Activation of innate immune system in response to lipopolysaccharide in chicken Sertoli cells

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Abstract

Sertoli cells (SCs) play an important physiological role in the testis, as they support, nourish, and protect the germ cells. As protection of the developing spermatozoa is an emerging aspect of reproductive physiology, this study examined the expression pattern of innate immune-related genes, including avian β -defensins (AvBDs), Toll-like receptors (TLRs), and cytokines, and investigated the time course of an inflammatory response in rooster SCs triggered by exposure to the bacterial endotoxin lipopolysaccharide (LPS). SCs were isolated from 6-week-old chicken, cultured *in vitro*, and stimulated with 1 μ g/ml LPS at different time courses (0, 6, 12, 24, and 48 h). Data on expression analysis revealed that all ten members of the chicken TLR family, nine members of the AvBD family, as well as eight cytokine genes were expressed in SCs. Quantitative real-time PCR analysis revealed that LPS treatment resulted in significant induction of the expression levels of six TLRs, six AvBDs, and four cytokine genes, while two cytokine genes were downregulated and two other genes were unchanged. The increasing interleukin 1 β (IL1 β) production was confirmed in the conditioned medium. Furthermore, the phagocytosis of SCs was increased after LPS treatment. In conclusion, these findings provide evidence that SCs express innate immune-related genes and respond directly to bacterial ligands. These genes represent an important component of the immune system, which could be integrated into semen, and present a distinctive constituent of the protective repertoire of the testis against ascending infections.

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Introduction

It is now well established that bacterial and viral infections of the male reproductive tract contribute significantly to impaired fertility. The testis is considered an immune-privileged site, and testicular Sertoli cells (SCs) have been identified as key players for conferring this immune privilege (Selawry 1994, Dufour *et al.* 2003). SCs mechanically segregate germ cell autoantigens by means of the blood–tubular barrier and create an effective immune-privileged environment that protects germ cells from invading pathogens (Riccioli *et al.* 2006, Mital *et al.* 2010). Therefore, the protection of germ cells from infections in the seminiferous tubules is of primary importance, because bacterial infections can seriously damage reproductive functions.

The innate immune system utilizes Toll-like receptors (TLRs) to recognize and bind pathogen-associated molecular patterns (PAMPs) (Akira *et al.* 2001). The best studied of the PAMPs is lipopolysaccharide (LPS) derived from Gram-negative bacteria. Binding of PAMPs leads to TLR activation, which, in turn, initiates MAPK- or nuclear factor kappa B (NF κ B)-dependent cascades that culminate in a proinflammatory response. This

response involves the secretion of cytokines and antibacterial substances, such as β -defensins (Froy 2005, Kogut *et al.* 2006). TLRs and β -defensins have been identified as the key components of innate immune recognition in the reproductive tract of male vertebrate species, providing the first line of defense against potential pathogens (Li *et al.* 2001, Com *et al.* 2003, Palladino *et al.* 2003, 2007, Avellar *et al.* 2004, Nishimura & Naito 2005, Rodrigues *et al.* 2008, Anastasiadou *et al.* 2011). Cytokines are essential for the activation, differentiation, and control of the immune system and also play a key role in initiating innate and adaptive immune responses (Hiscott & Ware 2011). Several cytokines have direct effects on testicular cell functions, and a number of these are produced within the testis even in the absence of inflammation or immune activation events (Hedger & Meinhardt 2003, Takeda & Akira 2005, Mogensen 2009).

Many studies have reported the involvement of TLRs, β -defensins, and cytokines in the interactions between the immune and reproductive systems, as well as the involvement of these molecules in the physiology of reproduction and their existence in the male reproductive tract in various vertebrate species (Jia *et al.* 1999,

Com *et al.* 2003, Riccioli *et al.* 2006, Anastasiadou *et al.* 2011). Furthermore, recent studies have reported that mammalian SCs express several *TLRs* and cytokines that can produce proinflammatory and anti-inflammatory effects (Hedger & Meinhardt 2003, Bhushan *et al.* 2009, Guazzone *et al.* 2009). It has also been reported that mouse SCs express several members of the *Tlr* family (Riccioli *et al.* 2006), and that stimulation of *Tlr2*, *Tlr3*, and *Tlr5* by agonists resulted in increased intercellular adhesion molecule 1 (*Icam1*) and monocyte chemoattractant protein 1 (*Mcp1*) expression, as well as the activation of the NF κ B pathway, adding further credential to a central role of the SCs in testicular immunology (Riccioli *et al.* 2006, Starace *et al.* 2008).

Although several microbial pathogens are able to invade and colonize male reproductive tract and semen, in livestock and particularly in avian species antimicrobial protection and function of the innate immune system in the rooster reproductive tract have not yet been studied extensively. Furthermore, immunological function and the defense to infections, particularly to bacteria, are poorly understood in the rooster testis at the molecular level. We have recently reported that several members of the *TLRs* family were significantly upregulated in the testis of sexually mature roosters infected with *Salmonella enteritidis* (Anastasiadou *et al.* 2011). It has also been reported that several members of the avian β -defensin (*AvBDs*) and *TLR* families were expressed in chicken sperm and the expression of four *AvBDs* was significantly increased in the sperm stimulated with LPS (Das *et al.* 2011).

