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# Vaccination inhibits TLR2 transcription via suppression of GR nuclear translocation and binding to TLR2 promoter in porcine lung infected with *Mycoplasma hyopneumoniae*

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ABSTRACT

Toll-like receptors (TLRs) and glucocorticoid receptor (GR) act respectively as effectors of innate immune and stress responses. The crosstalk between them is critical for the maintenance of homeostasis during the immune response. Vaccination is known to boost adaptive immunity, yet it remains elusive whether vaccination may affect GR/TLR interactions following infection. Duroc × Meishan crossbred piglets were allocated to three groups. The control group (CC) received neither vaccination nor infection; the non-vaccinated infection group (NI) was artificially infected intratracheally with *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*); while the vaccinated, infected group (VI) was vaccinated intramuscularly with inactivated *M. hyopneumoniae* one month before infection. The clinical signs and macroscopic lung lesions were significantly reduced by vaccination. However, vaccination did not affect the concentration of *M. hyopneumoniae* DNA in the lung. Serum cortisol was significantly decreased in both NI and VI pigs ($P < 0.01$), but only VI pigs demonstrated significantly diminished nuclear GR content. TLRs 1–10 were all expressed in lung, among which TLR2 was the most abundant and was significantly up-regulated ($P < 0.05$) in NI pigs, but not in VI pigs. Accordingly, GR binding to the GR response element on TLR2 promoter was significantly increased ($P < 0.05$) in NI pigs, but not in VI pigs. These results suggest that the inhibition of GR nuclear translocation and binding to the TLR2 promoter, which results in diminished TLR2 expression, is associated with the protective effect of vaccination on *M. hyopneumoniae*-induced lung lesions in the pig.



Abbreviations: 11β-HSD, 11β-hydroxysteroid-dehydrogenases; CCU, color changing unit; ChIP, chromatin immunoprecipitation; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HPA, hypothalamic–pituitary–adrenal, Mycoplasma hyopneumoniaeM. hyopneumoniae; NF-κ, Bnuclear factor κB, p38 MAPKp38 mitogen-activated protein kinase; QPCR, real-time quantitative PCR; TLR, Toll-like receptor; TNF-, alphatumor necrosis factor alpha.

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## 1. Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the etiological agent of mycoplasmal pneumonia of swine, which colonizes respiratory ciliated epithelial cells and induces inflammatory responses (Ross, 1973; Hwang et al., 2011). *M. hyopneumoniae*-induced characteristic pneumonic lesions have been documented in about 4 weeks post experimental infection (Woolley et al., 2012). Various studies have shown that *M. hyopneumoniae* vaccination activates adaptive immune responses against pneumonia (Ross, 1973; Djordjevic et al., 1997). Little information is available, however, regarding the innate immune responses to *M. hyopneumoniae* vaccination and infection.

Respiratory mucosa serves as a physicochemical barrier to protect the host against the invasion of airborne pathogens. The epithelium of the respiratory mucosa can express a class of receptors known as Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (Bals and Hiemstra, 2004). It was reported that *Mycoplasma pneumoniae* can modulate mucin expression via TLR2 signaling in human lung epithelial cells (Chu et al., 2005). Furthermore, TLR2 plays an important role in *M. hyopneumoniae*-activated inflammatory responses in porcine alveolar macrophages (Muneta et al., 2003). Mycoplasma lipopeptide-induced cytokine production is impaired in TLR2-deficient macrophages of mice (Takeuchi et al., 2000). Therefore, activation of *M. hyopneumoniae*-induced inflammation is thought to be mainly mediated by TLR2.

It is well known that the hypothalamic–pituitary–adrenal (HPA) axis is involved in the modulation of the innate immune response. Glucocorticoid receptor (GR), as the effector of stress responses, can interfere with TLR signaling and exert a positive or negative role in innate immune responses (Bornstein et al., 2006; Chinenov and Rogatsky, 2007). A number of studies indicate that GR inhibits TLR signaling to suppress inflammation (Hodgson et al., 2005; Ogawa et al., 2005), through a complex network of protein: protein and protein: DNA interactions (Biddie et al., 2012). Surprisingly, the GR is also found to exert a positive role in innate immune responses. Glucocorticoids (GCs) are reported to enhance *Haemophilus influenzae*-induced TLR2 expression in HeLa and primary human airway epithelial cells via inactivating p38 mitogen-activated protein kinase (p38 MAPK), the negative regulator for TLR2 expression (Imasato et al., 2002). Moreover, GCs cooperate with tumor necrosis factor alpha (TNF-alpha) to activate TLR2 promoter and induce TLR2 expression in lung epithelial cells through a complex regulatory machinery, which involves a 3′ nuclear factor κB (NF-κB) site, a STAT-binding element, and a 3′ glucocorticoid response element (GRE) (Hermoso et al., 2004). It is presumed that *M. hyopneumoniae* vaccination and infection may affect HPA activity and cortisol concentration, which may in turn modulate TLR2 expression through GR-mediated pathways. The nature of GR-mediated transcriptional regulation of TLR2 in the lung following *M. hyopneumoniae* infection with or without vaccination, however, has not been investigated in in vivo models.

Therefore, we used a pig model to describe, first, the expression profile of all the TLRs (1–10) in the porcine lung; second, to investigate the alteration of TLR2 and GR expression in the lung in response to *M. hyopneumoniae* infection with or without vaccination; and third, to clarify whether GR is involved in the transcriptional regulation of TLR2 in the lung of pigs exposed to *M. hyopneumoniae* infection with or without vaccination.

## 2. Materials and methods

### 2.1. M. hyopneumoniae and vaccine preparations

The strain used for challenge, JS strain, was isolated from pigs (showing clinical symptoms of *M. hyopneumoniae* infection such as chronic coughing and reduced productivity) in China in 1995 by Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, China. The virulence of the strain was determined in an infection experiment as previously described (Vicca et al., 2003), and the dose which induced characteristic symptoms of *M. hyopneumoniae* infection was used in the present study. The strain used for vaccination, NJ strain, was isolated from pigs (showing subclinical course of *M. hyopneumoniae* infection) in China in 2004 by Jiangsu Academy of Agricultural Sciences and preserved in China Center for Type Culture Collection (CCTCC) with the accession number CCTCC M2012286.

