Research Report

Constitutive expression of toll-like receptor genes and signaling pathways in stallion testis and epididymis

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Abstract

Toll-like receptors (TLRs) expression pattern and their associated molecules have not been described in stallions. Understanding the TLR expression and their responses to activation is key in interpreting the interactions between immune system and reproductive system in healthy and ill animals. Objective of this study was to describe the constitutive expression pattern of TLRs and their signaling pathways in stallion's testis and epididymis. It was hypothesized that all TLRs and downstream signaling molecules are constitutively expressed in these tissues in reproductively normal stallions. Transcriptome analysis was performed in testicular and epididymal samples from reproductively normal stallions (n = 8). A detectable constitutive expression of all TLRs, costimulatory molecules, and most downstream adaptors and effectors was identified in stallion reproductive tissues. Most significant pathways were associated with TLR3, TLR2/TLR1, TLR2/TLR6, and the effector molecules C-C motif ligand 5 (CCL5) and CD40. Most genes were overexpressed in the epididymis compared to testis. Widespread and abundant expression of these genes supports an important function of the innate immunity in testicular and epididymal defense. These TLRs may also have a physiological role in maintaining the tolerogenic environment, supporting posttesticular maturation, or removing defective sperm from the tubular system.

Keywords: Horse, testis, epididymis, toll-like receptor, innate immunity, transcriptome

Introduction

Equine toll-like receptor (TLR) family includes 12 reported transmembrane proteins localized on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11) or cytosolic endosomal membranes (TLR3, TLR7, TLR8, TLR9, TLR12).[1,2] These receptors are essential for the initiation of the innate immune response through recognition of conserved pathogen-associated molecular patterns (PAMPs). Although surface TLRs identify bacterial, fungal and protozoal peptides and lipopolysaccharides, cytosolic TLRs identify viral and bacterial nucleic acids. In mammals, most TLRs bind several ligands by forming homodimers; however, TLR2 forms heterodimers with TLR1 or TLR6. Dimer type influences the affinity and magnitude of the TLR response to its ligand. The heterodimer TLR2/1 has higher affinity and a more potent response to bacterial lipopeptides than TLR2/6 in the horse.[3]

Upon engagement with the ligand, TLR dimers initiate the myeloid differentiation protein 88 (MyD88)-dependent pathway. Recruitment of the adaptor proteins MyD88 and TIR domain containing adaptor protein (TIRAP) results in activation of the cytosolic nuclear factor kappa B (NF-kB), its translocation to the nucleus, and transcription of proinflammatory cytokine genes, such as TNFα, IL-6, IL-1, and IL-12.[4] The exception to this pathway is TLR3, whose activation triggers the Toll/IL-1R domain-containing adaptor inducing interferon β (TRIF)-dependent pathway. Recruitment of TRIF and TRIF-related adaptor molecule culminates with activation and nuclear translocation of interferon regulatory factor 3 and expression of type 1 interferons (IFN-α and IFN-β).[2,4] The TLR4 has the ability to activate both pathways and requires the presence of the co-stimulatory molecules CD14 and myeloid differentiation. Although the innate immunity has a critical role in the initial control of infectious agents, TLRs can also be activated by endogenous damage-associated molecular patterns (DAMPs), resulting in sterile inflammation.[5]

Equid peripheral leucocytes express the 12 TLRs, whereas equine thymus, lung, liver, jejunum, colon, kidney, and lymph nodes express TLR1 to TLR10.[2,6] There is also constitutive expression of TLR4 in the equine gingiva, hoof lamella, eye,

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skeletal muscle, cerebrum, cerebellum and adipose tissue, with increased expression induced by exercise, cortisol and heat in skeletal muscle and peripheral leucocytes, and by insulin resistance in adipose tissue.[7–11] In the mare reproductive tract, the chorioallantois, endometrial glandular and luminal epithelium, stromal cells, leucocytes, endothelium, and vascular smooth muscle express TLR2, TLR4, TLR6, and TLR7 constitutively.[12–16] Inducible expression increased in mares with endometritis and placentitis.[14–16] Expression of TLRs in the stallion reproductive tract has not been reported.