As SCs possess the machinery necessary to start an inflammatory response to microorganisms, the aim of this study was to investigate in rooster SCs the expression pattern of 36 innate immune-related genes, which constitute the main part of the innate immune mechanism, including ten *TLRs*, 14 *AvBDs*, and 12 cytokines, and to determine whether their expression was constitutive or induced as a response to bacterial LPS stimulation.

Materials and methods

Cell culture and challenge with LPS

Reagents used for cell culture preparation have been described previously (Guibert *et al.* 2011). SCs were obtained from 4- to 6-week-old (immature) laying breed chickens (ISA Brown, egg layer type, Institut de Selection Animale, Saint Brieuc, France). All birds were housed under a 14-h light:10-h darkness photoperiod, and feed and water were provided *ad libitum*. All procedures described herein were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Rooster SCs were isolated as described previously (Guibert *et al.* 2011). Cell preparation adopted for chicken species was based on rodent procedures as described in Mather & Phillips (1984) and

Odet *et al.* (2006). SCs were obtained from 4- to 6-week-old laying breed chickens (ISA Brown, egg layer type, Institut de Selection Animale, Saint Brieuc, France). Birds were housed under a 14-h light:10-h darkness photoperiod, and feed and water were provided *ad libitum*. All procedures described herein were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Four chickens were killed for each culture, then testes were recovered and conserved in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-buffered F12/DMEM (Sigma) supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin (Sigma). Testes were decapsulated, slightly minced, and incubated for 15 min at 37 °C with DNase (20 μ g/ml) and type Ia collagenase (0.4 mg/ml) (Sigma). Collagenase was removed by centrifugation and seminiferous tubules were allowed to sediment by gravity in order to separate them from Leydig cells in the supernatant. The pellet containing the seminiferous tubules was digested two times by collagenase (0.6 and 0.8 mg/ml for 15 min at 37 °C for each digestion) and added with DNase (20 μ g/ml). Finally, after centrifugation to remove collagenase, cells were incubated with 0.1% hyaluronidase (Sigma) for 10 min at 37 °C in order to eliminate peritubular cells.

SCs were counted and seeded at a density of 500 000 cells/well in a six-well plate containing F12/DMEM culture medium supplemented with antibiotics and 5% FCS at 37 °C in a humidified atmosphere of 5% CO₂ in air for the first 24 h. Then SCs were cultured in 2% FCS and 24 h later were stimulated with 1 μ g/ml LPS from *Escherichia coli* O111:B4 (Sigma) for 3, 6, 12, 24, or 48 h. LPS is a cell coat component of Gram-negative bacteria and is being used to mimic bacterial infections. The control condition was only culture medium. Conditions of stimulation are described in figure captions. The purity of SCs was ~90% after 48 h of culture. Few residual germ cells were detected (<5%) and the percentage of peritubular myoid cells, evaluated by alkaline phosphatase staining, was lower than 8% of the total cell population.

RNA isolation, RT-PCR, and quantitative real-time PCR analyses

Total RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion, Austin, TX, USA). RT-PCR and quantitative real-time PCR analyses were performed as described previously (Michailidis *et al.* 2010). Quantitative PCR amplification of *TLRs*, *AvBDs*, and cytokine genes was performed as described previously (Ebers *et al.* 2009, Michailidis *et al.* 2010, Abdelsalam *et al.* 2011), using the primers listed in Tables 1, 2 and 3. The cycling parameters were as follows: incubation at 95 °C for 3 min, followed by 45 cycles of incubation at 95 °C for 10 s, 56 °C for 8 s for *TLRs* and cytokines, and 58 °C for 8 s for *AvBDs*, 72 °C for 8 s, read at 60 °C, followed by a melt curve analysis from 65 to 95 °C with reads at intervals of 0.2 °C and 5 s to confirm amplification of single cDNA products.

Phagocytosis assay

The measurement of the phagocytic activity in the rooster SCs was performed using uptake of red fluorescent-labeled latex

Table 1 Primers used to amplify chicken *TLR* genes.

<i>TLRs</i>	Primer pair
<i>TLR1-1</i>	5'-TACTGCAATTGCTTGCAC-3' 5'-GGTTAGGAAGACCGTGTCCA-3'
<i>TLR1-2</i>	5'-CCCGTTCAAGTGTTCATGTG-3' 5'-GTTCGCTCAAGTCTTCTGG-3'
<i>TLR2-1</i>	5'-ACATGTGTGAATGGCCTGAA-3' 5'-TTGAGAAATGGCAGTTGCAG-3'
<i>TLR2-2</i>	5'-TCTCAACACGCTGAGGATTG-3' 5'-AGAGTGCAGAAGGTCCCTGA-3'
<i>TLR3</i>	5'-CCCTGATGGAGTGTTCGTT-3' 5'-CAGCTGTTGCTGCAATCTA-3'
<i>TLR4</i>	5'-GTTTGACATTGCTCGTCTC-3' 5'-GCTGCCTCCAGAAGATATGC-3'
<i>TLR5</i>	5'-TTTCAGGAACCTGCCAATC-3' 5'-TCCAGGATGGAATCTCCAAG-3'
<i>TLR7</i>	5'-GATGCAGTGTGTTTGTGG-3' 5'-AACCAAGCTCCTCCCTTGT-3'
<i>TLR15</i>	5'-ATCCCGAATACTCCATCC-3' 5'-TGGCAGGCAGGTTCTAGTC-3'
<i>TLR21</i>	5'-AGCTGGAGCTGTTGGACCTA-3' 5'-TTCACGTGCCATAGCATCTC-3'