The *M. hyopneumoniae* isolates were cultured in modified Friis medium. The vaccination strain was inactivated with thimerosal for 24 h and used for immunization. The vaccine was prepared by mixing $10^9$ color changing unit (CCU) of inactivated *M. hyopneumoniae* with 0.75 mL white oil, and adjusted to the volume of 1 mL.

### 2.2. Animals and experimental design

Fifteen 15-day-old Duroc × Meishan crossbred piglets from 5 litters (3 piglets per litter) were randomly assigned to control (CC), non-vaccinated infected (NI), and vaccinated infected (VI) groups. Each group consisted of 3 males and 2 females. All the pigs were obtained from a commercial herd verified free of several specific pathogens. The herd had been monitored regularly during the last 5 years for the infection of *M. hyopneumoniae* and PRRSV in sows and pigs of different age groups. No clinical and pathological evidences were found for the presence of these two pathogens. *M. hyopneumoniae* was detected by a nested-PCR and a commercial ELISA assay (IDEXX Laboratories, Westbrook, ME). PRRSV was checked with RT-PCR and a commercial ELISA assay (IDEXX Laboratories, Westbrook, ME). Porcine circovirus 2 was verified by PCR. The pigs were not vaccinated with *M. hyopneumoniae* vaccine. The piglets were acclimatized for a week and were fed a commercial feed with no antimicrobials throughout the trial. The VI pigs were injected intramuscularly in the neck with 1 mL *M. hyopneumoniae* vaccine. Pigs in the other two groups were mock-vaccinated with saline in the same manner. One month later, both VI and NI pigs were inoculated intratracheally with 2 mL of the challenge

**Table 1**
Nucleotide sequences of specific primers.

| Genes | GenBank accession no. | Primer sequences |
|---|---|---|
| P97 gene | CP002274.1 | Primer: 5′-CCAGAACCAAATTCCTTCGCTG-3′<br>5′-ACTGGCTGAACTTCATCTGGGCTA-3′<br>Probe: 5′-FAM-AGCAGATCTTAGTCAAAGTGCCCGTG-BHQ-3′ |
| TLR1 | NC_010450 | 5′-GTGTTGCCAATCGCTCAT-3′<br>5′-CAGATTTACTGCGGTGCT-3′ |
| TLR2 | NC_010450 | 5′-GACACCGCCATCCTCATTCT-3′<br>5′-CTTCCCGCTGCGTCTCAT-3′ |
| TLR3 | NC_010457 | 5′-TGCACTAAAACGTGAAGAACTT-3′<br>5′-ATGAAAACACCCTGGAGAGAAC-3′ |
| TLR4 | NC_010443 | 5′-TCTACATCAAGTGCCCCTAC-3′<br>5′-TAAATTCTCCCAAAACCAAC-3′ |
| TLR5 | NM_001123202 | 5′-AGATACCCCTTGTGTGCGA-3′<br>5′-TTCCTTGTGGTGTCCGCTG-3′ |
| TLR6 | NM_213760 | 5′-AGAAAGAAATCTTGAATTTGGA-3′<br>5′-AATGAAGGCTTATGACAGTAGG-3′ |
| TLR7 | NM_001097434 | 5′-TATGGGACCAGGAGCACACAA-3′<br>5′-AAAGAGAACTGCCGATAGGGA-3′ |
| TLR8 | NC_010461 | 5′-CGGTCGCTTCCCACATC-3′<br>5′-CCAGTCCCTCTCCTCCAAAC-3′ |
| TLR9 | NC_010455 | 5′-GGATGTGGGCTGAGGGAG-3′<br>5′-AGGCTTTTGGGGAGGTTG-3′ |
| TLR10 | NC_010450 | 5′-TGTGGTATTGTCATGTCAGTGC-3′<br>5′-AGTTGAAAAAGGAGGTTGTAGG-3′ |
| GAPDH | NC_010447 | 5′-CGTCCCTGAGACACGATGGT-3′<br>5′-CCCGATGCGGCCAAAT-3′ |
| GR | NC_010444 | 5′-TCTGTATGAAAACCTTACTGCT-3′<br>5′-TGTTCTTATCCAAAAATGTCTG-3′ |
| TLR2 promoter | | 5′-ATCCTTAACCCACTGAGCC-3′<br>5′-GCTGTTGCTTTGTAATCCC-3′ |

strain ($10^9$ CCU/mL), and the CC pigs were exposed to saline in the same manner. The pigs were randomly divided into pens in 3 separate rooms equipped with appropriate air filters, and the CC group was retained in a pen distant from the other groups. All pigs were provided with water and food ad libitum. Pigs from each group were euthanized 30 days post infection (dpi). During the study, episodes of coughing were counted daily for 15 min. At slaughter, macroscopic pneumonic lesions were determined using a previously described scoring system (Madec and Kobisch, 1982), with values ranging from 0 (no lesion) to a theoretical maximum of 28 (extensive lung lesions in all lobes). Clinical symptoms, macroscopic lung lesion score, and microscopic lesions were determined by the same person who was blinded to the experiment design. The animal experiment was undertaken following the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China and "the Regulation regarding the Management and Treatment of Experimental Animals" (2008) No.45 set by the Jiangsu Provincial People's Government.

### 2.3. Sample collection

The lobus apicalis of the right lung was removed and the samples were snap frozen in liquid nitrogen and stored at −70 °C. Blood was collected on 10, 20, 28 dpi from the precaval vein and transferred into 10 mL tubes. Blood samples were centrifuged at 1500 × *g* for 15 min at 4 °C and the serum samples were stored at −20 °C.

### 2.4. Serum cortisol levels

Serum cortisol concentrations were measured in duplicate using a commercial $^{125}$I-RIA kit (Beijing Research Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. The detection limit was 2 ng/mL, and the intra- and inter-assay coefficients of variations were 10% and 15%, respectively. All samples were measured in the same assay to avoid inter-assay variations.

### 2.5. Real-time quantitative PCR (QPCR)

Briefly, total RNA was extracted from lung tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. From each sample 1 μg total RNA was converted to cDNA by using PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China) that includes a step for the elimination of genomic DNA. All oligonucleotide primers for QPCR were synthesized by Invitrogen. The primers used are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as a reference gene for normalization. QPCR reactions were performed using the SYBR Green QPCR Master Mix (TAKARA, Japan) with a Mx3000P QPCR system (Stratagene, USA). A subsequent step to generate a dissociation curve was used to verify that a single amplicon was generated.

