微生物学报 *Acta Microbiologica Sinica*
49(2):239 – 245;4 February 2009
ISSN 0001 – 6209;CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn

Upregulation of TLR7 and TLR3 gene expression in the lung of respiratory syncytial virus infected mice

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Abstract: [Objective] TLR7 and TLR3 (Toll-like receptor, TLR) are important pattern recognition receptors (PRRs) that recognize the single-strand RNA and double-strand RNA of virus-origin. Respiratory syncytial virus (RSV) could be recognized by both TLR7 and TLR3. We studied the kinetics and profile of lung TLR7 and TLR3 expression during the early phase of the RSV infection, and explored their expression correlation with pulmonary inflammatory response. [Methods] We intranasally inoculated BALB/c mice with live RSV to induce acute lung inflammation, and detected the lung expression of TLR7 and TLR3 mRNA by semiquantitative RT-PCR analysis at day 1 after RSV infection. We also measured the transcription factor nuclear factor-kappaB (NF-κB) protein expression with western blot assay in nuclear extracts. Lung tissue sections were stained with hematoxylin and eosin, and examined under a light microscope for histopathological examination. [Results] SV infection could rapidly upregulate the lung expression levels of both TLR7 and TLR3 gene in a time-dependent manner. Furthermore, the response of TLR7 to RSV (1 h) was prior to that of TLR3 (4 h). It resulted in activation of NF-κB as soon as 4 h later in lung tissue. Moreover, RSV mediated early transcriptional responses of TLR7 and TLR3 were paralleling with the severe extent of RSV-induced pulmonary inflammation. [Conclusion] TLR7 and TLR3 were really involved in RSV-induced lung inflammation by detecting the viral RNA. This may allow to detect viral infections and initiate a proinflammatory response via multiple TLRs. This study may be useful in the development of tools to modulate the activities of therapeutic TLR ligands.

Keywords: toll-like receptor; respiratory syncytial virus; gene expression; inflammation; lung
CLC number: R392 **Document code**: A **Article ID**: 0001-6209(2009)02-0239-07

Toll-like receptors (TLRs) are newly discovered pattern recognition receptors (PRRs) binding microbial determinants called pathogen-associated molecular patterns (PAMPs). Currently, 13 TLRs (TLR1-13) have been identified[1]. Ligand engagement of TLR, and subsequently activation of NF-κB, leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity. The ligands for TLR7 and TLR3 were mainly considered to be viruses. Originally, two imidazoquinoline compounds, imiquimod and resiquimod (or R-848), were shown to activate murine macrophages in a TLR7-dependent manner[2]. More recently TLR7 was found to recognize the single-stranded (ss) RNA viruses, such as influenza virus and HIV[3-4]. TLR3 had been used to detect the double-stranded (ds) RNA of virus-origin, which could either be the genomic material of viruses themselves or derived from the replicative intermediates produced in the course of an ssRNA viral infection[5-6]. Therefore, respiratory syncytial virus (RSV), a single-stranded RNA virus, could be recognized by both TLR7 and TLR3, which then triggered the TLR-dependent response in lung and induced the inflammatory cascade resulting in enhanced lung pathology. TLR7 and TLR3 have already been demonstrated to associate with improving the lung microenvironment caused by RSV infection[7]. RSV, on

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Received: 18 July 2008/ Revised: 24 November 2008

the other hand, can interfere with the induction of the host response by modulating TLR3 and TLR7 signaling and virus induced IFN responses[8-11]. So, TLR7 and TLR3 may play important roles in the process of RSV infection. But no evidence in vivo was simultaneously described for the early expression patterns of TLR7 and TLR3, as well as NF-κB activation in the response of mouse lung to acute RSV infection. In this paper, using a mouse model, we will study the early transcript changes of TLR7, TLR3 and NF-κB activation during RSV inoculation, and the relationship between these two TLRs expression and lung inflammation.