beads of 1 μm (Sigma). Cells were seeded at a density of 5×10^4 cells/well in F12/DMEM medium supplemented with 5% FCS. The following day, chicken primary SCs were treated with 1 $\mu\text{g}/\text{ml}$ LPS for 48 h in F12/DMEM supplemented with 2% FCS, then incubated with fluorescent latex beads (1 μm diameter) for 3 h. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min. Quantification of engulfed beads was represented as a cell surface area occupied by fluorescent beads using the ImageJ Software, to analyze the immunofluorescence image (version 1.43, National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). The results were expressed as the mean value of three different preparations of immature SCs (4- to 6-week-old chicken).

Uptake of red fluorescent-labeled latex beads of 1 μm (Sigma) was used as a measurement of general phagocytic activity of SCs. Briefly, SCs were seeded in chamber slides at a density of 5×10^4 cells/well in F12/DMEM medium supplemented with 5% FCS. The following day, chicken primary SCs were treated with 1 $\mu\text{g}/\text{ml}$ LPS for 48 h in F12/DMEM supplemented with 2% FCS, then incubated with fluorescent latex bead (1 μm diameter) for 3 h. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min. Quantification of engulfed beads was represented as a cell surface area occupied by fluorescent beads using the ImageJ Software (<http://rsb.info.nih.gov/ij/>). At least 200 cells were counted in each condition, and the results were expressed as the mean value of three experiments.

Interleukin 1 β assay

Interleukin 1 β (IL β) concentration in the culture medium was measured using a chicken IL1 β ELISA Kit (CUSABIO, Interchim, France) according to the manufacturer's recommendations. All standards and samples were assayed in duplicate. The minimum detectable dose of chicken IL1 β is <0.5 pg/ml. Values for each condition are the mean $\pm$ S.E.M. of three different preparations of immature SCs (4- to 6-week-old chicken).

Luciferase reporter assays

Chicken primary SCs were infected with lentivirus in the presence of 6 $\mu\text{g}/\text{ml}$ polybrene for 12 h at a Multiplicity Of Infection (MOI) of 20 in HEPES-buffered F12/DMEM medium containing polybrene, overnight. The MOI is the number of transducing lentiviral particles per cell. Lentiviruses are Cignal Lenti NF κ B firefly luciferase reporter (luciferase, Cignal Lenti Reporters, SA Biosciences, Qiagen, Paris, France). After 72 h of cell infection, SCs were treated with 1 $\mu\text{g}/\text{ml}$ of LPS for 3, 6, 12, 24, and 48 h. SCs were trypsinized and counted, and luciferase activity in total cell lysates was measured in a 96-well plate luminometer using the Promega Luciferase Assay Kit (Promega Luciferase Assay Kit, Promega). The luciferase activity was quantified by the light produced and measured by a Luminoskan Ascent Microplate Luminometer (Thermo Scientific, Pittsburgh, PA, USA). Quantification was normalized by the number of cells counted in a well. Values were expressed as relative light units per 10^6 cells and represented as the percentage of control. Experiments were carried out with four different preparations of SCs.

Statistical analyses

Statistical analyses were performed using both parametric and nonparametric methods. The evaluation of the possible significant effects of the different time points of LPS stimulation (0, 6, 12, 24, and 48 h) on the expression of *TLRs*, *AvBDs*, and cytokines was performed using one-way ANOVA. In case of significance, differences between mean values of specific time points were estimated using Duncan's new multiple range test. The nonparametric Kruskal–Wallis and Mann–Whitney *U* tests

Table 2 Primers used to amplify chicken *AvBD* genes.