*M. hyopneumoniae* DNA was quantitated in lung samples by QPCR using specific TaqMan probe as described previously (Guimaraes et al., 2011). Briefly, genomic DNA was extracted from 25 mg lung tissue using a DNA

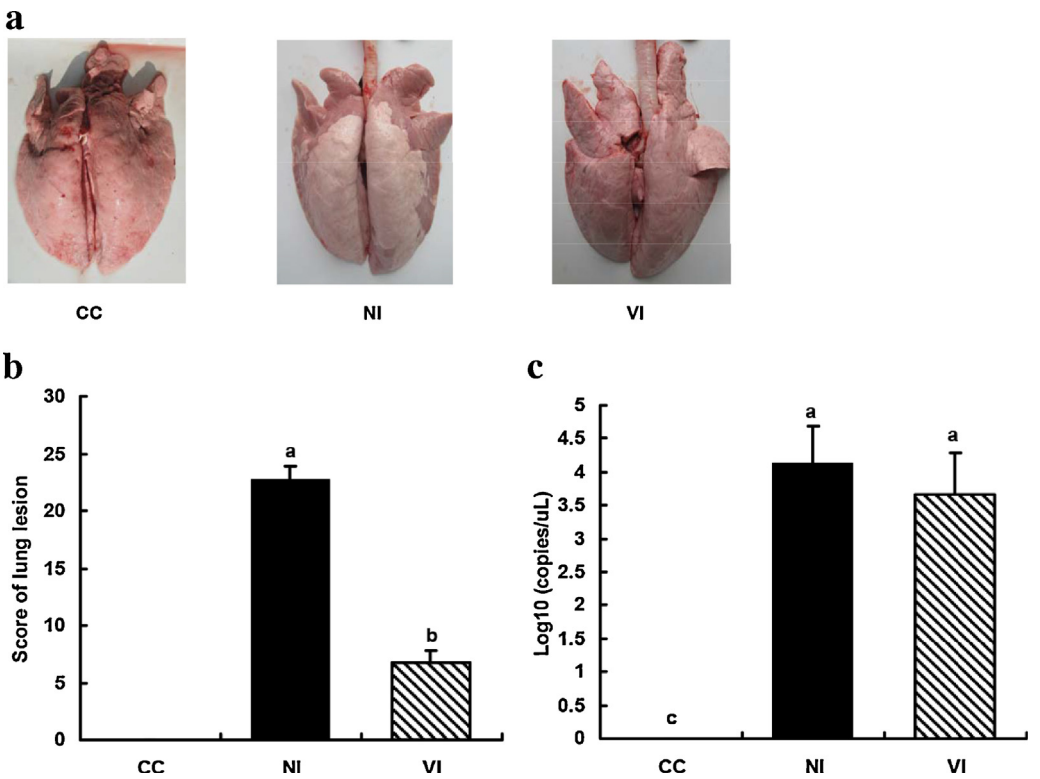


**Fig. 1.** Macroscopic lesions (a), macroscopic lesion score (b) and *M. hyopneumoniae* DNA concentration (c) in the lung tissue. Lung samples were collected on 30 days post infection, and macroscopic scoring was performed as previously described (Madec and Kobisch, 1982). *M. hyopneumoniae* DNA concentration was detected by QPCR. Means with different letters (a, b) are significantly different ($P < 0.05$) from each other, $n = 5$.

extraction kit (Tiangen, Beijing, China). The primers and the TaqMan probe were designed based on the conserved regions of *M. hyopneumoniae* P97 gene (Table 1) and synthesized by Genscript, Nanjing, China. QPCR was performed with the ABI 7500 PCR Instrument (Applied Biosystems, USA).

### 2.6. Nuclear and cytosolic protein extracts

Nuclear and cytosolic protein extracts were prepared from lung tissues of pigs as previously described (Rudiger et al., 2002), with the protease inhibitor cocktail (Roche Applied Science), prepared fresh and added to the lysate buffers. The protein concentration was measured by using Pierce BCA protein assay kit (Thermo Scientific, USA).

### 2.7. Western blot analysis

Nuclear or cytosolic protein extracts from lung tissues, were denatured by boiling for 5 min. Protein (60 μg) was electrophoresed in a 7.5% or 10% SDS-PAGE, transferred onto nitrocellulose membrane, blocked with

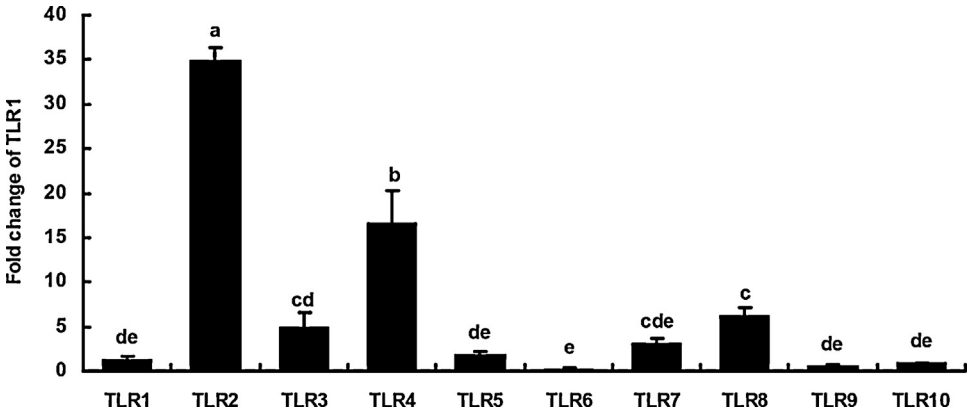


**Fig. 2.** Levels of TLR mRNA in normal porcine lung tissue. The mRNA abundance was determined by real-time QPCR. GAPDH mRNA was chosen as a reference for normalization. Means with different letters (a, b, c, d) are significantly different ($P < 0.05$) from each other, $n = 5$.

3% nonfat milk in Tris buffer (pH 7.6) with 0.1% Tween 20, then incubated overnight at 4 °C with a primary antibody GR (sc-1004, Santa Cruz Biotechnology, Santa Cruz, CA), or TLR2 (sc-166900, Santa Cruz Biotechnology, Santa Cruz, CA). Histone H1 (BS1656, Bioworld Technology, MN, USA) or β-actin (AP0060, Bioworld Technology, MN, USA) was used as a loading control. The membranes were incubated with a second horseradish peroxidase-linked antibody for 2 h, and detected using chemiluminescence.

