

# Developmental expression of Toll-like receptors in the guinea pig lung

LINGJIE MA<sup>1,2\*</sup>, JIALI YANG<sup>3\*</sup>, LI YANG<sup>4</sup>, JUAN SHI<sup>3</sup>, JING XUE<sup>1</sup>, YONG LI<sup>1,2</sup> and XIAOMING LIU<sup>1,3</sup>

<sup>1</sup>Key Laboratory of The Ministry of Education for The Conservation and Utilization of Special Biological Resources of Western China, Ningxia University, Yinchuan, Ningxia 750021; <sup>2</sup>Hangzhou Simo Pharmaceuticals Co., Ltd., Hangzhou, Zhejiang 310053; <sup>3</sup>Ningxia Key Laboratory of Clinical and Pathogenic Microbiology, General Hospital of Ningxia Medical University; <sup>4</sup>The Center of Experimental Animals, Ningxia Medical University, Yinchuan, Ningxia 750004, P.R. China

Received October 24, 2015; Accepted November 21, 2016

DOI: 10.3892/mmr.2017.6129

**Abstract.** The guinea pig is a useful model for investigating infectious and non-infectious lung diseases due to the sensitivity of its respiratory system and susceptibility to infectious agents. Toll-like receptors (TLRs) are important components of the innate immune response and are critical for lung immune function. In the present study, the differentiation of epithelial cells in the guinea pig lung was examined during gestation by studying anatomic morphology and the major epithelial cell types using cell type-specific markers. The developmental expression of all 9 TLRs and the TLR signaling adaptors myeloid differentiation factor 88 (MyD88) and tumor necrosis factor receptor associated factor 6 (TRAF-6) were investigated by reverse transcription-quantitative polymerase chain reaction and western blotting analysis. The formation of lung lobes in guinea pigs was observed at 45 days of gestation (dGA), along with the expression of the basal cell marker keratin 14 and the alveolar type II cell marker pro-surfactant protein. However, the cube cell marker secretoglobin family1A member 1 and ciliated cell marker b-tubulin IV were only detected in the lungs from 52 dGA onward. The expression levels of all TLRs, MyD88 and TRAF-6 were determined in lung tissues harvested from embryos, newborn, postnatal and adult animals. The expression levels of all TLR signaling components displayed similar dynamic expression patterns with gestation age and postnatal maturation time, except for TLR-4 and TLR-7. mRNA expression levels of TLR

components were significantly increased in the lungs at 45 and 52 dGA, compared with later developmental stages. These results suggest that TLR expression in the guinea pig lung is developmentally regulated, enhancing the understanding of lung biology in guinea pig models.

## Introduction

The guinea pig (*Cavia porcellus*) is a rodent species in the Caviidae family (1,2). Guinea pigs are a valuable animal model for the study of developmental biology and the pathogenesis of multiple diseases due to similarities between their biological characteristics and those of humans. These similarities appear in hormonal and immunologic responses, pulmonary physiology, exogenous vitamin C requirements and delayed-type hypersensitivity to infections such as tuberculosis (3). Similarities in the sensitivity of the respiratory system and susceptibility to infectious agents results in the broad use of this species as a model of respiratory diseases (4). With respect to the pathogenesis and the immune response to these diseases, guinea pigs are more representative of a human than models using other rodent species.

The lung is an organ directly exposed to the environment, resulting in continuous contact with a diverse array of environmental insults. To respond to the internal and/or external environment, resident myeloid cells and stromal cells of the lung express a full complement of Toll-like receptors (TLRs), which are a class of signaling pattern-recognition receptors (PRRs) that contribute to innate and adaptive immunity by recognizing pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) (5,6). In addition, TLRs are implicated in the pathogenesis of non-infectious pulmonary diseases, including pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and acute lung injury (ALI) (7). TLRs were originally identified in *Drosophila melanogaster*, and revealed to be transmembrane receptor proteins that are highly conserved between *D. melanogaster* and humans (8,9). Although a large body of studies have demonstrated the association of TLRs with various pulmonary disorders in adult animal models and adult patients (7), several studies on fetal animals, including

---

*Correspondence to:* Dr Xiaoming Liu or Dr Yong Li, Key Laboratory of The Ministry of Education for The Conservation and Utilization of Special Biological Resources of Western China, Ningxia University, 539 West Helanshan Road, Yinchuan, Ningxia 750021, P.R. China  
E-mail: lxm1966@nxu.edu.cn  
E-mail: liyong7732@126.com

\*Contributed equally

**Key words:** guinea pig, lung, Toll-like receptors, developmental expression, epithelial cell markers

fetal murine and preterm lamb lung tissue specimens, have suggested that steady-state TLR mRNA expression levels increase with gestational development (10,11). Distinct characteristics and expression patterns of TLRs have also been reported between mammalian species, tissues and developmental stages (12-17). Therefore, in order to improve understanding of the mechanisms involved in cell specificity and regulation of TLR signaling transduction pathways, there is a need to investigate the nature of TLRs and ligands in different species.

Given the importance of TLRs in the development and homeostasis of tissues, the present study examined the differentiation of guinea pig lung epithelial cells during embryonic development and maturation following birth, and profiled the expression of TLRs (TLR-1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-7, TLR-8, TLR-9 and TLR-10), as well as adaptors of the TLR pathway: Myeloid differentiation factor 88 (MyD88) and tumor necrosis factor receptor associated factor 6 (TRAF-6).