Most TLRs and signaling components are expressed in immune cells and somatic and germ cells of the testis, epididymis, vas deferens, and accessory sex glands of rodents.[17] Rodent Sertoli cells, Leydig cells, and testicular macrophages have the widest TLR expression patterns and express proinflammatory cytokines upon activation.[18] Microbial PAMPs and endogenous DAMPs released by germ cells induce inflammatory cytokine production by Sertoli cells through TLR activation.[17,19,20] Activation of inflammatory pathways in Sertoli cells inhibits their response to follicle stimulating hormone and androgens and disassembles junctional complexes of the blood-testis barrier.[17] The activation of TLRs in Leydig cells suppresses steroidogenesis, altering testicular function through decreased intratesticular testosterone concentrations, whereas the activation of TLR2 and TLR4 in germ cells directly induces germ cell apoptosis.[17,21] Activation of TLRs is also critical for the development of autoimmune orchitis in mice.[20,22]

Although the expression and activation pattern of TLRs in the rodent reproductive tract has been characterized, their expression in the genital tract of stallions has not been described. Understanding the expression patterns, ligands, and responses to activation is key to interpreting the interactions between the immune system and the reproductive system in healthy and ill animals. Objective of this study was to describe the constitutive expression of TLRs and their signaling pathways in stallion's testis and epididymis. It was hypothesized that the testis and epididymis of reproductively normal stallions constitutively express all TLRs and downstream signaling molecules.

Materials and methods

Animals and tissue processing

Testicular and epididymal samples were collected from 8 client-owned stallions presenting to the Veterinary Medical Center of the University of Georgia for routine castration as part of the clinical services provided by the hospital. Our Institutional Animal Care and Use Committee does not require an approved Animal Use Protocol for the use of discarded clinical samples. Stallions were 2-13 years American Quarter Horse (n = 5), Arabian (n = 2) and American paint horse breed (n = 1); 4 stallions were castrated in April and 4 in February. Testes and epididymides were placed on ice immediately after castration and processed within 10 minutes. Sperm were collected from the vas deferens. Sperm morphology was evaluated using a Hancock stain and light microscopy;[23] stallions' fertility was not known. Given that sperm morphology is associated with pregnancy rate, this parameter was chosen as an inclusion criterion.[23–25] Various threshold values have been suggested for fertile or highly fertile stallions, ranging from 30-60%.[26–30] The highest value was used here to include stallions assumed to have normal testicular and epididymal function; thus, tissues from stallions with ≥ 60% morphologically normal sperm were included.

Vaginal tunics and connective tissue were removed, and the organs were rinsed with sterile saline solution. A 1 x 1 cm tissue sample was collected with sterile instruments from the testicular parenchyma and the epididymal head, body, and tail; 1 testis and epididymis were included per stallion. The side (left versus right) was randomly selected. Each tissue was placed in individual tubes, snap-frozen in liquid nitrogen, and stored in liquid nitrogen until processing.

Total RNA was extracted using a commercial kit (RNeasy Mini Kit, Qiagen, Redwood City, CA, USA) following the manufacturer's protocol. Approximately 1 cm^3 of tissue from each sample was cryopulverized and homogenized with an OMNI TH homogenizer (VWR) in RLT lysis buffer containing 1% 2-mercaptoethanol. The tissue suspension was centrifuged at 11,000 × g for 1 minute. Supernatant containing the total RNA was loaded on a RNeasy spin column to remove any DNA contamination and to elute the total RNA. Total RNA was frozen at –80°C and shipped on dry ice to a commercial laboratory (Novogene, Sacramento, CA, USA) for quality control and RNA sequencing.