### 2.8. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to previous publications (Chen et al., 2004; Zheng et al., 2011) with some modifications. Briefly, approximately 200 mg frozen tissue from each lung sample was ground in liquid nitrogen and resuspended in PBS containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannhein, Germany). The suspended lung tissues were fixed with 1% formaldehyde at room temperature for 20 min. Fixation was stopped by adding glycine to the mixture. Thereafter the cross-linked lung tissues were washed with PBS and lysed with SDS lysis buffer containing protease inhibitors. The crude chromatin preparations were sonicated for 3 min (Sonics Vibra) on ice with 10 s on/off intervals to an average DNA length of approximately 500 bp. After preclearance for 1 h at 4 °C with 40 μL protein-A/G plus agarose beads (sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA), chromatin samples were immunoprecipitated overnight at 4 °C using 2 μg of antibody raised against GR (sc-1004x; Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Inc.). Preblocked protein-A/G plus agarose beads was added (40 μL) with chromatin and rotated for 2 h at 4 °C. Input and immunoprecipitated chromatin were incubated for 5 h at 65 °C in the presence of 200 mM NaCl to reverse the cross-links. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA templates precipitated by the GR antibody and normal rabbit IgG were subjected to QPCR. The primer pairs spanning the GR-binding sites of the proximal promoter of the TLR2 are listed in Table 1. The amount of GR precipitated DNA was calculated relative to the normal IgG precipitated DNA and presented as the fold change relative to the average value of the control group (CC).

### 2.9. Statistical analysis

Descriptive statistics were performed to check the normality and homogeneity of variances before using parametric analyses. *M. hyopneumoniae* DNA concentrations and cortisol concentrations were not normally distributed. Therefore, $Log_{10}$ transformation was performed for the two results before statistical analysis. Transformed cortisol concentrations were analyzed by repeated measures ANOVA in the General Linear Model (GLM) procedure of SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The model included the effects of treatment, time and treatment × time interaction. Statistical significance

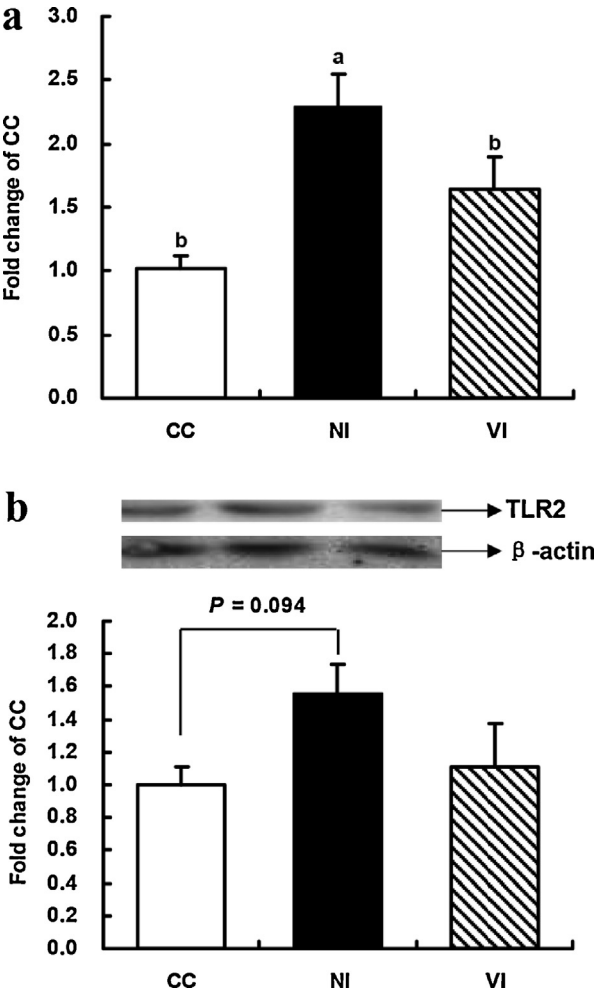


**Fig. 3.** Expression of TLR2 mRNA (a) and protein (b) in porcine lung tissue. β-Actin was chosen as a reference for normalization in the Western blot analysis. TLR2 mRNA and protein expression levels are presented as the fold change relative to the average values in the control group. Means with different letters (a, b) are significantly different ($P < 0.05$) from each other, $n = 5$.

of macroscopic pneumonic lesions, mRNA/protein expression, ChIP data and transformed *M. hyopneumoniae* DNA concentration, were assessed by one-way ANOVA. Bonferroni was used as post hoc test. Data of males and females in each group were pooled for all the statistical analyses. Significance level was set at 0.05. Data are expressed as mean ± SEM.

## 3. Results

### 3.1. Clinical symptoms, lung lesions and M. hyopneumoniae DNA concentrations

Pigs in NI group began coughing on 10 dpi. Dry and nonproductive coughing typical of *M. hyopneumoniae* infection was observed on 28 dpi in all the NI pigs. In contrast, coughing was observed in two of the five VI pigs on 28 dpi. None of uninfected control pigs showed any

