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 µg/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 1β (IL1β) 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 (NFκB)-dependent cascades that culminate in a proinflammatory response. This response involves the secretion of cytokines and antibacterial substances, such as β-defensins (Froy et al. 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).
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, 2009, Guazzzone 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 chemotactic protein 1 (Mcp1) expression, as well as the activation of the NFKB pathway, adding further credence 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-defensins (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. Four chickens were killed for each culture, then testes were recovered and conserved in 4-(2-hydroxyethyl)pipperazine-1-ethanesulfonic acid (HEPES)-buffered F12/DMEM (Sigma) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). Testes were decapsulated, slightly minced, and incubated for 15 min at 37 °C with DNase (20 μg/ml) and type la 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 μg/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 μg/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
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 μg/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 μg/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, 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).

### Table 1

<table>
<thead>
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<th>TLRs</th>
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<td>TLR1-1</td>
<td>5'-TTAGCTGCAAGCTGTTGCA-3' 5'-GCTGGGAGGCAGTTCCTTCA-3'</td>
</tr>
<tr>
<td>TLR1-2</td>
<td>5'-CTCAGAATGGTGCCAGGCG-3' 5'-CCGTAGAATGGTGCCAGGCG-3'</td>
</tr>
<tr>
<td>TLR2-1</td>
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</tr>
<tr>
<td>TLR2-2</td>
<td>5'-CTCAGAATGGTGCCAGGCG-3' 5'-CCGTAGAATGGTGCCAGGCG-3'</td>
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<td>TLR3</td>
<td>5'-CTCAGAATGGTGCCAGGCG-3' 5'-CCGTAGAATGGTGCCAGGCG-3'</td>
</tr>
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<td>TLR5</td>
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<td>TLR15</td>
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<tr>
<td>TLR21</td>
<td>5'-CTCAGAATGGTGCCAGGCG-3' 5'-CCGTAGAATGGTGCCAGGCG-3'</td>
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### Luciferase reporter assays

Chicken primary SCs were infected with lentivirus in the presence of 6 μg/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 NF2xB firefly luciferase reporter (luciferase, Cignal Lenti Reporters, SA Biosciences, Qiagen, Paris, France). After 72 h of cell infection, SCs were treated with 1 μg/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, Pittsburg, 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 were performed.

### Table 2

<table>
<thead>
<tr>
<th>AvBDs</th>
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<tr>
<td>AvBD1</td>
<td>5'-CGAAGAGAGCCAGTCG-3' 5'-GGTGAGGCTCTGACTG-3'</td>
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<tr>
<td>AvBD2</td>
<td>5'-AGTTTCTGCTTACGAGT-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD3</td>
<td>5'-CCTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD4</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD5</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD6</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD7</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD8</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD9</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
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<tr>
<td>AvBD10</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
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<tr>
<td>AvBD11</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
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<tr>
<td>AvBD12</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD13</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
</tr>
<tr>
<td>AvBD14</td>
<td>5'-CTCAGTGGCGAGATCAC-3' 5'-GGTGAGGCTCTGACTG-3'</td>
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**Interleukin 1β assay**

Interleukin 1β (IL1β) concentration in the culture medium was measured using a chicken IL1β 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 IL1β is <0.5 pg/ml. Values for each condition are the mean ± S.E.M. of three different preparations of immature SCs (4- to 6-week-old chicken).
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 ± 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 NFKB 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 * \( P < 0.05 \), ** \( P < 0.001 \), and *** \( P < 0.0001 \).

