

Dexamethasone alleviates allergic asthma immature rat through Toll like receptor 4

Z.-R. WANG¹, Q. WANG², Y. SUI¹, Z.-L. ZHANG¹, F.-J. JIA³, J. FAN², Z.-J. ZHANG⁴

¹Department of Pediatrics, Dezhou Pepole's Hospital, Dezhou, Shandong, China

²Department of Clinical Laboratory, Dezhou Pepole's Hospital, Dezhou, Shandong, China

³Department of Clinical Laboratory, Dezhou Hydropower thirteen bureau Hospital, Dezhou, Shandong, China

⁴Department of Ultrasonography, Dezhou Pepole's Hospital, Dezhou, Shandong, China

Abstract. – OBJECTIVE: The allergic asthma model induced by ovalbumin (OVA) was established in the immature rat. Dexamethasone (DXM) was adopted for intervention to analyze the treatment effect and to explore the relationship with toll-like receptor 4 (TLR4).

MATERIALS AND METHODS: Immature SD rat was treated by OVA to construct allergic asthma model and intervened by DXM. The rats were randomly divided into model group, experimental group, and control group. The changes in lung tissue were observed by light microscope. The EOS infiltration and reactivity of airway wall were compared. The expressions of TLR2 and TLR4 protein and mRNA in the lung tissue were tested by Western blot and RT-PCR.

RESULTS: The lung tissue in the model group was infiltrated by a lot of inflammatory cells, and mucous membrane edema was observed, compared with that in the control group. There were only a few inflammatory cells in the interstitial tissue and pulmonary alveoli in the experimental group compared with that in the model group. EOS count of airway wall and airway reactivity decreased in the experimental group. The levels of TLR2 and TLR4 were significantly elevated in the third week compared with the first week ($p < 0.05$).

CONCLUSIONS: The treatment of DXM can alleviate the pathological changes of the lung tissue in SD immature rat with allergic asthma, reduce EOS infiltration in the airway wall, decrease airway reactivity, and elevate expressions of TLR2 and TLR4.

is accounted for cell injury and inflammation¹. It is generally considered that the main pathogenesis of childhood asthma is associated with eosinophils (EOS)-induced airway inflammation². Toll-like receptor (TLR) belongs to the family of pattern recognition receptor that can timely recognize the pathogen-associated molecular pattern and further modulates the activation of the immune response of host. TLR contains a large category of molecular models and exists in various sorts of cells^{3,4}. The current study found that there are 11 types of TLR in the mammalian. All of them are characterized by biological function of pattern recognition receptor and participate in the regulation of multiple signaling pathways in the body⁵. Neutrophils, mast cells, and macrophages are stimulated by infection, immune, or inflammation may secrete cytokines, leading to the imbalance of Th1/Th2 immune response that deviates to Th2⁶. At present, the application of glucocorticoid is still the most effective reagent to treat asthma in clinic. Dexamethasone (DXM) is a kind of immunosuppressant and exerts to anti-inflammatory function⁷. However, it is still lack of report about the mechanism of glucocorticoid in regulating immune response. This study established allergic asthma model with immature rat and determined the curative effect of DXM and its relationship with TLR4.

Key Words

Dexamethasone, Allergic asthma, TLR4.

Introduction

Bronchial asthma is a type of common respiratory disease in children associated with multiple cells and components. Its pathological basis

Materials and Methods

Experimental Animals

A total of sixty female SD rats at three weeks old were bought from Shandong animal experiment center, Chinese Academy of Sciences (Jinan, Shandong, China). The rats were raised with temperature at 21 ± 1 °C, relative humidity at 50-70%, and 12 h day/night cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Dezhou People's Hospital.

Main Reagents

Ovalbumin (OVA) and DXM were got from Sigma (Temecula, CA, USA). TLR2 and TLR4 *in situ* hybridization kits were obtained from Boster (Wuhan, Hubei, China).