<i>AvBDs</i>	Primer pair
<i>AvBD1</i>	5'-CGAAAGAGTGGCTTCTGTGC-3' 5'-GGTGATGTCTGCTTGGG-3'
<i>AvBD2</i>	5'-AGGTTTCTCCAGGGTGT-3' 5'-TGCAATCCAAGGCCATT-3'
<i>AvBD3</i>	5'-CCACTCAGTGCAGAATAAGAG-3' 5'-AATTCAGGGCATCAACCTC-3'
<i>AvBD4</i>	5'-CATCTCAGTGTCTTCTCTGC-3' 5'-CGCATATCCAGTATGCATG-3'
<i>AvBD5</i>	5'-CTGCCAGCAAGAAAGGAACCTG-3' 5'-GTAATCTCGAGCAAGGGACA-3'
<i>AvBD6</i>	5'-AGGATTTCACATCCAGCCGTG-3' 5'-CGACATGGCCAGGAATGCAAG-3'
<i>AvBD7</i>	5'-TGGAGAAGGGAGACAGAAGGCA-3' 5'-CGAAGCCTACAAGTATCAAT-3'
<i>AvBD8</i>	5'-ACAGTGTGAGCAGGCAGGAGGGA-3' 5'-GAAGAGCTGCTTGGTCT-3'
<i>AvBD9</i>	5'-GCAAAGGCTATCCACAGCAG-3' 5'-GGAGCACGGCATGCAACAA-3'
<i>AvBD10</i>	5'-TGGGGCAGGCAGTCCACAAC-3' 5'-CATGCCCCAGCAGGAGCAAG-3'
<i>AvBD11</i>	5'-ACTGCATCCGTTCCAAAGTCTG-3' 5'-GTCCAGCTGTTCTCCAG-3'
<i>AvBD12</i>	5'-CCCAGCAGGACCAAGCAATG-3' 5'-AGTACTTAGCCAGGTATTC-3'
<i>AvBD13</i>	5'-CATCGTTGTCTTCTCTCTC-3' 5'-GGTGGAGAAGCTGAGCAGCG-3'
<i>AvBD14</i>	5'-ATGGGCATATTCCTCTGT-3' 5'-CACTTGGCAGTCCATTGT-3'

Table 3 Primers used to amplify chicken cytokine genes.

Cytokines	Primer pair
<i>IL1β</i>	5'-GCATCAAGGGCTACAAGCTC-3' 5'-CAGGCGGTAGAAGATGAAGC-3'
<i>IL2</i>	5'-AACTGGGACACTGCCATGAT-3' 5'-TCCTGGGTCTCAGTTGGTGT-3'
<i>IL4</i>	5'-GAGAGGTTCTCGTCAAG-3' 5'-TGACGCATGTTGAGGAAGAG-3'
<i>IL6</i>	5'-CTCCTCGCCAATCTGAAGTC-3' 5'-GGATTGTGCCCCAACTAAAA-3'
<i>IL8</i>	5'-GATTGAACCTCGATGCCAGT-3' 5'-TCCACATTCTTGCACTGAGG-3'
<i>IL10</i>	5'-GGAGCTGAGGGTGAAGTTTG-3' 5'-TAGAAGCGCAGCATCTCTGA-3'
<i>IL12</i>	5'-CTGTGGCTCGCACTGATAA-3' 5'-GAACATCTCAGTCGGTGGT-3'
<i>IL15</i>	5'-CAGCCAACACAAAACCTCTGC-3' 5'-GGCAAAGAAATGGCTGTC-3'
<i>IL16</i>	5'-ATCGCGAAGCTTCCACTTA-3' 5'-GCGGATGACACCTGGTTACT-3'
<i>IL17</i>	5'-TGCCTAACCCAAAAGATGG-3' 5'-CCAGTGAGCGTTTGCTGATA-3'
<i>IL18</i>	5'-AGAGCATGGGAAAATGGTTG-3' 5'-TCTTCTCAAAGGCCAAGAA-3'
<i>IFNγ</i>	5'-AGCCGCACATCAACACATA-3' 5'-TCCTTTGAACTCGGAGGA-3'

were also applied where the variability or the form of the population distribution was violated, or for the estimation of differences between the mean values of specific time points respectively. Results are expressed as the mean $\pm$ S.E.M.

The paired *t*-test was used to compare treated cells with their corresponding controls (phagocytosis assay). One-way ANOVA was also applied to investigate the effects on *IL1β* secretion and NFκB reporter, while the means were compared by Newman's test where ANOVA revealed significant effects, with $P < 0.05$ considered significant. In all applications, differences were considered significant at $P < 0.05$ and represented with different letters or by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Transcriptional profiling of TLRs, AvBDs, and cytokines in chicken SCs

Expression of *TLRs*, *AvBDs*, and cytokines in SCs was determined by RT-PCR. Expression analysis revealed that all ten members of *cTLR* family, namely *TLR1-1*, *TLR1-2*, *TLR2-1*, *TLR2-2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15*, and *TLR21* (Fig. 1A), nine members of the *AvBD* family, namely *AvBD1-5*, *AvBD9*, *AvBD10*, *AvBD12*, and *AvBD14* (Fig. 1B), as well as eight cytokines, namely *IL1β*, *IL6*, *IL8*, *IL12*, *IL15*, *IL17*, *IL18*, and *IFNγ* (Fig. 1C) were expressed in the rooster SCs.

Time course effect of LPS treatment on gene expression

Quantification of the expression levels of the 27 expressed genes (ten *TLRs*, nine *AvBDs* and eight cytokines) were performed in SCs after 0, 6, 12, 24, or 48 h stimulation with LPS by real-time PCR analysis.