## Materials and methods

**Animals and tissue processing.** All experimental procedures were carried out according to ethical guidelines established by Ningxia University and were approved by the Ethnic Committee for Scientific Research of General Hospital of Ningxia Medical University (NXMU-H-2014-250; Yinchuan, China). A total of 51 three months old outbred Hartley-Duncan guinea pigs of both sexes (45 females and 6 males; 300±50 g) were obtained from the Animal Facility of Ningxia Medical University (Yinchuan, China). Total of 51 animals (45 females and 6 males) were used. Individuals were housed in the animal facility under clean, non-specific pathogen free conditions with a constant temperature of 24-26°C and humidity of 30-50% (12/12 h light/dark cycle), provided a proper balance of pellets, hay and fresh vegetables and free access to water, according to the Housing and Husbandry Guidelines for Laboratory Animals of Ningxia Medical University. The appearance of a vaginal plug in the morning following breeding was designated the first day of gestation (dGA). Animals were terminally euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg) in the facility. Embryos were harvested from pregnant animals for lung isolation at 45, 52 and 59 dGA (embryos implanted in uterus with normal development were confirmed as alive, and those only showing implantation sites but without an embryo or a normally developed embryo were defined as dead), and lungs were harvested directly from pups 0, 7, 14 and 28 days following parturition (dPN). Lungs were also harvested from mature animals (6 weeks old). The trachea and lungs were removed and fixed in 10% neutral buffered formalin, then processed for embedding in a Sakura Tissue Tek Paraffin Tissue Processor (Model VIP E150; Sakura Finetek USA, Inc., Torrance, CA, USA).

**Immunohistochemistry (IHC).** Immunohistochemical staining was performed on 6 µm thick paraffin sections. Paraffin sections were dewaxed in xylene and incubated in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min to inactivate endogenous peroxidase. Sections were subsequently rehydrated with graded ethanol according to histological standards. The sections were boiled in citrate buffer (pH 6.0) at 95°C for 20 min for antigen retrieval

followed by slow cooling for 2 h at room temperature. Sections were then blocked using a blocking buffer (5% horse serum in PBS; Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 2 h prior to incubation with a primary antibody (diluted in blocking buffer; Table I) in a humid chamber at 4°C overnight. The primary antibodies used in the present study are listed in Table I. Following washing three times for 5 min in PBS, a mixture of biotinylated horse anti-rabbit IgG or anti-mouse IgG (cat. no. PK-6100; 1:200; Vector Laboratories, Inc.) secondary antibodies (Table I for detail) was applied at room temperature for 2 h. The antigen was detected with an elite ABC kit (Vector Laboratories, Inc.) and developed with a DAB peroxidase substrate kit (Vector Laboratories, Inc.). The stained sections were counterstained with hematoxylin for 10 sec (Gill's formula; Vector Laboratories, Inc.). Sections were subsequently rinsed in running tap water for 5 min, dehydrated with gradually increased concentration of ethanol (30-100% v/v), cleared sections by submerging slides in xylene for 1 min during mounting sections with Permount (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hematoxylin and eosin (H&E) staining was performed using H&E staining kit from Thermo Fisher Scientific, Inc.. Periodic Acid Schiff's staining was conducted using Schiff's reagents from Sigma-Aldrich; Merck Millipore. Staining was visualized under a light microscope (TCS SP8; Leica, Wetzlar, Germany) at magnifications of x200, x400 and x630, and images were captured using a Leica DFC300 F camera (Leica Microsystems, Inc., Buffalo Grove, IL, USA). For all antibodies tested, mouse positive and negative tissues were utilized as positive and negative controls, respectively.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from each tissue sample using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. RNA quality was assayed by calculation of the RNA integrity number (RIN) (18). High quality RNA (RIN value >7.5) was used to synthesize first strand cDNA using M-MLV reverse transcriptase (Takara Bio, Inc., Otsu, Japan). qPCR was performed in the Roche LightCycler 2.0 (Roche Diagnostics, Basel, Switzerland) using a SYBR-Green I kit (Takara Bio, Inc.). The thermal cycling conditions were as follows: 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 20 sec, followed by 40°C for 20 min. The primer sets used for β-actin control, TLRs, MyD88 and TRAF-6 are listed in Table II. To assess primer efficiency, standard curves for each primer pair were generated using serially diluted transcribed cDNA samples. PCR efficiency was calculated from the slope of the standard curves. β-actin internal controls were always included to normalize each reaction with respect to RNA integrity, sample loading and inter-PCR variations. The relative expression ratio was calculated from the real-time PCR efficiencies and the crossing point deviation of experimental samples vs. controls (19). The resulting threshold cycle (C<sub>q</sub>) values were normalized to the endogenous control, β-actin (ΔC<sub>q</sub>=C<sub>q</sub> value of target gene-C<sub>q</sub> value of β-actin). Dissociation analysis of amplification products was performed at the end of each PCR to confirm the specificity of the amplicon. The fold change in TLR gene expression during lung development was calculated by the 2<sup>-ΔΔC<sub>q</sub></sup> method (20).

Table I. Primary antibodies used for immunohistochemical staining and western blotting.

| Antibody   | Host   | Dilution | Supplier     | Catalog number | IHC or IB |
|------------|--------|----------|--------------|----------------|-----------|
| Keratin 14 | Rabbit | 1:500    | Thermo Sci   | RB-9020        | Both      |
| Tubulin IV | Mouse  | 1:2,000  | BioGenex     | MU178-UC       | Both      |
| pro-SPC    | Rabbit | 1:5,000  | Millipore    | AB3786         | Both      |
| CCSP       | Rabbit | 1:5,000  | USBiological | C5828-03       | Both      |
| TLR-3      | Rabbit | 1:1,000  | Millipore    | NG1593494      | IB        |
| TLR-6      | Rabbit | 1:1,000  | Abcam        | AB37072        | IB        |
| TLR-9      | Rabbit | 1:1,000  | Millipore    | AB10011        | IB        |
| MyD88      | Rabbit | 1:1,000  | Abcam        | AB2068         | IB        |
| TRAF-6     | Rabbit | 1:1,000  | Millipore    | 04-451         | IB        |
| GAPDH      | Goat   | 1:1,000  | Santa Cruz   | Sc-20357       | IB        |

Thermo Sci, Thermo Fisher Scientific, Inc., Waltham, MA, USA; BioGenex, BioGenex, Fremont, CA, USA; Millipore, Merck Millipore, Darmstadt, Germany; Abcam, Abcam, Cambridge, UK; Santa Cruz, Santa Cruz Biotechnology, Inc., Dallas, TX, USA.