Experimental Methods

Ova Allergic Asthma Model

Establishment

The rats were randomly equally divided into three groups. The rats were intraperitoneally injected with 0.5 ml normal saline containing 10 µg OVA and 1 mg Al(OH)₃ on the first day. Next, the rats were treated by 0.5 ml normal saline containing 10 µg OVA aerosol for 30 min inhalation. At last, the rat received 50 µl OVA aerosol via inhalation at 2 mg/ml for continuous fifteen days.

Experimental group: The rat received 1 ml normal saline containing 100 µg DXM via intraperitoneal injection at 8 am every day for continuous three weeks.

Model group: The rat received 1 ml normal saline by intraperitoneal injection at 8 am every day for continuous three weeks.

Control: The healthy rat received equal amount of normal saline during model rat establishment, and then received 1 ml normal saline by intraperitoneal injection at 8 am every day for continuous three weeks.

Airway Reactivity Measurement

The trachea was routinely incised and connected to the T-branch pipe. One pipe was connected to the breathing machine, while the other was connected to piezometer tube. The PBS was

injected to the trachea cannula with 0.01 g/L histamine. The pressure was monitored.

Sample Collection

The rat was fixed on the table and the eyelash was cut off. A capillary tube was vertically inserted to the inner canthus to collect the blood. The head of the SD rat was inverted to obtain more blood. At last, the blood was stored at -80 °C and the lung tissue was collected and saved at -80 °C.

Lung Tissue Morphology Changes

The lung tissue was embedded and sectioned. After stained by hematoxylin and eosin, the slice was dehydrated and hyalinized to be observed under the microscope.

Airway Wall EOS Counting

The airway wall with integrated structure was observed under the microscope. Mean number of EOS on the airway wall within five visual fields were counted.

Western Blot

Total protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blocked. Next, the membrane was incubated with primary antibody (TLR2 and TLR4 1:200, β-actin 1:500) for 30 min and then incubated in secondary antibody (1:2000) for 1 h (Abcam, Cambridge, MA, USA). After developed by chemiluminescence kit (Bio-rad, Hercules, CA, USA), the result was analyzed.

RT-PCR

Total RNA was extracted from the lung tissue. The RNA was reverse transcribed to cDNA for PCR amplification (TaKaRa, Kusatsu, Shiga, Japan). The primers used were listed in Table I. The PCR reaction contained 95 °C for 5 min, followed

Table I. Primers sequences.

Gene	Sequence	Product length
TLR2		370bp
Forward	5'-AAA CGG TAA CAA TAC GGA G-3'	
Reverse	5'-TGA CAA CTG TCG GGC ATA-3'	
TLR4		410bp
Forward	5'-CAG AGC CGT TGG TGT ATC-3'	
Reverse	5'-CCC TGT GAG GTC GTT GA-3'	
U6		150bp
Forward	5'-AGT TGC GTT ACA CCC TTT C-3'	
Reverse	5'-CAC CTT CAC CGT TCC AGT-3'	

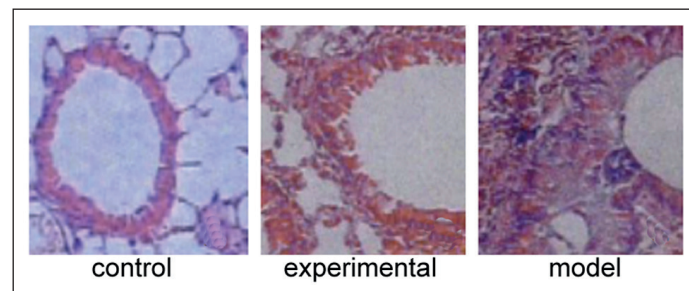


Figure 1. Lung tissue morphological changes (×400). **A**, Control. **B**, Experimental group. **C**, Model group.

by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and 72 °C for 10 min at last.

Data Analysis

SPSS 17.0 software was applied for statistical analysis. The enumeration data were compared by chi-square test, while the measurement data among groups were compared by one-way ANOVA followed by Fisher's LSD tests. $p < 0.05$ was considered as statistical significance.