Regarding *TLRs*, six genes were upregulated following LPS stimulation, compared with the control (unstimulated cells, 0 h; Fig. 2). *TLR15* mRNA was increased significantly at 12 h after LPS stimulation and remained significantly ($P < 0.05$) higher compared with the control till 48 h. *TLR4* and *TLR7* showed significantly ($P < 0.05$) higher expression in response to 1 µg/ml LPS at 24 and 48 h, while *TLR1-1*, *TLR2-2*, and *TLR21* mRNA expression was increased significantly ($P < 0.05$) only at 48 h after stimulation with LPS. The highest changes in the expression levels were observed for *TLR1-1* and *TLR15* at 48 h after stimulation, with 8.4- and 8-fold higher expression respectively. *TLR1-2*, *TLR2-1*, *TLR3*, and *TLR5* mRNA expressions remained at the same levels in LPS-stimulated SCs over time.

Stimulation with 1 µg/ml LPS also resulted in a significant upregulation of six *AvBD* genes (Fig. 3). In particular, *AvBD3* mRNA increased significantly ($P < 0.05$) at 6, 12, 24, and 48 h after 1 µg/ml LPS stimulation. *AvBD1*, *AvBD10*, and *AvBD12* mRNAs were significantly ($P < 0.05$) increased after 24 h of LPS stimulation, while *AvBD2* and *AvBD9* mRNA expression was increased significantly ($P < 0.05$) only at 48 h after stimulation with LPS. The highest changes in the expression levels were observed for *AvBD1* and *AvBD9* at 48 h after stimulation, with 11.3- and 10.3-fold higher expression respectively. LPS stimulation did not affect the expression levels of the expressed *AvBD4*, *AvBD5*, and *AvBD14* genes significantly ($P > 0.05$).

Expression levels of cytokines were also affected in response to LPS treatment (Fig. 4). Three cytokine genes, namely *IL1β*, *IL8*, and *IL12*, significantly ($P < 0.05$) upregulated their expression levels at 6 h following the stimulation with LPS and remained constantly higher till 48 h, while expression of *IL6* was significantly increased in response to 1 µg/ml LPS at 24 and 48 h. The highest

Figure 1 Expression pattern of *TLRs*, *AvBDs*, and cytokine genes in chicken primary Sertoli cells (SCs) using RT-PCR. (A) Expression pattern of *TLR* genes. (B) Expression pattern of *AvBD* genes. (C) Expression pattern of cytokines. RT-PCR analysis was performed using RNA extracted from immature SCs (4- to 6-week-old chicken).

Figure 2 Changes in the expression of *TLR* genes in chicken primary Sertoli cells treated with 1 µg/ml LPS for 0, 6, 12, 24, and 48 h. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean $\pm$ S.E.M. ($n=6$). The same letters indicate that the difference was not significant. Different letters indicate that the difference was significant ($P<0.05$).

upregulation was detected for *IL1 β* and *IL12* with 20.6- and 18.3-fold higher expression, respectively, after 48 h of LPS stimulation compared with the control. The increase in *IL1 β* protein secretion by chicken SCs in culture medium was confirmed at 24 h after LPS stimulation (Fig. 5).

Furthermore, a significant ($P<0.05$) downregulation in the expression levels of *IL17* and *IFN γ* was observed at 6 h after stimulation and remained at significantly lower levels compared with the control unstimulated cells until 48 h (Fig. 4). The mRNA levels of *IL15* and *IL18*, in response to LPS, were not significantly affected when compared with the control.

Interestingly, we observed an upregulation of the luciferase activity controlled by *NF κ B* response element 3 h after LPS stimulation and later, suggesting an activation of the *NF κ B* pathway as described in mammals after activation of *TLR* receptors by LPS (Fig. 6).

LPS-regulated phagocytosis in SCs

SCs present the property of phagocytosis in order to eliminate the residual cytoplasm of germ cells during spermiogenesis (Carr *et al.* 1968). Phagocytosis was evaluated in chicken SCs by their ability to engulf latex fluorescent beads (1 µm) as observed in Fig. 7A. The number of beads engulfed in 3 h was increased by ~1.4-fold after LPS stimulation for 48 h, compared with unstimulated cells (Fig. 7B).

Discussion

Recent studies have reported on the immunological role of SCs in testicular defense and in the initiation of testicular innate immune responses (Riccioli *et al.* 2006, Starace *et al.* 2008, Wu *et al.* 2008, Sun *et al.* 2010). In avian species, an improved understanding of the innate immune-related gene function and their importance to

Figure 3 Changes in the expression of *AvBD* genes in chicken primary Sertoli cells treated with 1 µg/ml LPS for 0, 6, 12, 24, and 48 h. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean $\pm$ s.e.m. ($n=6$). The same letters indicate that the difference was not significant. Different letters indicate that the difference was significant ($P<0.05$).

infectious diseases such as Salmonellosis is emerging. In this study, we investigated the time course of an inflammatory response in chicken SCs triggered by exposure to the bacterial LPS, which is present in the bacterial membrane wall and acts as a well-established PAMP to the innate immune system. As the interaction of *TLRs* and their ligands induces cellular responses (including synthesis of proinflammatory cytokines and antimicrobial peptides), we used an *in vitro* model to investigate *TLR*, *AvBD*, and cytokine signaling in rooster SCs.