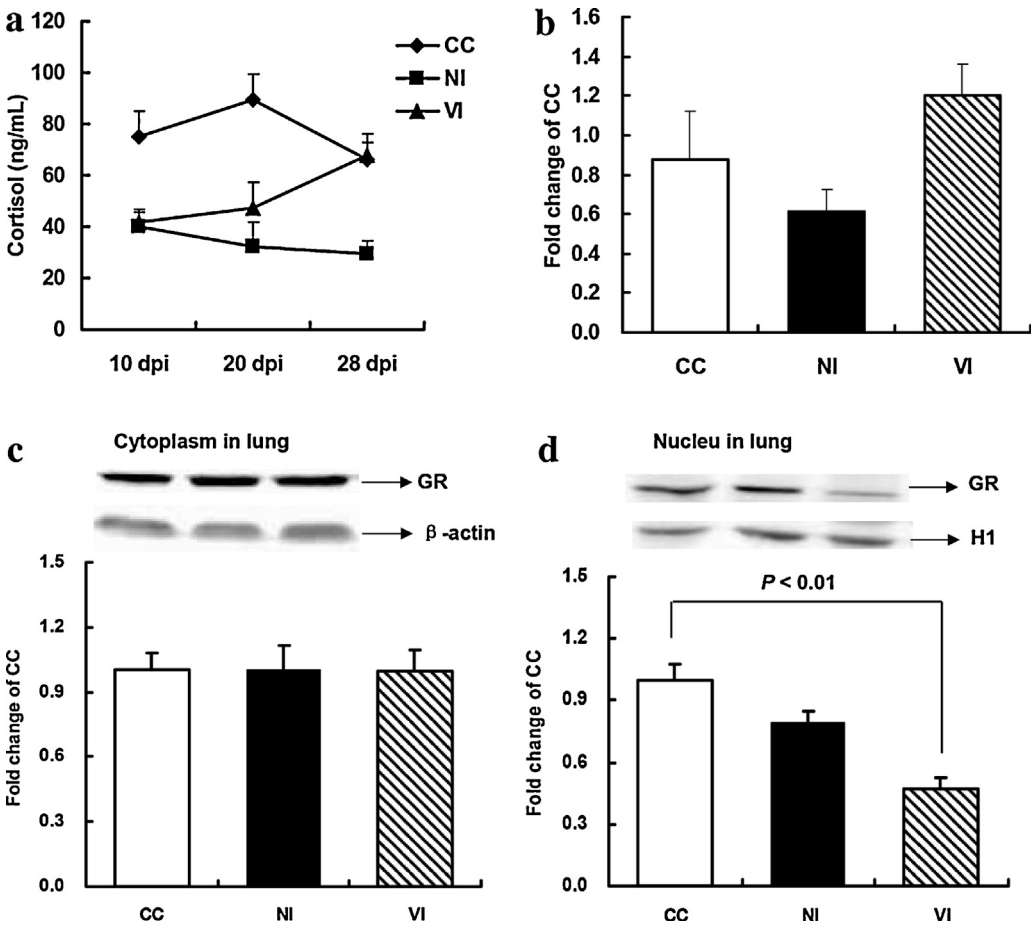


**Fig. 4.** Serum cortisol concentrations (a); GR mRNA (b), cytoplasmic (c) and nuclear (d) GR protein content in porcine lung tissue. Serum was collected on 10, 20, and 28 dpi. Serum cortisol concentrations were measured using a commercial $^{125}$I-RIA kit. The *P*-values represent the main effects of treatment and sampling time, as well as and the interaction between treatment and time. β-Actin was chosen as a loading control for the normalization of cytoplasmic protein, while histone H1 was used for normalizing the loading of nuclear protein. GR mRNA and protein expression levels are presented as the fold change relative to the average values in the control group.

clinical signs. As shown in Fig. 1a, no visible pneumonia was observed in CC group and pigs in VI group showed significantly reduced mycoplasmal lesions, whereas pigs in NI group manifested more severe pneumonia lesions typical of mycoplasmal infection consisting of well-demarcated purple to gray areas of cranioventral consolidation. In line with the macroscopic observations, the lung lesion score was significantly higher ($P < 0.05$) in NI pigs as compared with VI pigs (Fig. 1b). QPCR did not detect any mycoplasmal DNA in CC pigs, and both NI and VI pigs showed significantly higher ($P < 0.01$) concentration of *M. hyopneumoniae* DNA in lung samples ($10^{4.14}$ and $10^{3.67}$ copies/μL, respectively) as compared to CC pigs (Fig. 1c).

### 3.2. TLR mRNA and protein levels in pulmonic tissues

All the 10 TLRs (TLR1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) were found to be expressed in pig lung tissue (Fig. 2). TLR2 was the most abundantly expressed TLR, which was followed by TLR4. TLR2 mRNA expression was significantly up-regulated in NI pigs ($P < 0.01$), which was suppressed by vaccination in VI pigs ($P < 0.05$; Fig. 3a). Western blot analysis demonstrated a similar trend of changes ($P = 0.094$) in TLR2 protein (Fig. 3b).

### 3.3. Serum cortisol concentration

*M. hyopneumoniae* infection, regardless of vaccination pretreatment, caused significant decrease ($P < 0.01$) in serum cortisol levels, compared to the CC group (Fig. 4a). Serum cortisol in VI pigs was significantly lower ($P < 0.01$) compared with CC pigs (52.2 vs. 76.8 ng/mL), yet the lowest cortisol level was detected in NI pigs (33.8 ng/mL, $P < 0.01$ vs. VI group; $P < 0.01$ vs. CC group). No significant interactions between treatment and time were observed on cortisol concentrations.

### 3.4. GR mRNA and protein levels in pulmonic tissues

No significant differences were seen in GR mRNA expression (Fig. 4b) or cytoplasmic GR protein content (Fig. 4c) in lung tissue among all the three groups. The nuclear content of GR (Fig. 4d), however, was significantly

decreased ($P < 0.01$) in VI group, indicating an inhibitory effect of vaccination on GR nuclear translocation.

### 3.5. Binding of GR to the TLR2 promoter

A GRE was predicted in the TLR2 promoter by TRANSFAC® Public 6.0 (Fig. 5a). The putative binding site (5′-tgaacacattaagcc-3′) was located from −1152 to −1138 bp before the first exon. ChIP analysis revealed a significantly increased ($P < 0.05$) GR binding to the sequence containing the GRE on the TLR2 promoter in NI pigs, but not in VI pigs (Fig. 5b).

## 4. Discussion

Clinical observations, as well as macroscopic studies, demonstrated that models of *M. hyopneumoniae* infection and effective vaccine protection were successfully established in the present study. Vaccinated pigs showed minor lung lesions after *M. hyopneumoniae* challenge, which is in agreement with previous publication that inactivated vaccine was effective in protecting against *M. hyopneumonia*-induced lung lesions (Reynolds et al., 2009). It is noted that vaccination was not able to inhibit the multiplication of *M. hyopneumoniae*, as the concentration of *M. hyopneumoniae* DNA in lung samples was not affected. Similar results were reported in a previous publication (Reynolds et al., 2009). Actually, a time-dependent trend of increase in antibody titers was detected in both NI and VI pigs in the present study (data not shown). This observation supports the previous notion that antibody response against *M. hyopneumoniae* was not associated with severity of the lung lesions (Djordjevic et al., 1997).

TLR1, 6, 10 (Shinkai et al., 2006), TLR2 (Alvarez et al., 2008), TLR4 (Thomas et al., 2006) and TLR9 (Tohno et al., 2006) have been reported to be expressed in porcine lungs in various studies. Although the tissue distribution for each TLR mRNA has been recently reported in pigs (Veeresh et al., 2012), the profile of all the 10 TLRs expressed in a specific tissue is not clear. Here we describe, for the first time, the expression profile of all the 10 TLRs in porcine lung, using semi-quantitative real time PCR. TLR2 was found to be the most abundantly expressed TLR in porcine lung, indicating its important role in regulating the innate immunity in the lung. It should be noted, however, that lung tissue contains various cell types, including lymphocytes and macrophages. Therefore, the profile of TLRs expression shown here represents a collective state of TLRs expression in the pulmonic tissue with mixed cell types.