Library preparation for transcriptome sequencing

Degradation and contamination of the RNA were monitored on 1% agarose gels. Purity of the RNA was evaluated using the NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA). The RNA integrity and quantification was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). A total amount of 1 µg of RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (Illumina, San Diego, CA, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB and next in first strand synthesis reaction buffer (5 x). First strand cDNA was synthesized using random hexamer primer and M-MuLV reverse transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEB and next adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150-200 base pair (bp) in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Brea, CA USA); 3 µL of USER Enzyme (NEB, USA) were used with size-selected, adaptor-ligated cDNA at 37°C for 15 minutes, followed by 5 minutes at 95°C before PCR. Then PCR was performed with Phusion high-fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. The PCR products were purified (AMPure XP system), and the library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina), according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 125 bp/150 bp paired-end reads were generated.

Sequencing data analysis

Raw data (raw reads) of fastq format were first processed and clean data (clean reads) were obtained by removing reads containing adapters, reads containing ploy-N, and low-quality reads from the raw data. All the downstream analyses were based on the clean data with high quality. The reference genome (*Equus caballus*, Equcab3) and gene model annotation files were directly downloaded from the genome website. The index of the reference genome was built using hisat2 2.1.0 and paired-end clean reads were aligned to the reference genome using HISAT2 (Johns Hopkins University, Baltimore, MD, USA). FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. The expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (fpkm) of each gene was calculated based on the length of the gene and reads count mapped to this gene.[31] Differential expression analysis was performed comparing the testicular tissue to each segment of the epididymis using the DESeq2 R package (1.14.1, R Core Team, Vienna, Austria). DESeq2 provided statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The p values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate. Genes with an adjusted p value < 0.05 by DESeq2 were considered differentially expressed.[32] The differential expression of TLRs was evaluated within each tissue sample by comparing mean fpkm among TLRs. From the entire data set of transcripts, genes involved in the toll-like receptor pathway (https://www.genome.jp/pathway/hsa04620) were selected and reported.

Reverse transcription and real time polymerase chain reaction

Real time polymerase chain reaction (RT-PCR) was performed in a subset of 3 stallion testicular samples to confirm expression of TLRs 1-8 for quality control of the sequencing analysis. Tissue collected from equine lymph nodes was used as positive control. Total RNA yield and purity were estimated spectrophotometrically at 260 nm using a Nanodrop (Thermofisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from 1 µg total RNA using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) and nuclease-free water, following the manufacturer's instructions. Reverse transcription was performed at 25°C for 5 minutes, 42°C for 30 minutes, followed by 90°C for 5 minutes. The cDNAs obtained were stored at –20°C until further investigation. Reactions without reverse transcriptase were run in parallel to RT to confirm the absence of any genomic DNA or contamination. The TLR gene-specific primers (Table) were designed using sequences published in the National Center for Biotechnology Information database (NCBI, Bethesda, MD, USA) with the aid of Primer3 software and purchased from Sigma. Amplification of cDNA was performed using the following conditions: an initial denaturation at 94°C for 5 minutes; followed by 39 cycles of 94°C for 30 seconds, and annealing at the temperature specified for each gene for 60 seconds; with a final extension at 72°C for 4 minutes. A dissociation curve was added for 15 seconds at 95°C to ensure the presence of a single amplicon. Gene expression was analyzed using SYBR green quantitative RT-PCR, and expression data for the different genes were obtained in the form of threshold cycle (Ct) values.

