like receptor pathway: Sustaining inflammation or promoting tolerance? Int J Biochem Cell Biol 2007;39(9):1582-92.
10. Wang M, Li L, Xiao S, Chen W, Hu F, Li F, *et al.* The association of TLR2, TLR3, and TLR9 gene polymorphisms with susceptibility to talaromycosis among han Chinese AIDS patients in Guangdong. Front Cell Infect Microbiol 2021;11:625461.
11. Jez M, Bas T, Veber M, Kosir A, Dominko T, Page R, *et al.* The hazards of DAPI photoconversion: Effects of dye, mounting media and fixative, and how to minimize the problem. Histochem Cell Biol 2013;139:195-204.
12. Chen Y, Que R, Lin L, Shen Y, Liu J, Li Y. Inhibition of oxidative stress and NLRP3 inflammasome by Saikosaponin-d alleviates acute liver injury in carbon tetrachloride-induced hepatitis in mice. Int J Immunopathol Pharmacol 2020;34:2058738420950593.
13. Cristóbal I, Santos A, Rojo F, García-Foncillas J. The PP2A pathway plays a crucial role in controlling cardiac physiology. Am J Physiol Heart Circ Physiol 2022;323(1):H65-6.
14. Miranda-Hernandez S, Gerlach N, Fletcher JM, Biros E, Mack M, Korner H, *et al.* Role for MyD88, TLR2 and TLR9 but not TLR1, TLR4 or TLR6 in experimental autoimmune encephalomyelitis. J Immunol 2011;187(2):791-804.
15. Tang Y, Xu Z, Chen X, Wang N, Deng X, Peng L, *et al.* Effects of enalapril on TLR2/NF-κB signaling pathway and inflammatory factors in rabbits with chronic heart failure. Evid Based Complement Altern Med 2021;2021(3):1-7.