Data on expression analysis presented in this study revealed that all ten members of the *cTLR* family, nine members of the *AvBD* family, as well as eight cytokines were expressed in the rooster SCs. Previous studies have reported, in the rooster reproductive tract, the expression of most members of the *TLR* and *AvBD* families in the testis, epididymis, and sperm, while *S. enteritidis* infection or LPS stimulation resulted in significant induction of several of these genes in the testis, epididymis, and sperm of sexually mature roosters (Shimizu *et al.* 2008, Anastasiadou *et al.* 2011, 2013, 2014, Das *et al.* 2011, Watanabe *et al.* 2011, Zhang *et al.* 2012). Furthermore, administration of LPS upregulated

the expression of *IL1 β* , *IL6*, and *CXCLi2* in the rooster testis and epididymis by 3–6 h after injection (Zhang *et al.* 2012). These data suggest that rooster reproductive organs synthesize immune-related genes to form innate immune system in chickens.

Regarding SCs, previous studies have reported that the immunoreaction products for immunoreactive *AvBD11* were found in the cytoplasm of rooster SCs (Watanabe *et al.* 2011), while mouse SCs express members of the *Tlr* family (*Tlr3*, *Tlr6*, and *Tlr7*, but not *Tlr8*, *Tlr9*, and *Tlr11*; Wu *et al.* 2008), which can initiate testicular local innate immune responses by increasing expression of cytokines (Riccioli *et al.* 2006, Starace *et al.* 2008, Wu *et al.* 2008, Zhang *et al.* 2013). However, as *TLR* expression changes in relation to the pathogen involved, the cellular patterns of *TLR* expression vary in species, and the results obtained after *TLR* stimulation in one species may not be predictive of what will occur in another species (Akira & Takeda 2004).

Furthermore, the expression of defensins and proinflammatory cytokines has been reported to be dependent on breed or cell, affected by the dose of LPS, and depended on the time of stimulation (Ebers *et al.* 2009, Blomkalns *et al.* 2011, Sorensen *et al.* 2011).

Figure 4 Changes in the expression of cytokines in chicken primary Sertoli cells treated with 1 μ g/ml LPS for 0, 6, 12, 24, and 48 h. The mRNA expression levels were examined using quantitative real-time PCR analysis. Values represent the mean $\pm$ S.E.M. ($n=6$). The same letters indicate that the difference was not significant. Different letters indicate that the difference was significant ($P<0.05$).

Therefore, we developed an *in vitro* model to investigate the timing of response to LPS on patterns of gene expression in rooster SCs.

In hens, it has been reported that the oviduct, as well as ovarian follicular and stromal cells, expresses *TLRs*, *AvBDs*, as well as proinflammatory cytokines and chemokines, and the expression of these molecules was upregulated by LPS stimulation (Subedi *et al.* 2007, 2008, Abdel Mageed *et al.* 2008, Ozoe *et al.* 2009, Woods *et al.* 2009, Abdelsalam *et al.* 2011, Nii *et al.* 2011). Similar to the female reproductive tract, the gene expression data presented in this study revealed that rooster SCs responded to LPS stimulation. Interestingly, some genes, such as *IL1 β* , *IL8*, *IL12*, *IL17*, and *IFN γ* , changed their expression 6 h after induction and could be considered as 'early response gene'. Other genes, such as some *TLRs* or *AvBDs*, were upregulated after 24 h of stimulation and could be noted as 'later genes', as shown in Fig. 8.

In particular, quantification data on expression analysis revealed that LPS stimulation resulted in a significant upregulation of six *TLRs*, six *AvBDs*, and four cytokine genes. We confirmed that *IL1 β* mRNA upregulation led to an increase in the protein production by measuring an increase in IL1 β protein levels secreted in the culture medium. It is therefore possible that the

upregulation of the gene expression levels could lead to an increase in protein secretion, even if SCs (prepared when they are 6 weeks old) are not completely differentiated and competent for protein synthesis. Hence, the whole mechanism of an immune response can occur in chicken immature SCs, even if the blood–testis barrier is not fully functional at this age. The downregulation of *IL17* and *IFN γ* expression detected in

Figure 5 Interleukin 1 β was measured in culture medium of chicken primary Sertoli cells (SCs) treated with 1 μ g/ml LPS for 3, 6, 12, 24, and 48 h. Experiments were carried out with three different preparations of immature SCs (4- to 6-week-old chicken). The same letters indicate that the difference was not significant. Different letters indicate that the difference was significant ($P<0.05$).