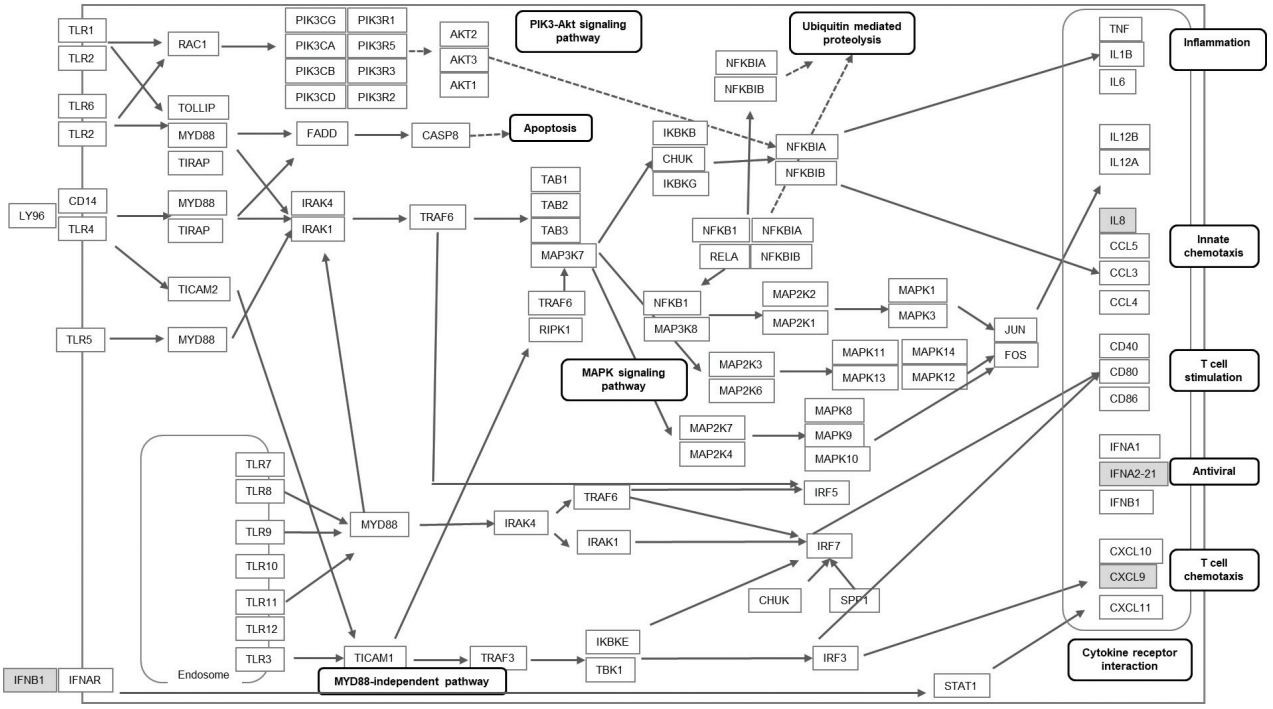

Figure 1. Toll-like receptors and their associated pathways, gray boxes represent genes that were not constitutively expressed (fpkm = 0) within the stallion's reproductive tract; fpkm = fragments per kilobase of transcript sequence per millions base pairs sequenced

Results

Gene expression was not affected by stallion age or season ($p > 0.05$); therefore, data from all stallions were grouped. Detectable constitutive expression of all TLRs, costimulatory molecules, and most downstream adaptors and effectors was identified in stallion reproductive tissues, including the ortholog gene TLR12 (Supplemental Table; Figure 1).

Within the testes, transcription of all TLRs and most of their associated adaptors and effectors was identified (Supplemental Table and Figure 2). However, not all genes were equally expressed. The testicular expression of TLR3 (fpkm 1.57 ± 0.4) was higher ($p = 0.002$) than all other TLRs, followed by TLR2 (0.57 ± 0.1), TLR6 (0.28 ± 0.05), and TLR1 (0.24 ± 0.05). The transcription of TLR4 (0.01 ± 0.01) and 10 (0.03 ± 0.01) was minimal (Figure 2). Furthermore, there was an active expression of most adaptor protein genes and downstream mediators from all signaling pathways, except for phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5). Genes for most of the resulting cytokines and effector proteins were also expressed in the testis, except for interleukin 8 (IL8), interferon alpha 2 (IFNA2), interferon beta 1 (IFNB1), and C-X-C motif ligand 9 (CXCL9) (Figure 1 and Supplemental Table). The most significantly expressed effector chemokine was CCL5 (Supplemental Table).