1 MATERIALS AND METHODS

1.1 Materials

RSV-Long strain was gifted from CDC of HuBei Province, China. Female BALB/c mice were purchased from Shanghai (Shanghai SLAC Laboratory Animal Co. LTD, China). Trizol reagent for RNA extraction and all primers were purchased from Invitrogen Co. (USA). Reverse transcription (RT) kit was obtained from Promega Co. (USA). Specific mouse monoclonal antibody anti-NF-κBp65 and anti-C23 (nucleolin) were products of Santa Cruz Biotechnology, Inc. (USA). Enhanced chemiluminescence (ECL) kit was purchased from Pierce Co. (USA).

1.2 RSV preparations

The RSV-Long strain was grown on HeLa cells and purified by polyethylene glycol precipitation, followed by centrifugation on 35% - 65% discontinuous sucrose gradients as described previously[12]. Viral pools were aliquoted, quick frozen, and stored at -80℃ until used. The viral titer of purified RSV pools was 10^7 PFU/mL, determined by a methylcellulose plaque assay in HeLa cells.

1.3 Mice and experimental protocol

Female 6-to 8-week-old BALB/c mice (n = 8 per group) were housed in specific pathogen-free environment. Each mouse was slightly anesthetized and inoculated intranasally with 10^7 PFU/mL of RSV in 0.1 mL. After 0, 1, 4, 8, 16 and 24 h of RSV infection, mice were sacrificed and the lung tissues were harvested for experiments. Control mice were exposed to the same volume of PBS. Equal amount of lung tissue in weight was used for RNA extraction.

All animals' treatments were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their sufferings.

1.4 Semiquantitative RT-PCR

Total RNA from lung tissue was extracted by using Trizol reagent according to the manufacturer's instructions. Reverse transcription (RT) of 1 μg RNA into cDNA was performed with oligo (dT) 15 primer, AMV reverse transcriptase and 1xPCR buffer. The cDNA was amplified by PCR (Mycycler, Bio-Rad, American) in a total volume of 25 μL. Reaction mixtures were heated at 94℃ for 5 min, and PCR amplification for TLR7 was performed with 34 cycles of 45sec at 94℃, 40sec at 57℃, and 1 min at 72℃. The PCR amplification for TLR3 was performed with 34 cycles of 45sec at 94℃, 40sec at 56℃, and 1 min at 72℃. All reactions were subjected to a final extension at 72℃ for 10 min. As an internal control, mouse β-actin cDNA was also amplified for 28 cycles with annealing temperature at 55℃.

Specific primers for mouse TLR7, TLR3 and β-actin were used for RT-PCR analysis as shown in Table 1. The table 1 also showed the Refgene numbers used for the primers designed with DNASTAR software by ourselves.

PCR products were electrophoresed through a 1.5% agarose gel and stained with ethidium bromide. The density of the bands was quantitated with a Labworks software imaging densitometer. Densitometry was normalized to β-actin expression and was presented as the fold increase in mRNA expression, i.e., the ratio of experimental to control.

Table 1 Nucleotide sequences of the primers used for RT-PCR

Primer	Sequence (5′→3′)	RefGene accession No.	Size/bp
TLR3	TGGATTCTTCTGGTGTCTTCC (upper)		
	AGTTCTTCACTTCGCAACGC (lower)[13]		521
TLR7	CCACCAGACCTCTTGATTCC (upper)		
	TCCAGATGGTTCAGCCTA GG (lower)[13]		315
β-actin	GTGGGCCGCTCTAGGCACCAA (upper)		
	CTCTTTGATGTCACGCACGATTTC (lower)	GI49867	540