Figure 6 Chicken primary Sertoli cells (SCs) were infected by lentiviruses expressing luciferase under the control of *NFκB* responsive element (luciferase, Cignal Lenti Reporters, Qiagen). Then, SCs were treated with 1 µg/ml LPS for 3, 6, 12, 24, and 48 h. Luciferase assay was performed, and promoter activity values are expressed as relative light units per 10^6 cells and normalized as the percentage of control. Experiments were carried out with four different preparations of immature SCs (4- to 6-week-old chicken). The same letters indicate that the difference was not significant. Different letters indicate that the difference was significant ($P < 0.05$).

this study could be due to an increase in cytokine levels with an anti-inflammatory activity. For example, *IL10* inhibits *IFNγ*, *IL2*, and *IL17* expression in mice (Hansen *et al.* 2013). By contrast, mice lacking the ability to respond to *IL10* present an increase in *IFNγ* and *IL17* levels (Gonzalez-Lombana *et al.* 2013).

IL1β, *IL6*, and *IL8* are known to be mediators of inflammation and physiological mediators of the acute-phase reaction. These cytokines are induced directly by LPS or other cytokines, which comprised the cell wall of Gram-negative bacteria. *In vitro* stimulation of rodent SCs by inflammatory mediators such as *IL1* or LPS in turn stimulates integrin ligand expression and lymphocyte adhesion by SCs (Gérard *et al.* 1992, Riccioli *et al.* 1995). *IL8* increases the metabolism of reactive oxygen species and stimulates *IL1* expression of adhesion molecules and chemotaxis. Two previous studies suggested that *IL1* is secreted by SCs in the lumen of seminiferous tubules and is detected in the efferent duct fluid (Syed *et al.* 1988, Cudicini *et al.* 1997). It could be noted that these authors have measured a high *IL1* production only after 3 h of incubation of SCs with LPS, and 12 h for *IL6* (Cudicini *et al.* 1997). In chicken SCs, we have found the same difference to induce *IL6* expression, because *IL1* expression was stimulated sixfold after 6 h of LPS stimulation and 24 h were required to increase *IL6* expression by fourfold. This result is concordant with the observation that *IL1* can induce *IL6* production (Sironi *et al.* 1989). Similarly, the synthesis of *IL8* could be increased by *IL1* (Standiford *et al.* 1990). It is known that *IL12* production is induced by bacteria, bacterial products, and parasites, which increases expression of *IFNγ*. In our study, expression of *IL15* and *IL18* was not induced by LPS, despite other observations reporting that LPS can stimulate *IL15*

(Lee *et al.* 2013). *IL15* is known to play a role in the innate response and adaptive immunity (Lodolce *et al.* 2002). Despite the fact that *IL18* is a pro-inflammatory cytokine biologically and structurally related to *IL1β*, their expression is differentially regulated (Puren *et al.* 1999) and could explain the difference in their expression in chicken SCs after LPS stimulation.

IL17 and *IFNγ* are also involved in inflammation (stimulate neutrophil recruitment). *IFNγ* induce the transcription of genes regulated by interferon response sequence. The downregulation of *IL17* and *IFNγ* expression in 6 h (reduced by approximately fivefold) could be due to an increase in the cytokine levels with an anti-inflammatory activity. For example, *IL10* inhibits *IFNγ*, *IL2*, and *IL17* expression in mice (Gonzalez-Lombana *et al.* 2013, Hansen *et al.* 2013).

We also observed an increase in the nuclear *NFκB* activity at 3 h after LPS stimulation. This result is in accordance with the similar data described in other cell types, where nuclear *NFκB* activity is induced by exposure to a wide variety of bacteria and bacterial products such as LPS. In addition, LPS is known to activate the *NFκB* via activation of *Tlr4* (Kawai & Akira 2007) or *Tlr3* even in mouse SCs (Wu *et al.* 2008). The active *NFκB* transcription factor promotes the expression of over 150 target genes and the majority of proteins encoded by *NFκB* target genes participate in the host immune response, including TLRs and cytokines (Pahl 1999, Kawai & Akira 2007). TLRs recognize a range of microbial molecular patterns and generate intracellular

Figure 7 Chicken primary Sertoli cells (SCs) were treated with 1 µg/ml LPS for 48 h, then incubated with fluorescent latex beads (1 µm diameter) for 3 h. After washing with PBS, the percentage of phagocytosis was estimated. (A) Representative microscopic fields of the phagocytic activity of chicken SCs. (B) Quantification was realized as cell surface area occupied by fluorescent beads. *Indicates that the difference in the expression levels are significantly different ($P < 0.05$). Experiments were carried out with three different preparations of immature SCs (4- to 6-week-old chicken).