### 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 \( \mu \)g/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 \( \mu \)g/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 \( \mu \)g/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 \( \mu \)g/ml LPS at 24 and 48 h. The highest

### Table 3 Primers used to amplify chicken cytokine genes.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Primer pair</th>
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<tbody>
<tr>
<td>IL1β</td>
<td>5'-GCATCAAGCTCAGAACGCTC-3' 5'-CAGCCGGTGAAGATGAGTAG-3'</td>
</tr>
<tr>
<td>IL2</td>
<td>5'-AACCTGGAACATCTGCAGAT-3' 5'-TCCTGGCTCAAGGAGAAGAG-3'</td>
</tr>
<tr>
<td>IL4</td>
<td>5'-GGAGGTTTCTCTGCTGTAAG-3' 5'-ATCGCGAAGCTTTCCACTTA-3'</td>
</tr>
<tr>
<td>IL6</td>
<td>5'-GATGGACTCTCAGCTGCGA-3' 5'-TGATCTTTCGAGCTGAGG-3'</td>
</tr>
<tr>
<td>IL8</td>
<td>5'-GATTGACTCTCAGCTGCGA-3' 5'-TCATTCTTTCGAGCTGAGG-3'</td>
</tr>
<tr>
<td>IL10</td>
<td>5'-GGACCTTCTGAGGTGTAAGTG-3' 5'-TAGAAGCAGCGACATCTGA-3'</td>
</tr>
<tr>
<td>IL12</td>
<td>5'-CTGTGGCTGGACAGT-3' 5'-GAACTCTCTCAGGCTGCTG-3'</td>
</tr>
<tr>
<td>IL15</td>
<td>5'-GCGCAACAAACAAAATCTGG-3' 5'-GGCAAAAGAAATGGCTGTA-3'</td>
</tr>
<tr>
<td>IL16</td>
<td>5'-ATGGGAAAGCTTCTCACTA-3' 5'-GCGGAGAAGCTCTGCTAT-3'</td>
</tr>
<tr>
<td>IL17</td>
<td>5'-TCCTCACCAAAAAAAATCG-3' 5'-ATCGAGAGAGCTGTCTA-3'</td>
</tr>
<tr>
<td>IL18</td>
<td>5'-AGACCTGGAAAGATTGGTGT-3' 5'-TCTTCCTCAAGCCTCTGTA-3'</td>
</tr>
<tr>
<td>IFNγ</td>
<td>5'-AGCCCGACATCAACACAATA-3' 5'-TCTTTTGGAAACTCTGGAGA-3'</td>
</tr>
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</table>

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).
upregulation was detected for IL1β 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 IL1β 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 IFNG 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 NFkB response element 3 h after LPS stimulation and later, suggesting an activation of the NFkB 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 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 ± 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 IL1β, IL6, and CXCL12 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 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 ± 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 IL1β, IL8, IL12, IL17, and IFNγ, 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 IL1β mRNA upregulation led to an increase in the protein production by measuring an increase in IL1β 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 IFNγ expression detected in

Figure 4 Changes in the expression of cytokines 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 ± 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).

Figure 5 Interleukin 1β was measured in culture medium of chicken primary Sertoli cells (SCs) treated with 1 μg/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).
observations reporting that LPS can stimulate IL15 synthesis of IL6 result is concordant with the observation that expression, because IL1 by bacteria, bacterial products, and parasites, which and IL15 et al. (Gonzalez-Lombana et al. 2013). By contrast, mice lacking the ability to suggested that molecules and chemotaxis. Two previous studies and 12 h for IL6 (Cudicini et al. 2013). It is known that these authors have measured a high IL1 expression was stimulated directly by 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 IL10, IL2, and IL17 expression in mice (Gonzalez-Lombana et al. 2013).

We also observed an increase in the nuclear NFkB activity at 3 h after LPS stimulation. This result is in accordance with the similar data described in other cell types, where nuclear NFkB 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 NFkB via activation of Tlr4 (Kawai & Akira 2007) or Tlr3 even in mouse SCs (Wu et al. 2008). The active NFkB transcription factor promotes the expression of over 150 target genes and the majority of proteins encoded by NFkB 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
signals through NfkB-dependent pathways to induce defensin and cytokine expression that activates a range of host responses (Zaremba & 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 Tlr genes, such as Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, Tlr6, Tlr7, and Tlr13 (Wu et al. 2008). Interestingly, Tlr1 (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, Tlrβ, and Ile6 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 (Iwanszko 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 (Iwanszko 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 LPS inhibits phagocytosis in macrophages (Michlewskia 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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