Table II. Sequences and parameters of reverse transcription-quantitative polymerase chain reaction.

| Genes   | Sequence (5'-3')                                       | Anneal temperature | Product size | Ensembl               | ID |
|---------|--|--------------------|--------------|-----------------------|----|
| TLR1    | F: GTCCCAAGTTAGCCCATTTT<br>R: CTTTCAGCCCAAGAGCAAGTAT   | 60.0°C             | 240 bp       | ENSCPOT00000005143    |    |
| TLR2    | F: AGGAAAAGTTGGAGCGGTTT<br>R: GGCAAAGGCAAAGAAAGTGA     | 60.0°C             | 225 bp       | ENSCPOG00000025374    |    |
| TLR3    | F: GCAACAACAACATAGCCAACA<br>R: AGAAAATGAACAGGACCACCA   | 60.0°C             | 133 bp       | ENSCPOT00000004982    |    |
| TLR4    | F: GTTATCGTTGTGGTGTCTCAGC<br>R: TTCCCATCCAGGTAAGTGTTT  | 60.0°C             | 198 bp       | ENSCPOT00000015306    |    |
| TLR6    | F: TTTGTCAGGGCTCAACATTCT<br>R: AAATCACCACAGGGTAGCAG    | 60.0°C             | 147 bp       | ENSCPOT00000005143    |    |
| TLR7    | F: ACTCCTTGGGGATAGATGGTTT<br>R: AATAGTGAGGGTGAGGTTGGTG | 60.0°C             | 154 bp       | ENSCPOT00000015254    |    |
| TLR8    | F: AGATGTGATTTGTGCCAGTCC<br>R: GGGATGTGGAAAGAGACCTGT   | 60.0°C             | 233 bp       | ENSCPOT00000004812    |    |
| TLR9    | F: GGGAAGCAGTGACCAGAAC<br>R: TCCAAGGTGAATGGGATGTT      | 60.0°C             | 121 bp       | X M 0 0 3 4 7 6 7 2 0 |    |
| TLR10   | F: GAGGAGATGGGCTTCGACTAC<br>R: CTCAGTTCCAACAGCACGTC    | 60.0°C             | 169 bp       | ENSCPOT00000015432    |    |
| MyD88   | F: GAGGAGATGGGCTTCGACTAC<br>R: CTCAGTTCCAACAGCACGTC    | 60.0°C             | 164 bp       | ENSCPOT00000027931    |    |
| TRAF6   | F: ATCACTTGGCACGACACCTAC<br>R: GGGACAGAGAGGCAGAATCAT   | 60.0°C             | 138 bp       | ENSCPOT00000004556    |    |
| β-actin | F: GGACTTTGAGCAGGAGATGACT<br>R: CTGGAAAATGGCTTCAGGAC   | 60.0°C             | 127 bp       | ENSCPOT00000011124    |    |

F, forward; R, reverse; TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; TRAF-6, tumor necrosis factor receptor associated factor 6.

**Western blot analysis.** Tissue extracts were prepared by homogenizing the cells in a lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40; pH 7.5) for 60 min on ice. Lysates were subsequently centrifuged at 10,000 x g for 10 min at 4°C,

and the supernatants were collected as whole-cell extracts. The soluble protein concentration was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using bovine serum albumin (BSA; Thermo Fisher

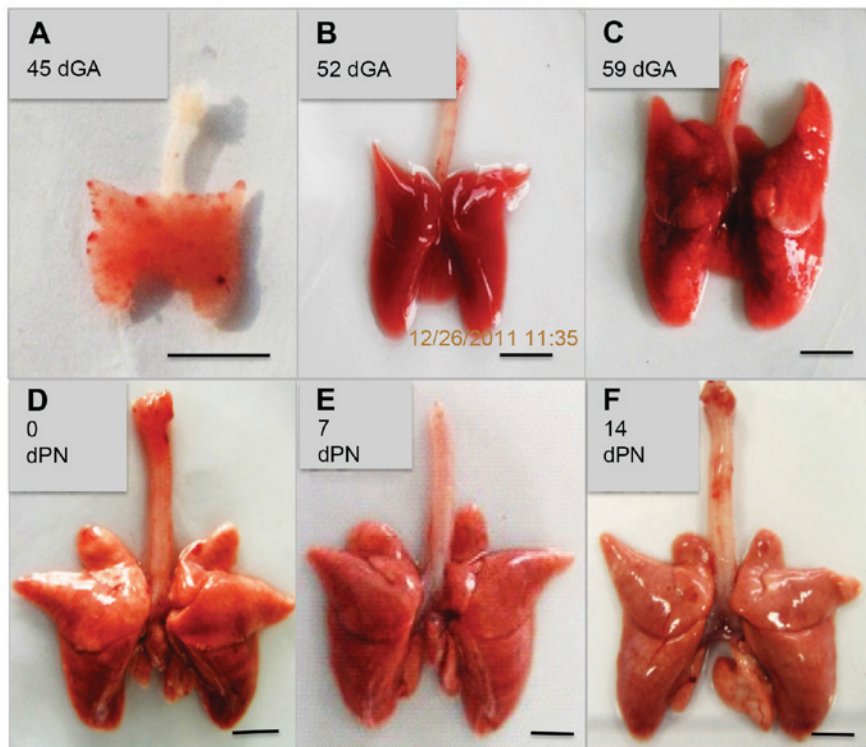


Figure 1. Representative anatomic images guinea pig lungs. (A-F) Gestation and postnatal age of the lungs are indicated in the respective images. Bar=1.0 cm. dGA, days of gestation; dPN, days following parturition.

Scientific, Inc.) as a standard. The cell extracts (50  $\mu$ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Merck Millipore). The membrane was blocked with 4% fat free dry milk in PBS containing 0.2% Tween-20 at room temperature for 2 h and incubated with primary antibodies (Table I) at 4°C overnight, followed by appropriate peroxidase-labeled secondary antibodies (cat. no. 108894; 1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. The blots were then developed using an enhanced chemiluminescence reagent (GE Healthcare, Chalfont, UK).