1.5 Western blot analysis

For immunodetection of NF-κB, we examined the NF-κB RelA/p65 subunit from nuclear extracts as an index of pulmonary NF-κB activity. Infected and uninfected mouse lung samples were first washed with ice cold phospate-buffered saline (PBS). Nuclear extracts were prepared from lung tissue using a modified method described previously[12]. Briefly, lung tissue was homogenized in 500 μL of ice-cold buffer A (10 mmol/L Hepes, 1.5 mmol/L $MgCl_2$ 10 mmol/L KCl, 0.5 mmol/L DTT, 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 mmol/L EDTA, 10 mmol/L NaF, 25% glycerol). The samples were left on ice for 30 min and lysed by adding NP-40 to a final concentration of 0.625% (V/V) after vortex for 10 sec. After being placed on ice for 5 min, the nuclei were pelleted by centrifugation at 13000 × g for 5 min at 4℃. The supernatant was carefully removed and the nuclei lysed by homogenizing in 100 μL of buffer B (20 mmol/L Hepes, 1.5 mmol/L $MgCl_2$ 420 mmol/L NaCl, 0.5 mmol/L DTT, 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 mmol/L EDTA, 25% glycerol). The samples were kept at 4℃ on a shaker for 30 min, and the nuclear extracts were obtained by centrifugation at 15000 × g at 4℃ for 5 min. The supernatants containing 50 μg nuclear protein were run on a 12% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific sites were blocked with a Tris-buffered saline (TBS) solution containing 5% nonfat milk for 2 h at room temperature. Then the membrane was incubated with specific mouse monoclonal antibody NF-κBp65 at a 1∶500 dilution in Tris-buffered saline containing 5% nonfat milk for overnight at 4℃. After washing with TBS solution for three times, the membrane was incubated with anti-mouse IgG-HRP (1∶1000 dilution) for 2 h at room temperature. The blot was washed and protein was visualized on film using the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions. As an internal control, anti-C23 (nucleolin) mouse monoclonal antibody was used to detect the C23 expression in the same sample preparations.

The densities of the specific NF-κB bands were quantitated with a Labworks software imaging densitometer. Densitometry was expressed as fold increase of experimental compared with that of control.

1.6 Histopathologic examination

Formalin-fixed lungs were embedded in paraffin. Multiple transverse sections of 5 μm thick were stained with haematoxylin-eosin (H&E). In a blinded fashion, two independent pathologists examined the slides under light microscopy for peribronchiolitis (i.e., infiltration of inflammatory cells in the peribronchiolar space), alveolitis (i.e., infiltration of inflammatory cells in the alveolar wall), and perivasculitis (i.e., infiltration of inflammatory cells in the perivascular space) by enumerating the numbers of inflammatory cell infiltrates. The overall inflammation in the lung tissue was characterized by an excess of mononuclear cells (monocytes/macrophages), lymphocytes, and, to a lesser extent, neutrophils.

1.7 Statistical analysis

Results were presented as the mean ± S.E from three independent experiments. Significance was determined by one-way ANOVA (SPSS12.0 statistical analysis software). A p value of less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Induction of TLR7 and TLR3 mRNA in lung tissues of RSV-infected mice

We used semiquantitative RT-PCR to determine the expression levels of TLR7 and TLR3 mRNA in mouse lung in each group. Lung RT-PCR analysis of β-actin mRNA confirmed the quality of all RNA preparations. By comparing lung TLR7 gene expression in RSV-infected mice at different time points with that of control mice, we found that TLR7 message was continuously enhanced expression from 1 h to 24 hrs in RSV-infected groups. Except for the RSV-infected 0 h group, there was a remarkable difference between each infected group and control. Furthermore, the relative quantities of TLR7 mRNA expression was presented a time-dependent increase (Fig. 1). In addition to TLR7, TLR3 expression was increased 4 h post infection (Fig. 2). The relative quantities of TLR7 and TLR3 transcripts were both enhanced in a time-dependent manner.

In all our experimental groups, the β-actin mRNA expression in the same cDNA as for TLR7 and TLR3 analysis in each group was consistent and undifferentiated, indicating that the lung expression of TLR7 and TLR3 mRNA was specifically induced in response to RSV acute infection. The experiments were performed three times with the similar results.