Results

Lung Tissue Observation Under the Microscope

A large amount of inflammatory cells were found infiltrating in bronchus, alveolar space, and blood vessel of the rats in the model group. Edema and ruffle were shown in the mucosa. Epithelial cells fell off, together with a lot of mucus plugs. However, in the experimental group, there were relatively few inflammatory cells located in the bronchus, interstitial tissue, and alveolar space. No inflammatory cells infiltration was found in control (Figure 1).

Airway wall EOS Infiltration and Airway Reactivity Comparison

The EOS count of airway wall was significantly elevated in model group compared with control, whereas EOS count and airway reactivity were decreased in the experimental group compared with the model group ($p < 0.05$) (Table II).

Table II. Airway wall EOS infiltration and airway reactivity comparison ($\bar{x} \pm s$).

Group	EOS(/HP)	PC ₂₀
Control	3.24±1.11	0.446±0.010
Experimental group	63.21±4.11 ^{*#}	0.102±0.006 ^{*#}
Model group	75.45±6.42 [#]	0.036±0.011 [#]

TLR2 and TLR4 Protein Expressions Comparison

TLR2 and TLR4 protein levels were higher in experimental group than those in model group and control. Moreover, their expressions were markedly higher in the third week compared with that in the first week ($p < 0.05$) (Figure 2-3).

TLR2 and TLR4 mRNA Expressions Comparison

We also detected the expressions of TLR2 and TLR4 at mRNA levels. Our data showed

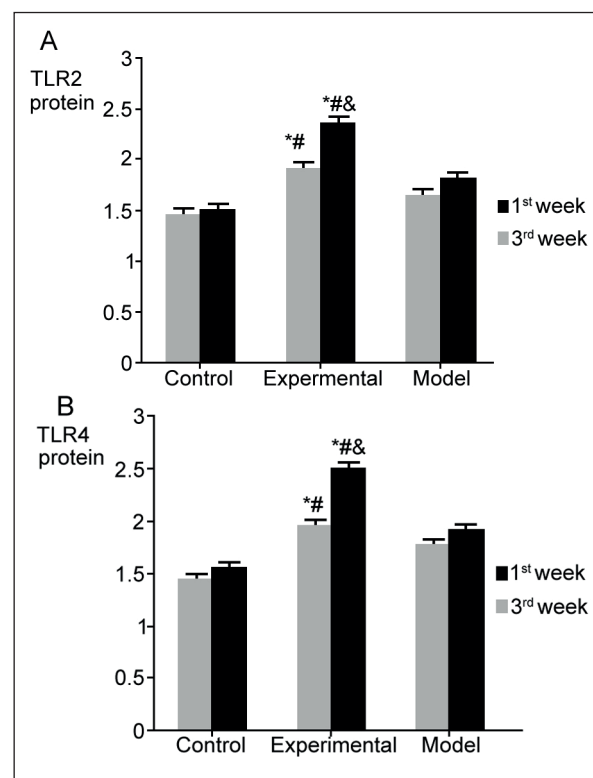


Figure 2. TLR2 and TLR4 protein expressions comparison. **A**, TLR2 protein. **B**, TLR4 protein. ^{*} $p < 0.05$, compared with model group. [#] $p < 0.05$, compared with control. [&] $p < 0.05$, compared with the first week.

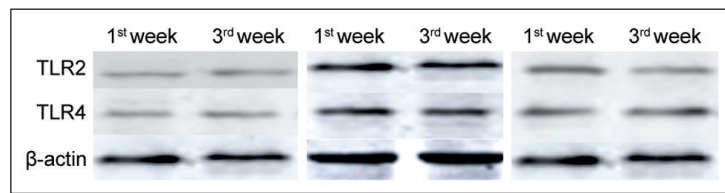


Figure 3. TLR2 and TLR4 protein expressions. **A**, Control. **B**, Experimental group. **C**, Model group.

that similar to the protein expressions, the levels of TLR2 and TLR4 mRNA were also apparently higher in experimental group than those in model group and control, the expressions of which were significantly higher in the third week compared with the first week ($p < 0.05$) (Figure 4).