**Statistical analysis.** All data collected in the present study were obtained from at least three independent experiments for each condition. SPSS 18.0 analysis software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Statistical evaluation of the data was performed by one-way ANOVA and Student's t-tests to compare differences between groups. Tukey's honestly significant difference test was employed for post hoc testing for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference and  $P < 0.01$  was considered to indicate a highly statistically significant difference. Data were expressed as the mean  $\pm$  standard deviation.

## Results

**Development of the guinea pig lung during embryogenesis.** The guinea pig gestation period spans 59–73 days, with an average of  $66 \pm 2$  days recorded in the present study. Lobe formation in the lung was displayed at 45 dGA, with all 7 lobes

(4 lobes in the right, 3 lobes in the left) formed at 59 dGA, as determined by whole mount anatomic lung morphological examination (Fig. 1). The lung continued to grow and mature, increasing in weight and size, with body growth (Fig. 1 and Table III). Histological analysis by H&E staining revealed that primary alveolar sacs had formed by 45 dGA, and bronchiolar structure was first observed in the distal lung of 52 dGA embryos (Fig. 2). Well-structured alveolar epithelia and bronchiolar epithelia were observed at 52 dGA and onward (Fig. 2). Epithelial cells in the trachea of 45 dGA embryos were poorly differentiated, despite the formation of a primary epithelial structure at this stage (Fig. 3). Abundant epithelial cells were observed on the surface of 52 dGA tracheas, but pseudostratified epithelial layers with ciliated cells were only observed in 59 dGA tracheas and onward (Fig. 3).

**Expression of epithelial cell type-specific markers in the developing guinea pig lung.** The expression of epithelial cell markers (21) in developing guinea pig lungs was examined using IHC analysis. At 45 dGA, keratin 14 (K14) was expressed in the basal layer of the tracheal epithelia (Fig. 4Aa) and pro-surfactant protein C (SPC) of alveolar type II cells was expressed in the lung (Fig. 4Ba), but there was no expression of  $\beta$ -tubulin IV (T4) of ciliary axonemes in the tracheal ciliated cells (Fig. 4C) or secretoglobin family1A member 1 (CCSP) of cube cells in the distal airway (Fig. 4D) at this gestation age (21). However, these 4 cell-specific markers were expressed in the lung of 52 dGA embryos and onward, particularly the CCSP was mainly expressed in epithelial cells of small airways in distal lung (Fig. 4), indicating that differentiation of varied epithelial cell types occurs during lung development in the guinea pig.



Table III. Gross parameters of the guinea pig lung at different developmental stages.

| Ages   | Body length (cm) | Body weight (g) | Lung length (cm) | Lung weight (g) | Trachea length (cm) |
|--------|------------------|-----------------|------------------|-----------------|---------------------|
| 45 dGA | 4.27±0.05        | 4.88±0.10       | 1.33±0.03        | 0.13±0.01       | 0.7±0.00            |
| 52 dGA | 7.70±0.09        | 37.63±3.68      | 3.08±0.13        | 0.78±0.09       | 1.58±0.13           |
| 59 dGA | 10.55±0.12       | 79.97±2.83      | 3.83±0.13        | 1.90±0.06       | 1.85±0.07           |
| 0 dPN  | 11.38±0.26       | 96.32±5.01      | 3.70±0.12        | 1.47±0.06       | 1.82±0.05           |
| 7 dPN  | 13.03±0.25       | 110.22±3.47     | 3.75±0.18        | 1.28±0.05       | 2.08±0.11           |
| 14 dPN | 15.23±0.87       | 177.58±21.15    | 4.28±0.19        | 1.72±0.21       | 2.23±0.17           |
| 28 dPN | 17.73±0.65       | 239.47±19.07    | 4.77±0.08        | 2.16±0.22       | 2.57±0.03           |

dGA, days of gestation; dPN, days following parturition.

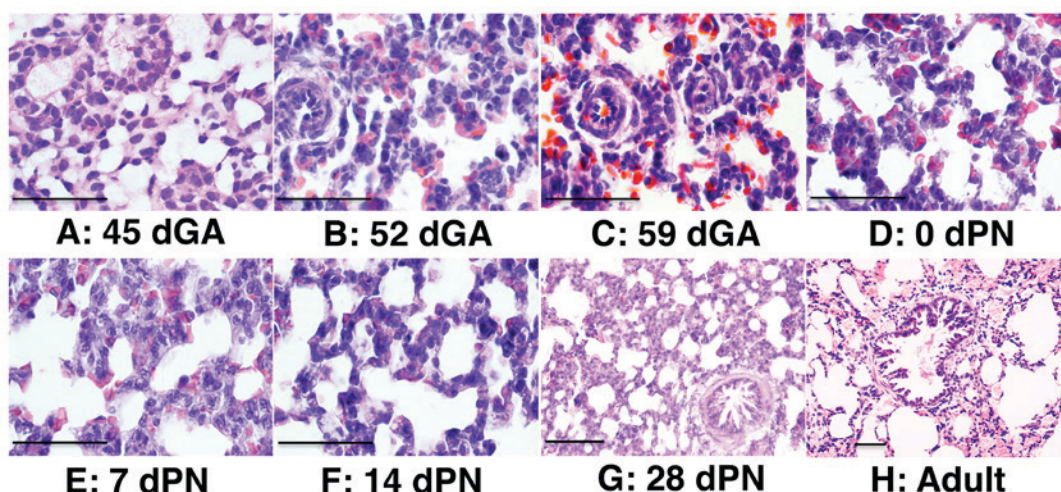


Figure 2. Hematoxylin and eosin staining of distal guinea pig lungs at different developmental stages. Gestation and postnatal ages are indicated in the respective images. Bar=50  $\mu$ m. dGA, days of gestation; dPN, days following parturition.