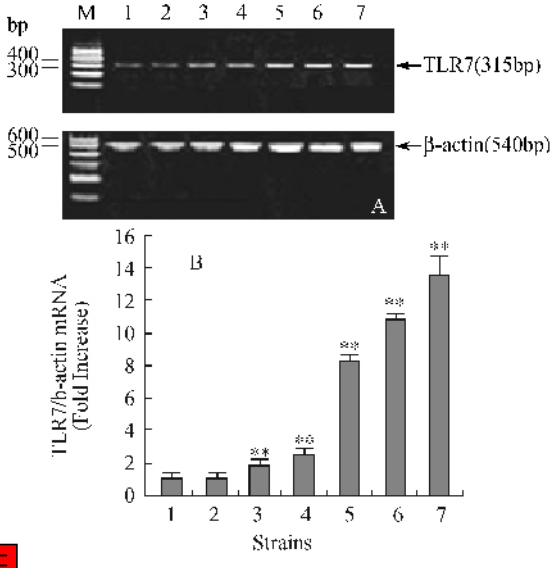

Fig. 1 Time course of lung TLR7 mRNA expression in RSV-infected mice. Total RNA from lung tissues at indicated time points was extracted and used for semiquantitative RT-PCR. (A) Lane 1 was the negative control, lanes 2 – 7 represented lungs collected at 0, 1, 4, 8, 16 and 24 h after RSV infection, respectively. TLR7 mRNA is shown rapid activation and increase at 1 h postinfection. (B) The mean ± S. E (experimental value/control value) from three independent experiments reflected the expression as fold increase. ** $P < 0.01$ compared with control, respectively.

2.2 Lung NF-κB activation induced by RSV

NF-κB in lung nuclear protein was measured by western blot. NF-κBp65 were constitutively expressed at the indicated time intervals (shown in Fig. 3).

2.3 Pulmonary histopathology

At various indicated time points of RSV postinfection, multiple H&E stained lung sections were observed. Lung lesions were evaluated for the total inflammatory infiltrates. At 1 h of RSV-infected mice, few infiltrating cells were found around bronchioles or vessels (Fig. 4-A). Cellular infiltration around bronchioles and vessels increased with increasing times of inoculated RSV (Fig. 4-B and 4-C), and had clear evidence of diffuse increase in the number of inflammatory cells in the alveolar spaces with some areas of more severe involvement. Additionally, the total infiltrating inflammatory cells were

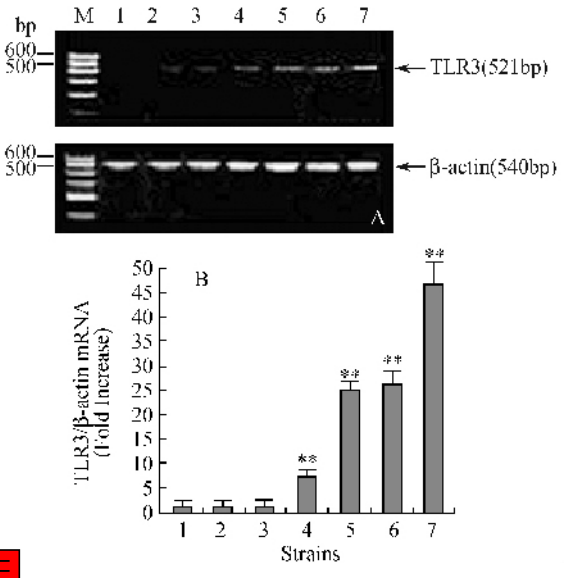

Fig. 2 Time course of lung TLR3 mRNA expression in RSV-infected mice. Total RNA from lung tissues at indicated time points was isolated and used for semiquantitative RT-PCR. (A) Lane 1 was the negative control, lanes 2 – 7 represented lungs collected at 0, 1, 4, 8, 16 and 24 h postinfection, respectively. TLR3 mRNA was first increased at 4 h. (B) The mean ± S. E (experimental value/control value) from three independent experiments reflected the expression as fold increase. ** $P < 0.01$ compared with control, respectively.