Discussion

Asthma is a common chronic airway inflammatory disease in clinic. Its incidence has been gradually increasing in children recently. The epidemiologic study proposed that the reduction of microbes exposure may be one of the important causes of asthma. It is confirmed that a large amount of inflammatory cells and components are involved in the occurrence of asthma^{8,9}. The pattern recognition function of innate immune system triggers the T cell-mediated immune response in the body, while TLR belongs to one type of PRR in innate immune system. It is pointed out that TLRs have a critical role in the pathogenesis of asthma by bridging the innate immunity to acquired immunity¹⁰. Up to now, there are 13 types of TLR found by the researchers. Among them, only TLR2 and TLR4 can trigger transcription factor translocation to activate immune-related genes and induce inflammatory response, which is associated with TLR level¹¹. External stimuli activate TLR to trigger downstream molecules, resulting in the occurrence and development of inflammation^{12,13}. Researchers suggested that TLR elicits the release of inflammation factors and aggravates the inflammatory response¹⁴.

Asthma mainly includes airway inflammation and reconstruction, which are different from other upper respiratory tract diseases¹⁵. In this study, OVA was used to establish animal model of allergic asthma. The rats in the model group present severe inflammatory response with a large amount of inflammatory cell infiltration in bronchus, alveolar space, and blood vessel. However, the treatment of DXM remarkably alleviated the pathological changes of the lung tissue, reduced

EOS infiltration in airway wall, and decreased airway reactivity caused by allergic asthma, along with the rise of TLR2 and TLR4 expressions.

Macrophages play the fastest biological function in the first line of defense against the pathogenic infection. As TLR2 and TLR4 participate in the innate immunity, their mRNA mostly express in macrophages^{16,17}. Once the specific pathogenic pattern was recognized by TLR2 or TLR4, macrophages are activated to release a large amount of proinflammatory factors, leading to the activation of the innate immune and acquired immune response¹⁸. Consistently, this study found that TLR2 and TLR4 protein and mRNA

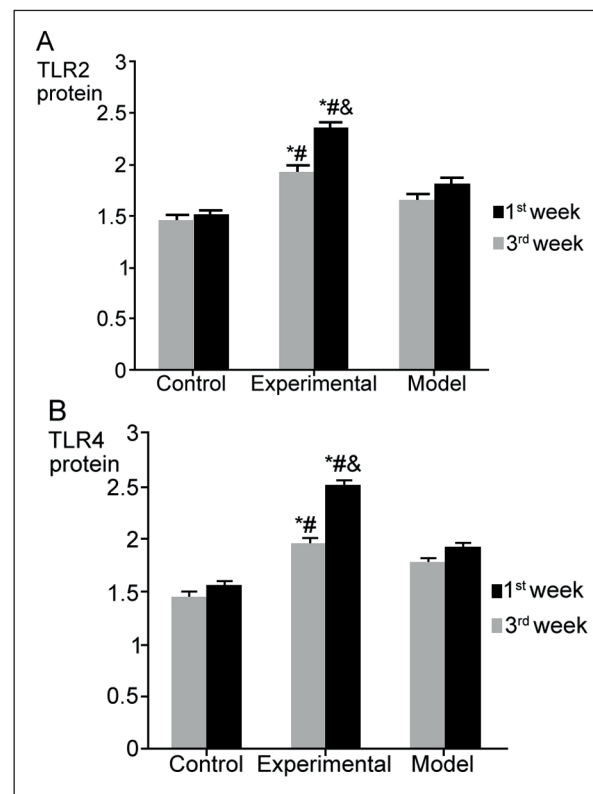


Figure 4. TLR2 and TLR4 mRNA expressions comparison. **A**, TLR2 protein. **B**, TLR4 protein. * $p < 0.05$, compared with model group. # $p < 0.05$, compared with control. & $p < 0.05$, compared with the first week.