*Mycoplasma pneumoniae*-induced activation of TLR2 signaling, which was tightly associated with the severity of the airway damage, has been documented in mice and

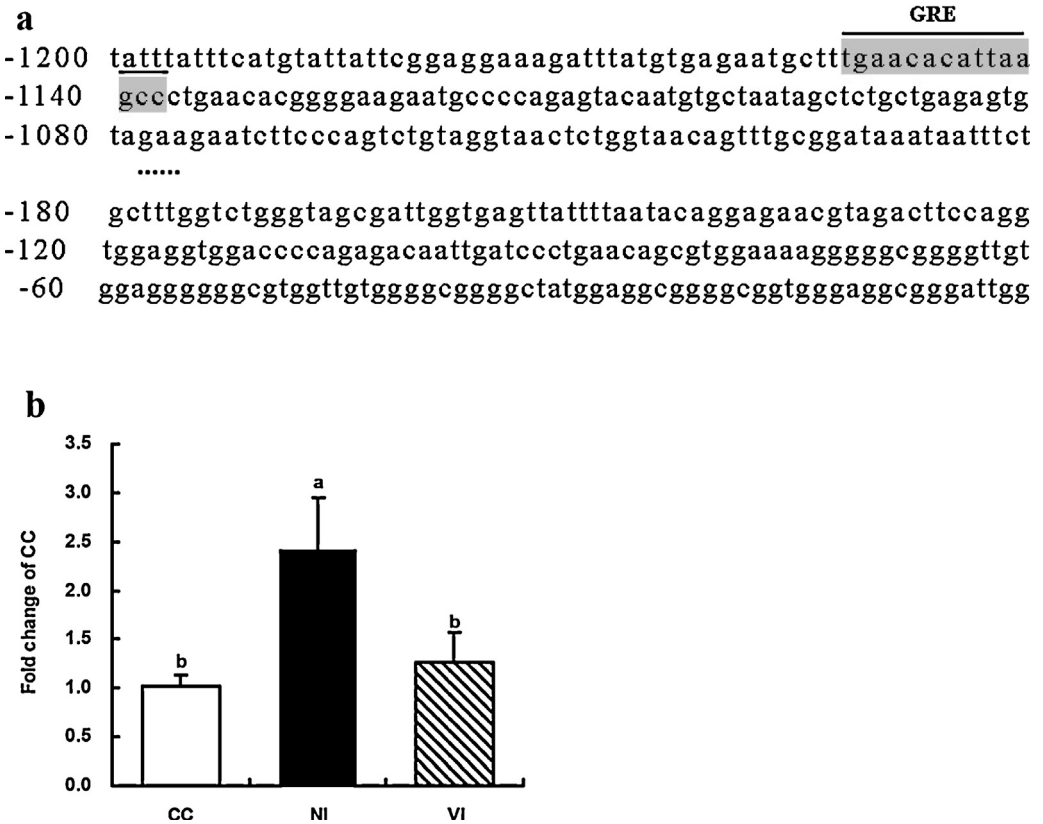


**Fig. 5.** Predicted GR binding site on the TLR2 gene promoter (a) and GR binding to the TLR2 promoter (b). The putative GR binding site (5′-tgaacacattaagcc-3′) is located from −1152 to −1138 bp before the first exon of TLR2 gene, and is underlined. The primer pairs for ChIP-PCR span the GR-binding site. The ChIP results were calculated relative to the normal IgG precipitated DNA and presented as the fold change relative to the average value of the control group (CC). Means with different letters (a, b) are significantly different ($P < 0.05$) from each other, $n = 5$.

human lung epithelial cells (Chu et al., 2005), as well as epithelial cells isolated from asthmatic patients (Kraft et al., 2008), Similarly, TLR2 was reported to be critical in *M. hyopneumoniae*-induced inflammatory responses in porcine alveolar macrophages (Muneta et al., 2003). As expected, *M. hyopneumoniae* infection significantly increased TLR2 mRNA expression in the mucosa of porcine lung. Vaccination prevented the transcriptional activation of TLR2 induced by *M. hyopneumoniae* infection in VI pigs. To our knowledge, this is the first report that vaccination prevented the *M. hyopneumoniae*-induced up-regulation of TLR2 mRNA expression. The vaccination-induced down-regulation of TLR2 expression may prevent the over-activation of inflammation, and thus protect the lung from *M. hyopneumoniae*-induced injury.

The HPA axis is known to be activated by a variety of bacterial, viral and inflammatory insults through immune-endocrine interactions. The resultant glucocorticoid secretion and GR-mediated action are critical for the maintenance of homeostasis in immune and inflammatory responses to pathogen invasion (Chinenov and Rogatsky, 2007). Although an interaction between infectious agents and GR signaling pathway has been reported (Bailey et al., 2003), the effect of pathogen infection or vaccination on the GR nuclear translocation has not been fully addressed. Here, we provide evidence that vaccination inhibited GR nuclear translocation which was indicated by significantly decreased nuclear content of the GR protein. Classically, GC mediated-GR nuclear translocation is a critical step for their anti-inflammatory effects, yet in the present study, opposite results were found. Decreased GR nuclear translocation is actually associated with enhanced protection from *M. hyopneumoniae*-induced lung injury in VI pigs. In this study, serum cortisol level was not closely coupled with GR nuclear translocation, which indicates the complexity of GC signaling. The secretion of GC is pulsatile in nature. In mice, plasma level of corticosterone peaks approximately every hour (Biddie et al., 2012). GR translocates into the nucleus upon GC exposure, and GR binds to GREs of its target genes when the GC level reaches the peak (Biddie et al., 2012). Therefore, the GC/GR interaction is highly dynamic. Single time point sampling, as in the present study, may produce high individual variations due to unsynchronized GC pulsatility. Moreover, cortisol in the peripheral circulation may be metabolized locally in the tissue by 11β-hydroxysteroid-dehydrogenases (11β-HSD) 1 and 11β-HSD2 which activates and inactivates GCs respectively (Holmes et al., 2003). Therefore, the serum cortisol level does not reflect the active cortisol concentration in its target tissues.