Level of expression of most genes differed ($p < 0.05$; Supplemental Table). Expression of TLR1, TLR2, TLR3, and TLR5-9 was downregulated in the testes compared to epididymis (Figure 2). Expression of TLR4 and TLR10-12 did not differ among tissues. Expression of most genes was upregulated in several segments of the epididymis compared to testis. The exception was some proteins of the PIK3-Akt and MyD88-dependent signaling pathways that were downregulated in the epididymis (Supplemental Table). Similar to testes, epididymal expression of TLR3 was higher than all other TLRs. The TLR1, TLR2, TLR5, TLR6, TLR7 and TLR10 were abundantly expressed (fpkm > 1) in at least 1 segment of the epididymis, whereas TLR4 and TLR11 had a minimal level of expression (fpkm < 0.1). There was also active expression of most adaptor proteins and downstream mediators from all signaling

Table. Forward and reverse primer sequences for TLR 1-8 genes for RT-PCR

Gene name	Gene symbol	Accession no.	Forward	Reverse	Product size (bp)
Toll-like receptor 1	*TLR1*	NM_001256899.1	GCCCATATGCAAAGAGTTTGGC	TCTGTGTTAAGGTGTCGAAGGC	183
Toll-like receptor 2	*TLR2*	NM_001081796.1	CATGCTTTGTGGACGGTGTG	AAGACTTTTCACAGCTGCCG	168
Toll-like receptor 3	*TLR3*	NM_001081798.1	GAGACTGTTGCCCTTTTGGG	CCGTTATGTTTGCTGGGAGG	125
Toll-like receptor 4	*TLR4*	NM_001099769.2	ACATCCCCACATCAACCAAGG	ATGGTTGAGGCCCTGATATGC	158
Toll-like receptor 5	*TLR5*	Kwon, et al. 2011	TCCATGGAGGGTTGTGATGA	CCCCGGAACTTTGTGACAAT	–
Toll-like receptor 6	*TLR6*	NM_001257142.1	GACTTGCCACCAGAAACCAAG	AGGTACCGGATGCTATTACGG	128
Toll-like receptor 7	*TLR7*	NM_001081771.2	ACTTCTGTAGCAGGTCACGG	ACTTAGGTCCAAGGTCTGCC	159
Toll-like receptor 8	*TLR8*	NM_001111301.1	GCCGTTTTGGAACTTGGTGG	GGCATCTGAAACACACGTCG	186

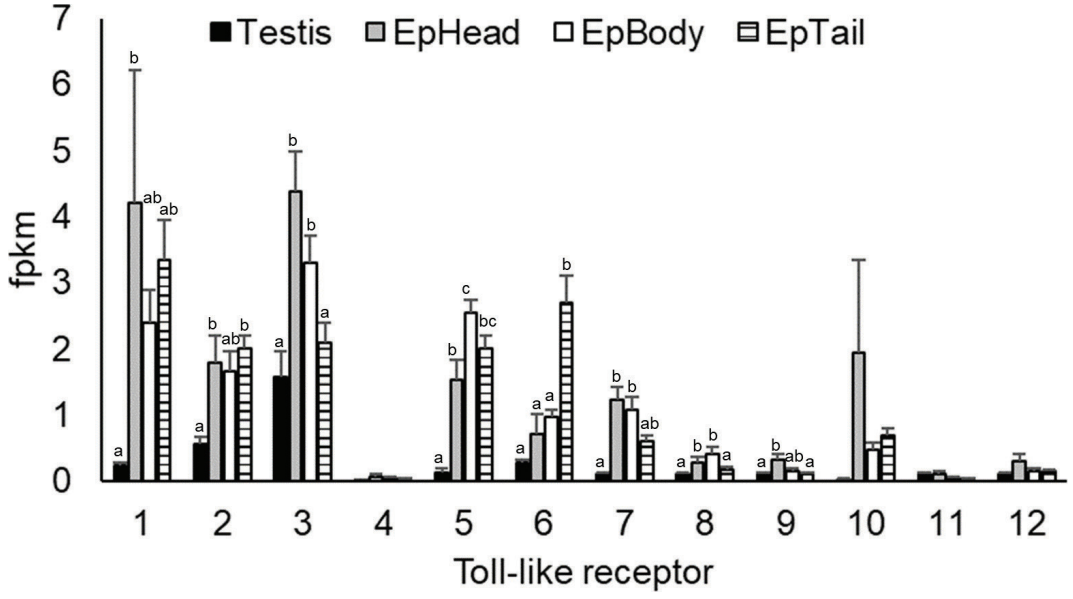

Figure 2. Differential expression of equine toll-like receptors in the testis, epididymal head (EpHead), body (EpBody), and tail (EpTail). Fpkm = fragments per kilobase of transcript sequence per millions base pairs sequenced; [a-c]Within a tissue, means without a common superscript differed ($p < 0.05$)