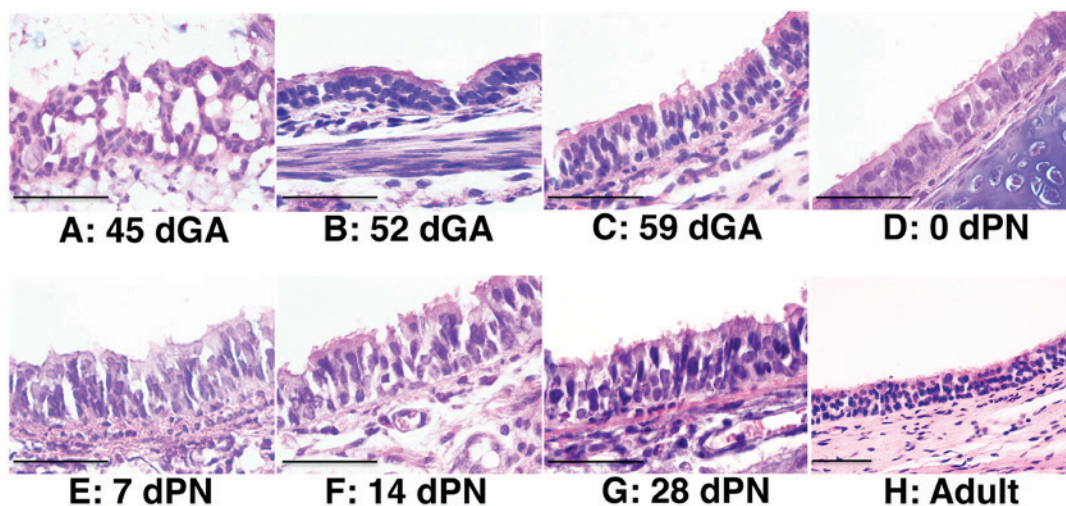


Figure 3. Hematoxylin and eosin staining of guinea pig trachea at different developmental stages. Gestation and postnatal age are indicated in the respective images. Bar=50  $\mu$ m. dGA, days of gestation; dPN, days following parturition.

*Developmental expression of TLRs in the lung of guinea pig.* In order to evaluate changes of mRNA and protein expression levels of TLR signaling components during organogenesis and maturation of the lung in guinea pigs, the relative abundance

of TLRs (-1, -2, -3, -4, -6, -7, -8, -9 and -10), MyD88 and TRAF-6 were investigated by RT-qPCR and western blotting, respectively, in distal lung tissues at 45, 52 and 59 dGA, 0, 7, 14 and 28 dPN, and in adult animals. RT-qPCR analysis