Despite all the complexities discussed above, we detected significantly increased GR binding to TLR2 promoter in NI pigs, which is associated with up-regulation in TLR2 expression in lung tissue. The positive role of GR in the regulation of TLR2 transcription found in this study agrees with a previous report that GR activated TLR2 gene promoter activity in cooperation with tumor necrosis factor alpha in human lung adenocarcinoma A549 cells (Hermoso et al., 2004). Additionally, GC enhances *Haemophilus influenza*-induced TLR2 expression in human epithelial cells (Shuto et al., 2002). As both previous studies were carried out on cell models in vitro, our findings provide the first in vivo evidence that the GR also plays a role in activating the innate immune response, in addition to its well established role of anti-inflammation and immune-depression. Interestingly, vaccination blocked *M. hyopneumoniae*-induced increase of GR binding to TLR2 promoter, and consequently attenuated the activation of TLR2 expression.

In this study, GR nuclear translocation was not altered in NI pigs, yet GR binding to the TLR2 gene promoter was significantly increased. The uncoupling of nuclear GR protein content and GR binding is, however, not a unique observation. It was reported that respiratory syncytial virus infection represses GR-mediated gene activation by inhibiting GR binding, without affecting GR nuclear translocation in A549 cells (Hinzey et al., 2011). Several possibilities may be considered to understand the uncoupling of GR nuclear translocation and binding to the TLR2 promoter. First, GR binding to the promoter of its target gene is dynamic and is dependent on the pulsatile pattern of GC secretion (Biddie et al., 2012). Second, GR is involved in transactivation or transrepression of a wide array of its downstream genes in the manner of GR-DNA binding or protein-protein interaction (Biddie et al., 2012). Therefore, *M. hyopneumoniae*-induced GR recruitment to the TLR2 promoter only represents a single event in GR signaling and certainly may not be closely correlated to the total GR content in the nucleus. Third, the active form of the GR in the nucleus is thought to be phosphorylated (Duma et al., 2006), while the GR determined in the present study represents the total GR. Finally, it is reported that *Toxoplasma gondii* inhibits TNF-alpha transcription by interfering with chromatin remodeling events (Leng et al., 2009) and some virally encoded enzymes have been documented to have chromatin remodeling actions (Wei and Zhou, 2010), which thereby regulates GR accessibility to DNA. Thus, there is a possibility that *M. hyopneumoniae* infection enhances GR-mediated TLR2 gene activation by disrupting the chromatin remodeling mechanism. Further studies are required to unravel these potential mechanisms.

In summary, we have shown that *M. hyopneumoniae*-induced lung lesions are associated with increased GR binding to TLR2 promoter and thereby the activation of TLR2 expression. Vaccination protected the pigs from *M. hyopneumoniae*-induced lung lesions, which is associated with diminished GR nuclear translocation and binding to TLR2 promoter, which result in the inhibition of TLR2 activation. Further investigations are needed to delineate the complex regulatory network of GR and TLR2 and its role in keeping the optimal balance in immune response to achieve the protection of pigs from *M. hyopneumoniae*-induced lung injury.

## Conflict of interest

None of the authors have a conflict of interest.

## Authors' contributions

Z.Y.S. and M.J.L. designed the set of experiments, analyzed data, and wrote the paper. H.F.Z., and X.L.

performed specific research protocols and analyzed data; G.Q.S. contributed to the design of the animal experiments. R.Q.Z. contributed to experimental concepts and design, provided scientific direction, analyzed and interpreted the results, and finalized the manuscript.

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## References

Alvarez, B., Revilla, C., Domenech, N., Perez, C., Martinez, P., Alonso, F., Ezquerra, A., Domiguez, J., 2008. Expression of toll-like receptor 2 (TLR2) in porcine leukocyte subsets and tissues. Vet. Res. 13–39.

Bailey, M., Engler, H., Hunzeker, J., Sheridan, J.F., 2003. The hypothalamic–pituitary–adrenal axis and viral infection. Viral Immunol. 16, 141–157.

Bals, R., Hiemstra, P.S., 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur. Respir. J. 23, 327–333.

Biddie, S.C., Conway-Campbell, B.L., Lightman, S.L., 2012. Dynamic regulation of glucocorticoid signalling in health and disease. Rheumatology 51, 403–412.

Bornstein, S.R., Ziegler, C.G., Krug, A.W., Kanczkowski, W., Rettori, V., McCann, S.M., Wirth, M., Zacharowski, K., 2006. The role of toll-like receptors in the immune-adrenal crosstalk. Ann. N. Y. Acad. Sci. 1088, 307–318.

Chen, H., Pan, Y.X., Dudenhausen, E.E., Kilberg, M.S., 2004. Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. J. Biol. Chem. 279, 50829–50839.

Chinenov, Y., Rogatsky, I., 2007. Glucocorticoids and the innate immune system: crosstalk with the toll-like receptor signaling network. Mol. Cell. Endocrinol. 275, 30–42.

Chu, H.W., Jeyaseelan, S., Rino, J.G., Voelker, D.R., Wexler, R.B., Campbell, K., Harbeck, R.J., Martin, R.J., 2005. TLR2 signaling is critical for *Mycoplasma pneumoniae*-induced airway mucin expression. J. Immunol. 174, 5713–5719.

Djordjevic, S.P., Eamens, G.J., Romalis, L.F., Nicholls, P.J., Taylor, V., Chin, J., 1997. Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Aust. Vet. J. 75, 504–511.

Duma, D., Jewell, C.M., Cidlowski, J.A., 2006. Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J. Steroid Biochem. Mol. Biol. 102, 11–21.

Guimaraes, A.M., Vieira, R.F., Poletto, R., Vemulapalli, R., Santos, A.P., de Moraes, W., Cubas, Z.S., Santos, L.C., Marchant-Forde, J.N., Timenetsky, J., Biondo, A.W., Messick, J.B., 2011. A quantitative TaqMan PCR assay for the detection of *Mycoplasma suis*. J. Appl. Microbiol. 111, 417–425.

Hermoso, M.A., Matsuguchi, T., Smoak, K., Cidlowski, J.A., 2004. Glucocorticoids and tumor necrosis factor alpha cooperatively regulate toll-like receptor 2 gene expression. Mol. Cell. Biol. 24, 4743–4756.