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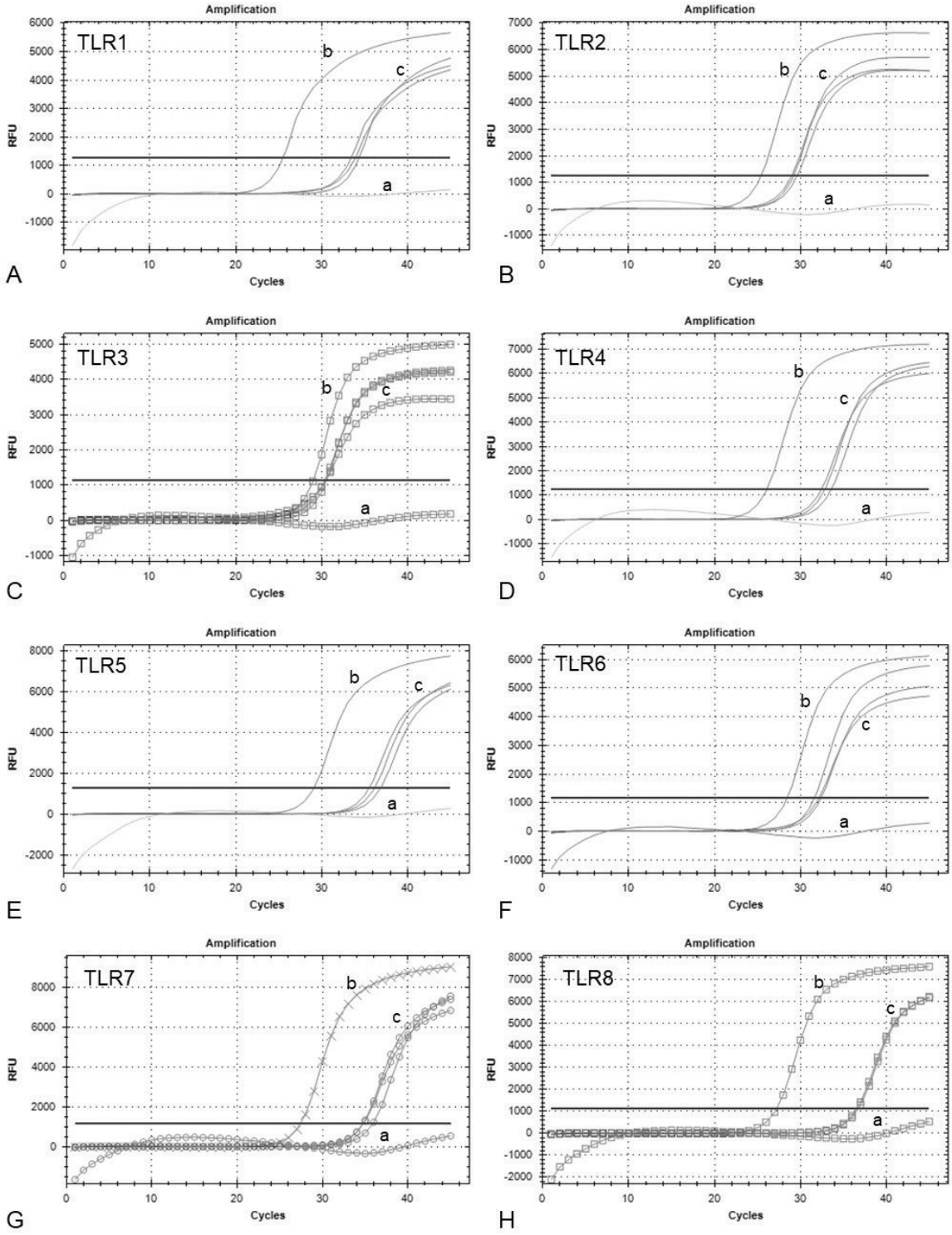