levels were significantly higher in experimental group than those in model group and control, and increased in a time-dependent manner. Kumar et al¹⁹ proposed that serum TLR2 and TLR4 levels were significantly higher in children with asthma, especially in acute phase than that in children at remission phase or healthy children. Moreover, TLR4 exhibited certain regulatory effect in promoting the release of proinflammatory factors, such as interleukin. Accumulative evidence found that IL-6 and TNF- α expressions were decreased after the expression of TLR4 was inhibited^{20,21}. It showed that the impact of TLR4 on asthma may be related to the expression of IL-6 and TNF- α . DXM can enhance TLR2 expression in the airway epithelial cells under the effect of interferon γ . The previous study indicated that DXM can suppress TLR4 mRNA expression and elevate TLR2 mRNA level in the spleen cells^{22,23}. It was pointed out that DXM can improve the airway remodeling in the lung tissue via the downregulation of TLR4 expression. TLR activation in the body can induce Th1 immune response. As a common and powerful immunosuppressant, DXM plays an effective role in anti-inflammation, suppressing immune response, regulating TLR2 and TLR4 expressions, and controlling the balance of Th1/Th2 immune response in asthma. Intriguingly, our result demonstrated that DXM remarkably relieved the symptom of asthma through activation of TLR2 and TLR4, although its specific usage and dosage still need further investigation. This work provides theoretical basis for the clinical application of DXM in asthma, while further in-depth investigation is needed to clarify its specific mechanism.

Conclusions

We showed that the treatment of DXM can alleviate lung tissue pathological changes, reduce EOS infiltration, impede airway reactivity, and induce TLR2 and TLR4 expressions in the allergic asthma SD rat.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- 1) MALMHALL C, BOSSIOS A, RÄDINGER M, SJÖSTRAND M, LU Y, LUNDBÄCK B, LÖTVALL J. Immunophenotyping of circulating T helper cells argues for multiple functions and plasticity of T cells *in vivo* in humans--possible role in asthma. *PLoS One* 2012; 7: e40012.
- 2) RAHMAN MS, YAMASAKI A, YANG J, SHAN L, HALAYKO AJ, GOUNNI AS. IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J Immunol* 2006; 177: 4064-4071.
- 3) LIU Y, ZHU L, FATHEREE NY, LIU X, PACHECO SE, TATEVIAN N, RHOADS JM. Changes in intestinal Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* 2009; 297: G442-450.
- 4) LI GX, LN INCOMPLETE. Toll like receptors on intestinal barrier is protection or damage. *Chin J Gastrointest Surg* 2009; 12: 540-541.
- 5) CHUNG SH, CHOI SH, CHO KJ, JOO CK. Toll-like receptor 4 signaling attenuates experimental allergic conjunctivitis. *Clin Exp Immunol* 2011; 164: 275-281.
- 6) GRIBAR SC, SODHI CP, RICHARDSON WM, ANAND RJ, GITTES GK, BRANCA MF, JAKUB A, SHI XH, SHAH S, OZOLEK JA, HACKAM DJ. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. *J Immunol* 2009; 182: 636-646.
- 7) BIELINSKA AU, GERBER M, BLANCO LP, MAKIDON PE, JANCZAK KW, BEER M, SWANSON B, BAKER JR JR. Induction of Th17 cellular immunity with a novel nanoemulsion adjuvant. *Crit Rev Immunol* 2010; 30: 189-199.
- 8) HONGJIA L, QINGLING G, MEIYING L, WEIXUAN W, LIHONG Z, YONGSHENG G, YANLI L, JINXIANG W, LIANG D. House dust mite regulate the lung inflammation of asthmatic mice through TLR4 pathway in airway epithelial cells. *Cell Biochem Funct* 2010; 28: 597-603.
- 9) WILSON RH, MARUOKA S, WHITEHEAD GS, FOLEY JF, FLAKE GP, SEVER ML, ZELDIN DC, KRAFT M, GARANTZIOTIS S, NAKANO H, COOK DN. The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nat Med* 2012; 18: 1705-1710.
- 10) KLEIN KLOUWENBERG P, TAN L, WERKMAN W, VAN BLEEK GM, COENJAERTS F. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. *Crit Rev Immunol* 2009; 29: 531-550.
- 11) ZHANG W, GU Y, CHEN Y, DENG H, CHEN L, CHEN S, ZHANG G, GAO Z. Intestinal flora imbalance results in altered bacterial translocation and liver function in rats with experimental cirrhosis. *Eur J Gastroenterol Hepatol* 2010; 22: 1481-1486.
- 12) DAVICINO RC, ELICABE RJ, DI GENARO MS, RABINOVICH GA. Coupling pathogen recognition to innate immunity through glycan-dependent mechanisms. *Int Immunopharmacol* 2011; 11: 1457-1463.
- 13) AKIRA S. Pathogen recognition by innate immunity and its signaling. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; 85: 143-156.
- 14) CHAMBERLAIN ND, VILA OM, VOLIN MV, VOLKOV S, POPE RM, SWEDLER W, MANDELIN AM, 2ND, SHAHRARA S.