Hinzey, A., Alexander, J., Corry, J., Adams, K.M., Claggett, A.M., Traylor, Z.P., Davis, I.C., Webster Marketon, J.I., 2011. Respiratory syncytial virus represses glucocorticoid receptor-mediated gene activation. J. Endocrinol. 152, 483–494.

Hodgson, P.D., Aich, P., Manuja, A., Hokamp, K., Roche, F.M., Brinkman, F.S., Potter, A., Babiuk, L.A., Griebel, P.J., 2005. Effect of stress on viral-bacterial synergy in bovine respiratory disease: novel mechanisms to regulate inflammation. Comp. Funct. Genomics 6, 244–250.

Holmes, M.C., Yau, J.L., Kotelevtsev, Y., Mullins, J.J., Seckl, J.R., 2003. 11 Beta-hydroxysteroid dehydrogenases in the brain: two enzymes two roles. Ann. N. Y. Acad. Sci. 1007, 357–366.

Hwang, M.H., Damte, D., Lee, J.S., Gebru, E., Chang, Z.Q., Cheng, H., Jung, B.Y., Rhee, M.H., Park, S.C., 2011. *Mycoplasma hyopneumoniae* induces pro-inflammatory cytokine and nitric oxide production through NFkappaB and MAPK pathways in RAW264.7 cells. Vet. Res. Commun. 35, 21–34.

Imasato, A., Desbois-Mouthon, C., Han, J., Kai, H., Cato, A.C., Akira, S., Li, J.D., 2002. Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable *Haemophilus influenzae*-induced expression of toll-like receptor 2. J. Biol. Chem. 277, 47444–47450.

Kraft, M., Adler, K.B., Ingram, J.L., Crews, A.L., Atkinson, T.P., Cairns, C.B., Krause, D.C., Chu, H.W., 2008. *Mycoplasma pneumoniae* induces airway epithelial cell expression of MUC5AC in asthma. Eur. Respir. J. 31, 43–46.

Leng, J., Butcher, B.A., Egan, C.E., Abi Abdallah, D.S., Denkers, E.Y., 2009. *Toxoplasma gondii* prevents chromatin remodeling initiated by TLR-triggered macrophage activation. J. Immunol. 182, 489–497.

Madec, F., Kobisch, M., 1982. Bilan lesionnel de poumons de porcs charcutiers a l'abattoir. J. Rech. Porcine 14, 405–412.

Muneta, Y., Uenishi, H., Kikuma, R., Yoshihara, K., Shimoji, Y., Yamamoto, R., Hamashima, N., Yokomizo, Y., Mori, Y., 2003. Porcine TLR2 and TLR6: identification and their involvement in *Mycoplasma hyopneumoniae* infection. J. Interferon Cytokine Res. 23, 583–590.

Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R.K., Westin, S., Hoffmann, A., Subramaniam, S., David, M., Rosenfeld, M.G., Glass, C.K., 2005. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. Cell 122, 707–721.

Reynolds, S.C., St Aubin, L.B., Sabbadini, L.G., Kula, J., Vogelaar, J., Runnels, P., Peters, A.R., 2009. Reduced lung lesions in pigs challenged 25 weeks after the administration of a single dose of *Mycoplasma hyopneumoniae* vaccine at approximately 1 week of age. Vet. J. 181, 312–320.

Ross, R.F., 1973. Pathogenicity of swine mycoplasmas. Ann. N. Y. Acad. Sci. 225, 347–368.

Rudiger, J.J., Roth, M., Bihl, M.P., Cornelius, B.C., Johnson, M., Ziesche, R., Block, L.H., 2002. Interaction of C/EBPalpha and the glucocorticoid receptor in vivo and in nontransformed human cells. FASEB J. 16, 177–184.

Shinkai, H., Muneta, Y., Suzuki, K., Eguchi-Ogawa, T., Awata, T., Uenishi, H., 2006. Porcine Toll-like receptor 1, 6, and 10 genes: complete sequencing of genomic region and expression analysis. Mol. Immunol. 43, 1474–1480.

Shuto, T., Imasato, A., Jono, H., Sakai, A., Xu, H., Watanabe, T., Rixter, D.D., Kai, H., Andalibi, A., Linthicum, F., Guan, Y.L., Han, J., Cato, A.C., Lim, D.J., Akira, S., Li, J.D., 2002. Glucocorticoids synergistically enhance nontypeable *Haemophilus influenzae*-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. J. Biol. Chem. 277, 17263–17270.

Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Muhlradt, P.F., Akira, S., 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J. Immunol. 164, 554–557.

Thomas, A.V., Broers, A.D., Vandegaart, H.F., Desmecht, D.J., 2006. Genomic structure, promoter analysis and expression of the porcine (*Sus scrofa*) TLR4 gene. Mol. Immunol. 43, 653–659.

Tohno, M., Shimosato, T., Moue, M., Aso, H., Watanabe, K., Kawai, Y., Yamaguchi, T., Saito, T., Kitazawa, H., 2006. Toll-like receptor 2 and 9 are expressed and functional in gut-associated lymphoid tissues of presuckling newborn swine. Vet. Res. 37, 791–812.

Veeresh, B.H., Hiremath, S.V., Patil, S.S., Hemadri, D., Shome, B.R., 2012. Tissues-specific mRNA expression pattern of swine Toll-like receptors (TLRs) 1–10. RRJoVST 1, 14–23.

Vicca, J., Stakenborg, T., Maes, D., Butaye, P., Peeters, J., de Kruif, A., Haesebrouck, F., 2003. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet. Microbiol. 97, 177–190.

Wei, H., Zhou, M.-M., 2010. Viral-encoded enzymes that target host chromatin functions. BBA-Gene Regul. Mech. 1799, 296–301.

Woolley, L.K., Fell, S., Gonsalves, J.R., Walker, M.J., Djordjevic, S.P., Jenkins, C., Eamens, G.J., 2012. Evaluation of clinical, histological and immunological changes and qPCR detection of *Mycoplasma hyopneumoniae* in tissues during the early stages of mycoplasmal pneumonia in pigs after experimental challenge with two field isolates. Vet. Microbiol. 161, 186–195.

Zheng, S., Rollet, M., Pan, Y.X., 2011. Maternal protein restriction during pregnancy induces CCAAT/enhancer-binding protein (C/EBPβ) expression through the regulation of histone modification at its promoter region in female offspring rat skeletal muscle. Epigenetics 6, 161–170.