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Sample	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8
Lymph node	25.5	25.4	29.0	26.1	29.1	28.4	27.3	27.2
Testis 1	34.3	29.2	30.6	32.9	35.5	31.6	34.7	36.8
Testis 2	33.4	29.6	30.4	33.6	36.1	32.4	35.9	36.5
Testis 3	33.9	29.0	30.5	32.4	36.8	32.1	34.8	NA

Figure 3. RT-PCR amplification curves of toll-like receptor (TLR) genes TLR1 (A), TLR2 (B), TLR3 (C), TLR4 (D), TLR5 (E), TLR6 (F), TLR7 (G), and TLR8 (H) and Cq values of individual samples (I). a = nontemplate negative control, b = positive control (lymph node tissue), c = testicular tissue samples

pathways, except for PIK3R5. The resulting cytokines involved in inflammation, chemotaxis, and stimulation of the immune system were upregulated in the epididymis, except for IL8, IFNA1, IFNA2, IFNB1, and CXCL9 that were not detected. The most significantly expressed effector chemokine was CCL5 (Supplement Table). Constitutive testicular expression of TLRs 1-8 was confirmed using RT-PCR (Figure 3).

Discussion

To our knowledge, this is the first description of constitutive expression of TLR genes and their downstream adaptors and effector cytokines in the stallion's reproductive tract, confirming that the equine testis and epididymis are fully equipped with the machinery needed for TLR stimulation and function. Limitations of our study are: first, unavailability of stallions' fertility history and second, histologic evaluation of the tissue. However, a strict criterion was used to select stallions with a high percentage of morphologically normal sperm that were assumed to have normal testicular and epididymal function.[26–30] In this group of stallions, TLR3 was the most abundant transcript within all segments of the stallion's genital tract evaluated. A third limitation is that we did not attempt to identify the cell types expressing each gene. In rodents, TLR3 was abundantly expressed in germ cells, Sertoli cells, and peritubular cells.[17,33] Toll-like receptor 3 also had the most widespread and abundant expression in the rodent and human testis, suggesting an important role in testicular function.[17,33,34] This cytosolic receptor recognizes viral and mammalian double-stranded DNA. It was speculated that physiological activation of TLR3 during phagocytosis of germ cells by Sertoli cells induced an inflammation-like activation of Sertoli cells that triggered the release of critical spermatogenic regulators.[35] During inflammation, TLR3 activates the TRIF-dependent pathway, resulting in release of interferons. Despite the abundance of TLR3 and its mediators, interferons were not abundantly transcribed in the reproductive tract of reproductively normal stallions, suggesting that TLR3 may indeed serve a physiological function in the absence of inflammation. Several SNPs were identified in the coding regions of the horse TLR3 gene, as well as TLR7 and TLR8, but their effect on pattern recognition or function is not known.[36]

The next most abundantly expressed receptors were TLR1, TLR2, and TLR6, together with their associated mediators of the PIK3-Akt signaling pathway. Toll-like receptor 2 was ubiquitously expressed in rodent germ cells, Leydig cells, and Sertoli cells, whereas TLR6 was only expressed in mouse Sertoli cells.[17,33] Cellular localization of TLR1 in the testis has not been described. These TLRs are located in cell membranes.[1] Heterodimers TLR1/TLR2 and TLR2/TLR6 recognize lipoproteins in Gram positive bacteria. Although their activation induces the release of several cytokines, those involved in innate chemotaxis, particularly CCL5, were the most abundantly expressed in stallion's testis and epididymis. Macrophages in the basal region of the epididymal epithelium constitutively express CCL5. These macrophages present antigens to lymphocytes and help eliminate abnormal sperm.[37] Concentration of CCL5 in seminal plasma was lower in men with immune-mediated infertility.[38] It was suspected that reduced CCL5 concentration decreased the chemoattraction of scavenger leukocytes involved in the physiological elimination of abnormal sperm during storage in the genital tract, leading to increased exposure to autoantigens and autoimmune reactions.[38] Furthermore, CCL5-positive macrophages are involved in the acidification of the epididymal luminal fluid. This low pH is necessary for posttesticular sperm maturation and inhibition of sperm motility during epididymal storage.[37] Role of CCL5 in the testicular medium is less well characterized; however, since the testicular luminal pH is also acidic, CCL5 may serve a similar function in the testis.[39]