Figure 8 Schematic summary of LPS induction on expression of genes involved in the immune system. It can be noted that LPS induces two responses in gene expression. 'Early response genes', such as *IL1β*, *IL8*, and *IL12*, were upregulated, while *IL17* and *IFNγ* were downregulated 6 h after LPS stimulation. Other genes, such as some *TLR* and *AvBD* genes, were upregulated after 24 h of LPS stimulation. LPS-induced *NFkB* activity since 3 h, with maximal expression after 24 h of stimulation in Sertoli cells. A consequence of LPS stimulation was an increase in the phagocytic activity by ~1.5-fold.

signals through *NFkB*-dependent pathways to induce defensin and cytokine expression that activates a range of host responses (Zarembler & Godowski 2002). Although the innate immune responses of the male genital tract have not been studied extensively, recent studies have reported that rat and mouse SCs express most of the *Tlrs* (*Tlr2*, *Tlr3*, *Tlr4*, *Tlr5*, *Tlr6*, *Tlr7*, and *Tlr13*; Wu *et al.* 2008). Interestingly, *TLR1-1* (*TLR6*) observed in mouse SCs is absent in rooster (Anastasiadou *et al.* 2011), and *TLR15*, not observed in mouse, is unique in the chicken (Temperley *et al.* 2008). Some of these *TLRs* are able to induce expression of immune genes such as *ILs* via *NFkB* (Riccioli *et al.* 2006, Starace *et al.* 2008, Hedger 2011). The upregulation of immune-related genes presented in this study is in agreement with these previous published data and suggests that *NFkB* activation through LPS stimulation induces innate immune responses in the rooster SCs.

It has been reported that mice SCs were able to potentially respond to a wide variety of bacteria through *Tlr* stimulation (Riccioli *et al.* 2006), while LPS stimulation resulted in the upregulation of *TLR4*, *IL1β*, and *IL6* in rooster testis (Zhang *et al.* 2012). Furthermore, we have previously reported that *S. enteritidis* infection resulted in the induction of *TLRs* (*TLR2-1*, *TLR4*, *TLR5*, *TLR15*, and *TLR21*) in the rooster testes (Anastasiadou *et al.* 2011). The data presented in this study indicate that the same *TLR* and cytokine genes were upregulated in the rooster SCs after LPS stimulation, suggesting that SCs may respond to bacterial infections activating different inflammatory pathways *in vivo*.

Interestingly, four genes, namely *AvBD3*, *IL1β*, *IL8*, and *IL12* were upregulated only after 6 h of LPS exposure, while others, such as *AvBD2* and *AvBD6*, *TLR1-1* and *TLR2-2*, showed a late response, after 48 h of LPS stimulation. Strangely, other *AvBDs* (*AvBD5*, *AvBD9*, *AvBD10*, and *AvBD12*) were also quickly upregulated (4 h) in chicken sperm after LPS incubation (Das *et al.* 2011). Although all *TLR* signaling pathways culminate in activation of the transcription factor *NFkB*, which controls the expression of an array of inflammatory cytokine genes, the kinetics of gene expression of *NFkB*-dependent genes are not activated simultaneously even if cell type and stimulus are constant. It has recently been reported that upon stimulation by pathogen-associated patterns, the kinetics of expression of *NFkB*-dependent genes differ relatively when *NFkB* translocates into the nucleus and the genes were referred as early, middle, and late genes (Iwanaszko *et al.* 2012).

It is therefore possible that the differences in timing presented in our study, are due, in part, to the number and type of transcription factor-binding sites of chicken immune-related gene promoter structures, as well as for the putative cofactors, in the early vs late genes, similar to what has been proposed for human, chimpanzee, cattle, and mouse (Iwanaszko *et al.* 2012). We can also hypothesize that the slow increase in *TLR* expression could be associated with the increase in *IL* levels, which occurs at 24 h after LPS incubation.

In addition, we have confirmed the hypothesis of the induction of immune system by analysis of a biological process, the phagocytic activity induced by LPS. Indeed, SCs show strong phagocytic activities toward specific substrates, such as apoptotic germ cells, residual bodies, or bacteria. Thus, both *in vivo* and *in vitro* studies have reported that rat and mouse SCs exhibit high phagocytic activity in response to LPS (Bourdon *et al.* 1999, Nakagawa *et al.* 2005). However, this response seems to be different in macrophages, where studies have reported that LPS inhibits phagocytosis in macrophages (Michlewska *et al.* 2009, Feng *et al.* 2011). In this study, we detected an increase in the phagocytic activity induced by LPS in rooster SCs, as observed by an increase in the surface occupied by engulfed latex beads after 48 h of LPS incubation. Similar results were observed in a mouse SC line (Bourdon *et al.* 1999).

These results suggest that in chicken as in mammals several molecular mechanisms involved in immune response are conserved and are functional even in immature SCs.

In conclusion, to our knowledge, this study provides the first evidence that rooster SCs express innate immune-related genes and that LPS induced a major increase in expression of several of these genes. These data are in accordance with the previously published data, where LPS stimulation resulted in the upregulation of several innate immune-related genes in various vertebrates (Herath *et al.* 2006, Ibeagha-Awemu *et al.*

2008, Yunhe *et al.* 2013). The production of inflammatory cytokines after activation of TLRs by their agonists is essential for antimicrobial responses. The results suggest that the LPS stimulation of rooster SCs induces the production of TLRs, AvBDs, and inflammatory cytokines that may mediate immune responses against pathogens in the testis. Our data expand previous understanding and provide novel insight into the function of TLRs, AvBDs, and cytokines in SCs. The data presented in this study suggest that these immune-related genes play important roles in the protection of rooster SCs and the seminiferous epithelium from invading pathogens and autoantigens.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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