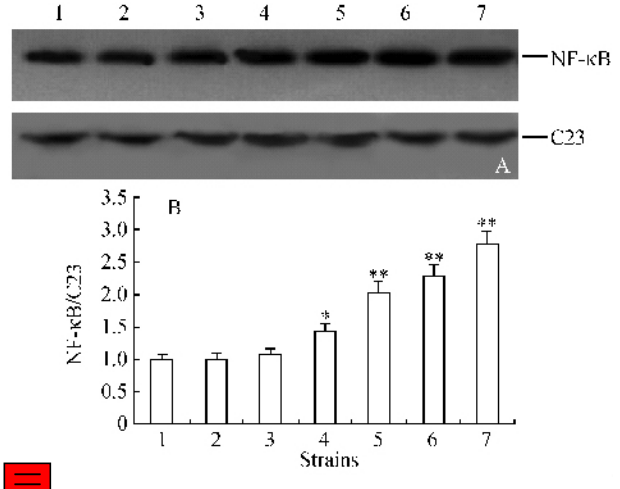

Fig. 3 Expression of lung NF-κB activity in mice infected with RSV. (A) As an indicator of NF-κB activation, nuclear NF-κB/p65 expression was detected by immunoblotting analysis. RSV-induced NF-κB activation was enhanced with increasing times of infection, as indicated (1: control, 2-7: RSV infection 0, 1, 4, 8, 16 and 24 h, respectively). (B) The relative level of NF-κB/p65 protein was determined by quantitation of the results in (A) from the three different experiments by using Labworks software. ** $P < 0.01$, * $P < 0.05$ in comparison with control group (n = 8).

enumerated to measure the inflammatory progress under light microscopy for 10 high power fields in each section. Table 2 showed the changes of inflammatory cell infiltrates per high power field in mouse lungs at different time points of RSV infection.