Effect of TLR2 stimulation depends on the relative expression of TLR1 and TLR6 that determines heterodimer predominates. Heterodimer TLR2/TLR1 was more predominant in stallion's epididymis than TLR2/TLR6; heterodimer is thought to have an antiinflammatory role by inducing the production of IL-10 and favoring the differentiation of regulatory T cells.[40] This immunosuppressive effect can be synergized by low level stimulation of CD40 in tolerogenic dendritic cells that leads to maintenance of the homeostasis of the immune system and a prevalence of a tolerogenic environment.[40–43] Of the costimulatory molecules evaluated here, CD40 was the most abundantly expressed. Altogether, it is possible that the prevalent TLRs and the effectors identified in our study have a physiological role maintaining the homeostasis of the immune system and removing abnormal sperm.

Conversely, the expression of TLR4 and its costimulatory molecule CD14 were minimal in the stallion's reproductive tract, contradicting findings in other species. Expression of TLR4 was high in rodent and human germ cells, and Sertoli cells.[17,33,34,44] Conversely, as in other species, TLR10 and TLR11 were also less abundant.[17,33,44] It is possible that this low level of expression indicates a less important role of these TLRs in testicular and epididymal immunity. Alternatively, these TLRs may be inducible, and may acquire a more active role in the face of infection. Furthermore, the low transcriptional levels may be due to the cellular distribution of these TLRs since the expression of TLR8, TLR9 and TLR11 was limited to myeloid cells within the rodent testis.[17,33]

The majority of the genes were overexpressed in the epididymis compared to testis. Despite the higher gene expression of the TLRs and adaptors, cytokine expression was also relatively low (fpkm < 1) within the epididymis. The overexpression emphasizes the importance of the innate immunity in controlling and preventing ascending infections of the reproductive tract. Conversely, the innate immunity may have a role in the removal of abnormal sperm from the epididymis, preventing their transport through the tubular system into the ejaculate. In the rat epididymis, all TLRs, except for TLR10, were localized in the epithelial cells.[44] Although seasonal variations were reported in the donkey epididymal epithelium morphology and presence of inflammatory cells, no differences in gene expression were identified in our study between samples collected in the spring versus winter.[45] Our study is the first to identify the expression of TLR12 in equine tissues. Expression of TLR12 was only recently described in equine peripheral leucocytes.[2] In mice, TLR12 was described as an intracellular receptor acting in cooperation with TLR11 in the recognition of *Toxoplasma gondii* profilin.[46] Equine TLR12 is poorly characterized, and its role in equine immunity is not known. Future studies should consider evaluating inducible expression of TLRs and associated genes in the testes and epididymis of stallions with testicular or epididymal dysfunction or suspected immune-mediated infertility to evaluate the role of these components of the innate immune system on the pathophysiology of these conditions.

In summary, constitutive gene expression of most TLRs and associated proteins was identified in stallion's testis and

epididymis. Their widespread expression supports an important function of the innate immunity in testicular and epididymal defense. The most significant pathways were associated with TLR3, TLR2/TLR1, and TLR2/TLR6, and the effector molecules CCL5 and CD40. These TLR pathways may have a physiological role maintaining the tolerogenic environment, supporting posttesticular maturation or removing defective sperm from the tubular system.

Conflict of interest

Authors have no conflict of interest that could be perceived as prejudicing against the impartiality of the research reported.

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