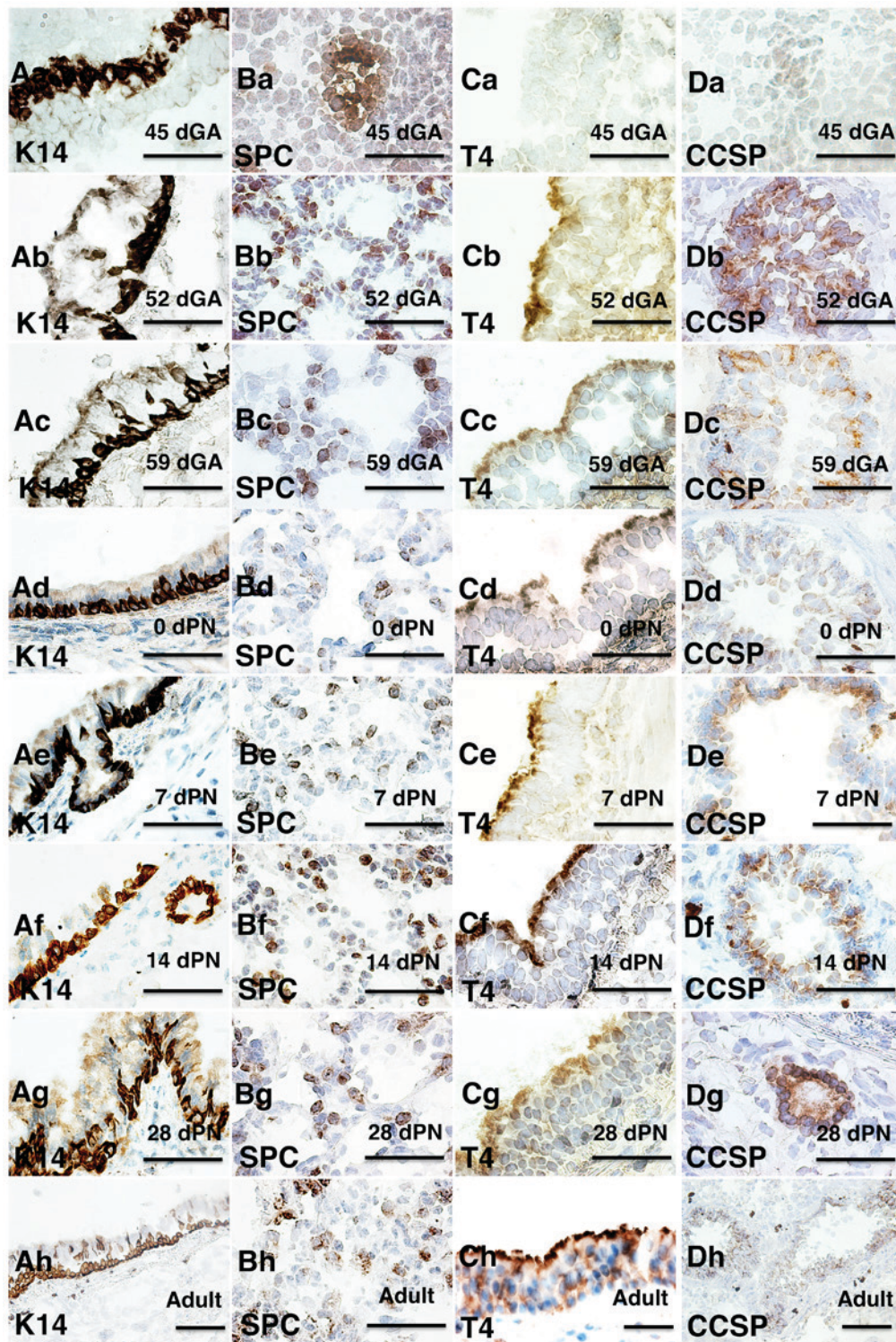


Figure 4. Immunohistochemical staining of major epithelial cell markers at different developmental stages in the guinea pig lung. Epithelial markers and developmental stages are indicated on the images. (A) K14 staining of tracheal epithelium; (B) SPC staining of lung tissue; (C) T4 staining of tracheal epithelium and (D) CCSP staining of lung tissue at developmental stages of (a) 45 dGA, (b) 52 dGA, (c) 59 dGA, (d) 0 dPN, (e) 7 dPN, (f) 14 dPN, (g) 28 dPN and (h) adult. Bar=50  $\mu$ m. dGA, days of gestation; dPN, days following parturition; K14, keratin 14; T4,  $\beta$ -tubulin IV; CCSP, secretoglobin family1A member 1; SPC, pro-surfactant protein C.

demonstrated that the mRNA expression levels of all tested TLR signaling components was dynamic, with a similar expression pattern, in which transcripts were more abundant in the 45 dGA and 52 dGA lungs compared with later stages (Fig. 5A-D). Notably, TLR-2, -3, -6, -7, -8, -9 and -10 mRNA expression levels were higher in 45 dGA lungs compared with

52 dGA lungs (Fig. 5A and C), while TLR-1, -4, MyD88 and TRAF-6 mRNA expression levels were higher in 52 dGA lungs compared with 45 dGA lungs (Fig. 5A, B and D). In addition, increased TLR-7 mRNA expression levels were observed in 7 dPN lungs as compared with TLR-8, 9 and 10 at this stage ( $P=0.000$ ; Fig. 5C). The results of western blotting

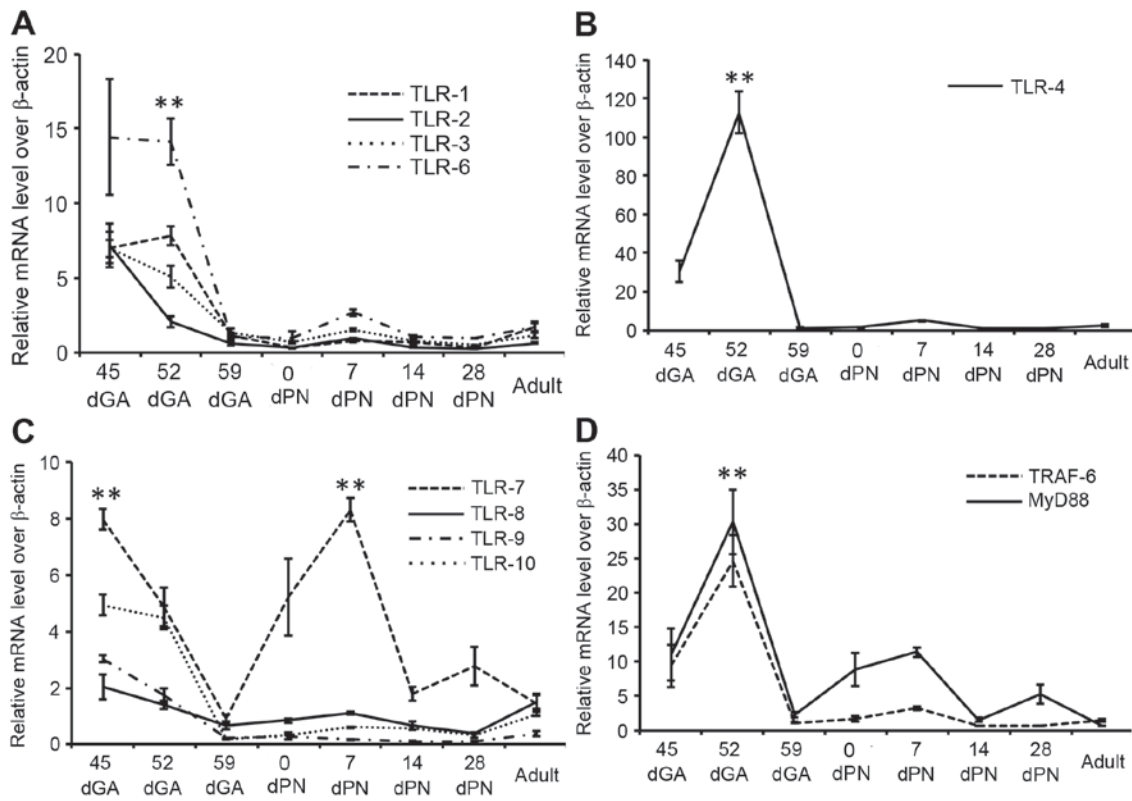


Figure 5. Differential expression of TLR mRNA in the guinea pig lung. The relative expression levels of TLR mRNAs in lung tissues at the indicated gestation and postnatal ages were determined by reverse transcription-quantitative polymerase chain reaction assays. (A) Fold-changes of TLR-1, -2, -3 and -6 mRNA expression levels. (B) Fold-changes of TLR-4 mRNA expression levels. (C) Fold-changes of TLR-7, -8, -9 and -10 mRNA expression levels. (D) fold-changes of MyD88 and TRAF-6 mRNA expression levels. Data are expressed as the mean  $\pm$  standard deviation of fold-changes over the internal control ( $\beta$ -actin) from 6 animals for each time point. \*\* $P < 0.01$ : TLR-1 vs. TLR-2, -3 and -6 at 56 dGA in A; 52 dGA vs. other developmental stages for TLR-4 in B; TLR-7 vs. TLR-8, -9 and -10 at 45 dGA and 7 dPN in C; 52 dGA vs. other developmental stages for MyD88 and TRAF-6 in D. dGA, days of gestation; dPN, days following parturition, MyD88, myeloid differentiation factor 88; TRAF-6, tumor necrosis factor receptor associated factor 6.