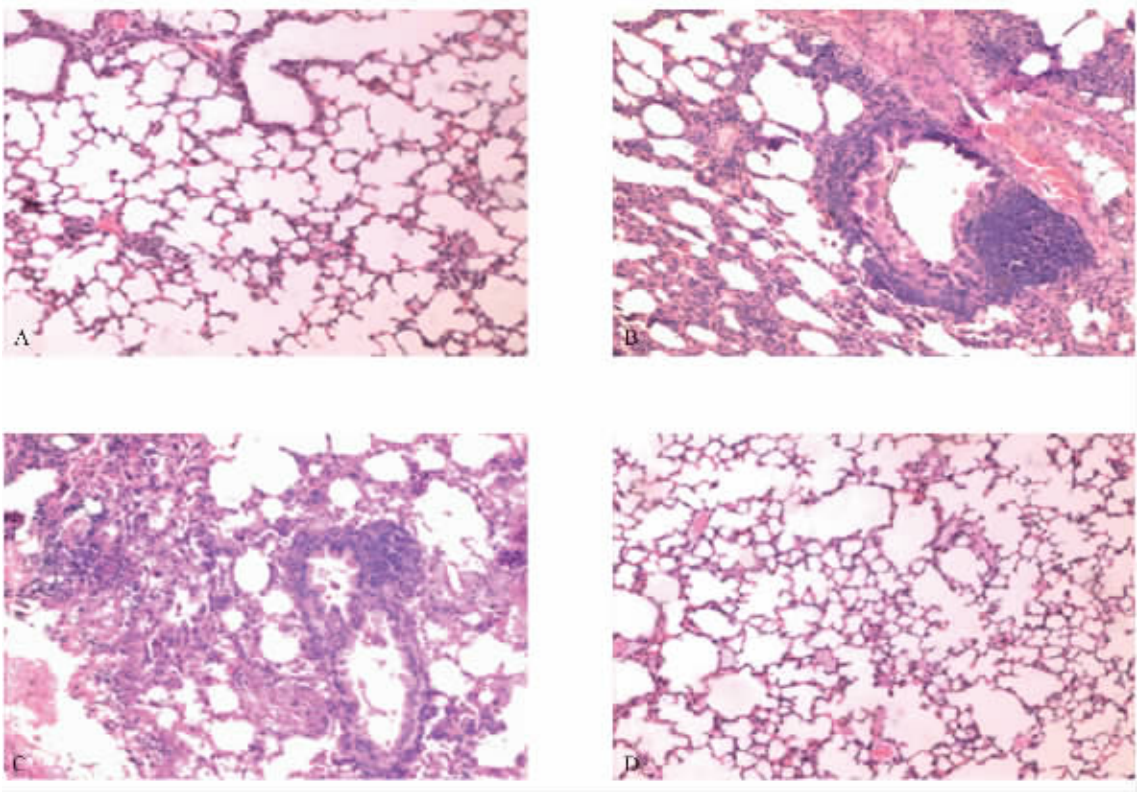

Fig. 4 Typical result of time course of lung inflammation in RSV-infected mice. Mice were inoculated intranasally with live RSV. At different infectious time points, mice were killed and lungs were removed to perform the histopathologic examination. At 1 h (A), 16 h (B), and 24 h (C) of RSV infection, mouse lung showed a slight inflammation, moderate inflammation, and severe inflammation, respectively. (D) control group. Original magnification, 10 × 10.

Table 2 Alteration of the numbers of infiltrating inflammatory cells in mouse lungs

Groups	Numbers of infiltrating inflammatory cells (/high power field, mean ± S.E)		
	1 h	16 h	24 h
control	27.50 ± 7.25	32.84 ± 8.55	26.79 ± 6.72
RSV	88.10 ± 10.32[a]	215.90 ± 26.18[a,b]	378.40 ± 47.39[a,b]

a: $P < 0.01$ compared with control group; b: $P < 0.01$ compared with preceding RSV-infected group.

3 DISCUSSION

RSV, a common cause of severe lower respiratory tract infection in children, infects nearly all infants by age 3 in worldwide[14] which pathogenesis remains to be elucidated. The newly discovery of TLRs has revolutionized our understanding of RSV-induced respiratory tract inflammatory disease. TLRs play a crucial role in activating NF-κB and inducing proinflammatory cytokines such as TNF-α, IL-1β among others that lead to lung inflammation by detection of different PAMPs.

TLR4 had been reported to mediate inflammatory response to RSV via recognition fusion glycoprotein[15]. Groskreutz et al.[8] showed that infection with RSV could up-regulate TLR3 and PKR (dsRNA dependent protein kinase) in A549 lung epithelial cells and human bronchial epithelial cells, and increased expression of TLR3 mRNA, protein. A different group, however, reported that RSV replication was independent of TLR3 when RSV infected HEK293 cells (human embryonic kidney, HEK) transfected with TLR3 gene and express TLR3 steadily[16]. Our results of up-regulated pulmonary TLR3 mRNA in a time-dependent fashion by RSV early challenge confirmed and extended the data published by others mentioned above[7-8]. TLR7 is the receptor of ssRNA. Evidences were provided that TLR7 was required for pDC and B cell response to ssRNA virus by recognition genomic RNA, such as influenza virus and vesicular stomatitis virus (VSV)[3,17]. Heil et al.[18] suggested that TLR7 recognizes viral PAMPs in endosomal or lysosomal compartments inside cells, since ssRNA virus would reach the endosome through receptor-mediated uptake of a viral particle. In our experiments, we found that RSV infection could directly trigger the lung expression of TLR7 time-dependently, which supported the idea that viral nucleic acid would be most likely to be detected by TLRs within an infected cell in