- TLR5, a novel and unidentified inflammatory mediator in rheumatoid arthritis that correlates with disease activity score and joint TNF-alpha levels. *J Immunol* 2012; 189: 475-483.
- 15) MANSSON KVARNHAMMAR A, TENGROTH L, ADNER M, CARDELL LO. Innate immune receptors in human airway smooth muscle cells: activation by TLR1/2, TLR3, TLR4, TLR7 and NOD1 agonists. *PLoS One* 2013; 8: e68701.
 - 16) CHEN K, XIANG Y, YAO X, LIU Y, GONG W, YOSHIMURA T, WANG JM. The active contribution of Toll-like receptors to allergic airway inflammation. *Int Immunopharmacol* 2011; 11: 1391-1398.
 - 17) KLAASSEN EM, THÖNISSEN BE, VAN EYS G, DOMPELING E, JÖBSIS O. A systematic review of CD14 and toll-like receptors in relation to asthma in Caucasian children. *Allergy Asthma Clin Immunol* 2013; 9: 10.
 - 18) LEE WI, YAO TC, YEH KW, CHEN LC, OU LS, HUANG JL; PATCH Study Group. Stronger Toll-like receptor 1/2, 4, and 7/8 but less 9 responses in peripheral blood mononuclear cells in non-infectious exacerbated asthmatic children. *Immunobiology* 2013; 218: 192-200.
 - 19) KUMAR V, SHARMA A. Mast cells: emerging sentinel innate immune cells with diverse role in immunity. *Mol Immunol* 2010; 48: 14-25.
 - 20) SATURNINO SF, PRADO RO, CUNHA-MELO JR, ANDRADE MV. Endotoxin tolerance and cross-tolerance in mast cells involves TLR4, TLR2 and FcepsilonR1 interactions and SOCS expression: perspectives on immunomodulation in infectious and allergic diseases. *BMC Infect Dis* 2010; 10: 240.
 - 21) YAO H, CAI ZY, SHENG ZX. NAC attenuates adriamycin-induced nephrotic syndrome in rats through regulating TLR4 signaling pathway. *Eur Rev Med Pharmacol Sci* 2017; 21: 1938-1943.
 - 22) CAMATEROS P, TAMAOKA M, HASSAN M, MARINO R, MOISAN J, MARION D, GUIOT MC, MARTIN JG, RADZIOCH D. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am J Respir Crit Care Med* 2007; 175: 1241-1249.
 - 23) HOMMA T, KATO A, HASHIMOTO N, BATCHELOR J, YOSHIKAWA M, IMAI S, WAKIGUCHI H, SAITO H, MATSUMOTO K. Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *Am J Respir Cell Mol Biol* 2004; 31: 463-469.