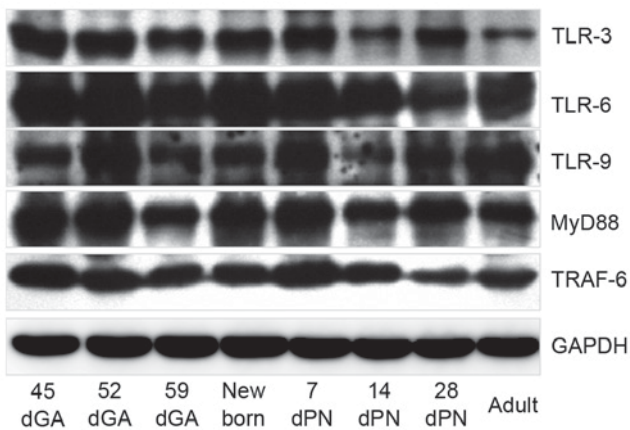


Figure 6. Expression of TLR proteins determined by western blotting. TLR-3, TLR-6, TLR-9, MyD88 and TRAF6 protein levels were determined in guinea pig lung tissues at the indicated developmental stages. TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; TRAF-6, tumor necrosis factor receptor associated factor 6; dGA, days of gestation; dPN, days following parturition.

demonstrated similar expression patterns to the RT-qPCR analysis for TLR-3, -6, -9, MyD88 and TRAF-6 proteins, with differential expression in guinea pig lungs during different developmental stages (Fig. 6). These data imply that TLR expression in guinea pig lungs is developmentally regulated.

## Discussion

In the present study, the morphogenesis and maturation of the guinea pig lung and the expression of TLR ligands and TLR signaling adaptors MyD88 and TRAF-6 were investigated from 45 dGA to maturation. Lung development was observed as early as 45 dGA, along with expression of the basal cell marker K14 and alveolar type II cell marker SPC, but the cube cell marker CCSP and ciliated cell marker T4 were only observed in the lungs from 52 dGA onwards. Notably, the expression of all 9 examined TLRs and the TLR signaling adaptors MyD88 and TRAF-6 were detected at the transcriptional level in all lung tissues harvested from 45, 52 and 59 dGA, 0, 7, 14 and 28 dPN, and in adult animals. Transcript levels of all TLR signaling components formed similar dynamic expression patterns with gestation age and maturation time following parturition, with TLRs significantly more abundant in the lungs of 45 and 52 dGA embryos compared with later stages. These data imply that TLR expression in guinea pig lungs may be developmentally regulated.

TLRs are a family of mammalian proteins first observed in *D. melanogaster* (5). To date, 11 TLRs have been identified in humans (5). Different TLR ligands have been demonstrated to be involved in the regulation of both innate and adaptive immune responses, in part through binding to different specific ligands. TLR-2 forms heterodimers with TLR-1 and



TLR-6 upon binding to different lipopeptide and lipoprotein structures (22); TLR-2 and TLR-4 recognize lipids including lipopolysaccharide (LPS); TLR-3 and TLR-7-9 are activated by nucleic acids derived from viruses or bacteria, and TLR-5 primarily recognizes the protein flagellin from flagellated bacteria (23). In particular, TLR-9 recognizes various types of unmethylated cytosine-guanine repeat dideoxynucleotides (24). TLR-10, an orphan receptor with an unidentified ligand, is the most recent human TLR to be identified, and has been demonstrated to form heterodimers with TLR-1 and TLR-2 (22,25). TLR-10 mRNA expression was detected in guinea pig lungs in the present study.

In addition to immunoregulation, TLRs are involved in development and homeostasis, sensing endogenously derived materials during the processes of organogenesis and tissue generation. For example, hyaluronan (HA) is a breakdown product of extracellular matrix components, and the recognition of different forms of HA is essential for the maintenance of homeostasis and epithelial integrity during tissue regeneration following an injury and the restoration of normal tissue architecture (26-28). Several lines of evidence have demonstrated the developmental involvement of TLRs in mammalian and non-mammalian species during embryogenesis. For example, Kannaki *et al* (29) demonstrated differential expression patterns of TLR mRNAs during chicken embryological development, suggesting the potential involvement of TLRs in the regulation of chicken embryo development. This is supported by the initial discovery of Toll in *D. melanogaster*, the first member of the TLR family, as it was initially characterized as a developmental protein prior to the establishment of its immunological involvement (8,15). This is further supported by the discovery of the developmental involvement of TLR-7 and TLR-9 in the vertebrate brain (16).

The immunological and developmental involvements of TLRs in embryonic organogenesis have also been reported in mammalian lungs and other epithelial tissues (17,30). For example, in a study evaluating the developmental expression of TLR-2 and TLR-4 in preterm baboon lungs, Awasthi *et al* (30) demonstrated that the expression of these TLRs was significantly lower at 125 and 140 dGA compared with adult baboons, only reaching comparable levels at 175 dGA. This indicates that that TLR expression in baboon lungs is developmentally regulated. This developmental expression pattern was consistent with previous findings in fetal murine lung tissues (10) and in preterm lamb lung tissues (11), with TLR expression increasing with the length of gestation. This expression pattern may be different to that observed in the mature lung of adult animals and humans, where the expression of TLRs is dependent on immune cells including macrophages, dendritic cells and lung epithelial cells (7,31). In addition, developmentally regulated expression of TLRs was observed in fetal and neonatal human and mouse colonic epithelium (17). The results of the present study were consistent with these previous findings, with the expression of TLRs also being developmentally regulated in the guinea pig lung. Altered TLR signaling may lead to compromised structural development in the lung, as previously demonstrated in mice (32). An injection of LPS into the amniotic fluid of 15 dGA mice led to increases in the luminal volume density and decreases in distal branching of the lungs at 17 and 18 dGA,

induced by activation of functional TLR-4 (32). Notably, in the present study, TLR mRNA expression levels were highest in guinea pig lungs at 45 and 52 dGA, and dramatically decreased as lung organogenesis and maturation progressed. Adult levels were reached by 59 dGA. The implications of such a dramatic change in TLR expression in the guinea pig lung warrants further investigation.