vitro. Additionally, we here found that TLR7 sensing ssRNA in response to RSV was prior to that of TLR3 sensing dsRNA, since there was no evident TLR3 PCR product in RSV-infected 1 h group, while mRNA encoding lung TLR7 was evidently increased from 1 h post infection. It might contribute to different emerging time between ssRNA and dsRNA during RSV infection, RSV is an ssRNA virus that may require more time to replicate and then form dsRNA. But this observation that expression of TLR3 is lower than that of TLR7 is because the PCR amplification efficiency of TLR3 is much lower than that of TLR7 due to target selection, primer design, etc., needs a further exploration. Collectively, as an ssRNA virus, RSV is likely to be the viral trigger of the TLR-dependent response and NF-κB activation in lung. Moreover, RSV mediated early transcriptional responses in the present paper are consistent with the severe extent of RSV-induced pulmonary inflammatory response. Our observation of RSV-induced inflammation is correspondent with Haeberle and his colleagues' report[19], but is inconsistent with another group's report[20], probably due to difference in the use of RSV strain and virulence inoculated. However, perhaps there are other TLR recognition of RSV, such as TLR9 and intracellular helicases etc., and the relationship between RSV replicated titers and TLRs upregulation, needs to be further complicated. Murine TLR8 is thought to be nonfunctional[21].

It should be noted, however, that our findings were based on the measurements of mRNA levels rather than protein levels. Although internal control β-actin exhibited a strong signal, no TLRs protein could be detected in lung by Western blot plus ECL. The failure to detect TLRs protein might be partially because the times of RSV infection were too short to produce adequate proteins, perhaps in amounts not detectable with our method. The surface expression of TLRs may not necessarily correlate with the mRNA levels, and further studies are warranted on this matter.

In summary, our data suggest that RSV challenge indeed induces the rapid and prolonged transcription profiles of lung TLR7 and TLR3 through recognizing ssRNA and dsRNA of this virus. It is likely that RSV promotes a rapid activation of innate responses via multiple TLRs, at least in part, via the increased pulmonary expression of TLR7 and TLR3. This study will contribute to our understanding of RSV infection and disease, and might be useful in the development of tools to modulate the activities of therapeutic TLR ligands to prevent and treat RSV disease in the early stage of the RSV infection.

ACKNOWLEDGMENTS: We gratefully acknowledge associate Professor Sheng-quan Zhang for advice on molecular biology and techniques. We thank Prof. Jing-xian Chen (Columbia University Department of Microbiology) for the valuable discussion and support.

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呼吸道合胞病毒感染上调 Toll 样受体 7 和 3 基因的早期表达

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摘要：【目的】Toll 样受体(Toll-like receptor，TLR)7 和 3 是两个重要的模式识别受体，分别通过识别病毒的单股和双股 RNA 而活化细胞。呼吸道合胞病毒(RSV)能被 TLR7 和 TLR3 识别。在 RSV 感染致病的早期阶段，对肺中 TLR7、TLR3 的表达动力学和表达丰度进行研究，并探讨其表达与肺部炎症反应的关系。**【方法】**我们以活 RSV 滴鼻感染 BALB/c 鼠诱导急性肺炎，在 RSV 感染 0，1，4，8，16 和 24h 的不同时间点，用半定量 RT-PCR 方法检测鼠肺 TLR7、TLR3 的 mRNA 表达，用 western blot 法检测核转录因子 NF-κB 的蛋白表达，HE 染色观察肺的病理学改变。**【结果】**我们发现，RSV 感染早期能快速上调 TLR7 和 TLR3 的基因表达水平，与正常组相比，其升高有显著性差异，并与 RSV 感染之间存在时间依赖关系；TLR7 的反应(RSV 感染 1h)早于 TLR3(RSV 感染 4 h)。肺中 NF-κB 在 RSV 感染的 4 h 即可被活化。RSV 介导的 TLR7 和 TLR3 早期转录反应与 RSV 肺炎的严重程度是平行的。**【结论】**TLR7 和 TLR3 确实可通过识别病毒 RNA 参与 RSV 肺炎的发生和发展，表明感染的器官在识别病毒感染和激发前炎反应时，可能经由多个 TLRs。这将对开发制剂用以调节治疗性 TLR 配体的活性具有重要意义。

关键词：Toll 样受体；呼吸道合胞病毒；基因表达；炎症；肺

中图分类号：R392　　**文献标识码：**A　　**文章编号：**0001-6209(2009)02-0245-07

(本文责编：王晋芳)

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收稿日期：2008-07-18，**修回日期：**2008-11-24