To conclude, morphogenesis and developmental expression of TLR signaling components in the guinea pig lung were examined in the present study. The dynamic expression of TLRs during lung development and maturation in the guinea pig was demonstrated, further confirming the developmental involvement of TLRs in lung morphogenesis in this animal model. These data lay a foundation upon which further understanding of the immunoregulatory mechanisms in the guinea pig lung may be built.

### Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant nos. 31260615 and 31472191).

### References

1. D'Erchia AM, Gissi C, Pesole G, Saccone C and Arnason U: The guinea-pig is not a rodent. *Nature* 381: 597-600, 1996.
2. Graur D, Hide WA and Li WH: Is the guinea-pig a rodent? *Nature* 351: 649-652, 1991.
3. Padilla-Carlin DJ, McMurray DN and Hickey AJ: The guinea pig as a model of infectious diseases. *Comp Med* 58: 324-340, 2008.
4. Kashino SS, Napolitano DR, Skobe Z and Campos-Neto A: Guinea pig model of Mycobacterium tuberculosis latent/dormant infection. *Microbes Infect* 10: 1469-1476, 2008.
5. Takeda K and Akira S: TLR signaling pathways. *Semin Immunol* 16: 3-9, 2004.
6. Jimenez-Dalmaroni MJ, Gerswhin ME and Adamopoulos IE: The critical role of toll-like receptors-From microbial recognition to autoimmunity: A comprehensive review. *Autoimmun Rev* 15: 1-8, 2016.
7. Kovach MA and Standiford TJ: Toll like receptors in diseases of the lung. *Int Immunopharmacol* 11: 1399-1406, 2011.
8. Hashimoto C, Hudson KL and Anderson KV: The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 52: 269-279, 1988.
9. De Nardo D: Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine* 74: 181-189, 2015.
10. Harju K, Glumoff V and Hallman M: Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice. *Pediatr Res* 49: 81-83, 2001.
11. Meyerholz DK, Kawashima K, Gallup JM, Grubor B and Ackermann MR: Expression of select immune genes (surfactant proteins A and D, sheep beta defensin 1, and toll-like receptor 4) by respiratory epithelia is developmentally regulated in the preterm neonatal lamb. *Dev Comp Immunol* 30: 1060-1069, 2006.
12. Mestas J and Hughes CC: Of mice and not men: Differences between mouse and human immunology. *J Immunol* 172: 2731-2738, 2004.
13. Schneberger D, Caldwell S, Suri SS and Singh B: Expression of toll-like receptor 9 in horse lungs. *Anat Rec (Hoboken)* 292: 1068-1077, 2009.
14. Singh Suri S, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G and Singh B: Expression of toll-like receptor 4 and 2 in horse lungs. *Vet Res* 37: 541-551, 2006.
15. Kambris Z, Hoffmann JA, Imler JL and Capovilla M: Tissue and stage-specific expression of the Tolls in *Drosophila* embryos. *Gene Expr Patterns* 2: 311-317, 2002.
16. Kaul D, Habbel P, Derkow K, Krüger C, Franzoni E, Wulczyn FG, Bereswill S, Nitsch R, Schott E, Veh R, *et al*: Expression of Toll-like receptors in the developing brain. *PLoS One* 7: e37767, 2012.



17. Meng D, Zhu W, Shi HN, Lu L, Wijendran V, Xu W and Walker WA: Toll-like receptor-4 in human and mouse colonic epithelium is developmentally regulated: A possible role in necrotizing enterocolitis. *Pediatr Res* 77: 416-424, 2015.
18. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M and Ragg T: The RIN: An RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* 7: 3, 2006.
19. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
21. Li Y, Wang J, He HY, Ma LJ, Zeng J, Deng GC, Liu X, Engelhardt JF and Wang Y: Immunohistochemical demonstration of airway epithelial cell markers of guinea pig. *Tissue Cell* 43: 283-290, 2011.
22. Ospelt C and Gay S: TLRs and chronic inflammation. *Int J Biochem Cell Biol* 42: 495-505, 2010.
23. Takeda K and Akira S: Toll receptors and pathogen resistance. *Cell Microbiol* 5: 143-153, 2003.
24. Bauer S: Toll-like receptor 9 processing: The key event in Toll-like receptor 9 activation? *Immunol Lett* 149: 85-87, 2013.
25. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guet C, Briere F, Vlach J, Lebecque S, *et al*: Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* 174: 2942-2950, 2005.
26. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA, *et al*: Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature Med* 11: 1173-1179, 2005.
27. O'Neill LA: TLRs play good cop, bad cop in the lung. *Nature Med* 11: 1161-1162, 2005.
28. Sabroe I, Parker LC, Dower SK and Whyte MK: The role of TLR activation in inflammation. *J Pathol* 214: 126-135, 2008.
29. Kannaki TR, Reddy MR, Verma PC and Shanmugam M: Differential Toll-like receptor (TLR) mRNA expression patterns during chicken embryological development. *Animal Biotechnol* 26: 130-135, 2015.
30. Awasthi S, Cropper J and Brown KM: Developmental expression of Toll-like receptors-2 and -4 in preterm baboon lung. *Dev Comp Immunol* 32: 1088-1098, 2008.
31. Armstrong L, Medford AR, Uppington KM, Robertson J, Witherden IR, Tetley TD and Millar AB: Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. *Am J Respir Cell Mol Biol* 31: 241-245, 2004.
32. Prince LS, Dieperink HI, Okoh VO, Fierro-Perez GA and Lallone RL: Toll-like receptor signaling inhibits structural development of the distal fetal mouse lung. *Dev Dyn* 233: 553-561, 2005.