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Expression of Toll-like Receptors in Genital Tract Tissues from Normal and HIV-infected Men

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Abstract

Problem—Cells of the innate immune system use Toll-like receptors (TLRs) to recognize and respond to invading pathogens. This study was carried out to characterize TLR expression in the human male genital tract, an initial infection site for several sexually transmitted pathogens.

Method of Study—Immunohistochemistry was used to detect expression of TLRs 1–9 in genital tract tissues from HIV[−] and HIV⁺ men.

Results—In HIV[−] men, TLR1⁺ leukocytes were detected throughout the genital tract. Leukocytes in the penile urethra also expressed TLRs2, 3, 5, 7 and 9. Epithelial cells in most tissues did not express TLRs; exceptions were the prostate, where TLRs3 and 8 were observed on the apical surface of luminal epithelial cells, and the penile urethra, where epithelial cells expressed TLR9. In genital tissues from HIV⁺ men with AIDS, few TLR⁺ cells were detected.

Conclusion—Cells in the male genital tract can express a variety of TLRs. The penile urethra contained the highest number of TLR⁺ cells, indicating that this tissue plays a major role in the innate immune defense of the male genital tract. Overall, TLR expression was reduced in genital tissues from HIV⁺ men.

Keywords

HIV-1; innate immunity; male genital tract; sexually transmitted infections; Toll-like receptors; urethra

Introduction

The ability to discriminate self from non-self is a central feature of both acquired and innate immune defense. Innate immune recognition is principally mediated by cellular receptors known as pattern-recognition receptors (PRRs). These molecules detect pathologic microorganisms through recognition of invariant pathogen-associated molecular patterns (PAMPs) (reviewed in Ref. 1). PRRs are distributed throughout cell membrane and cytoplasmic compartments to formulate a comprehensive line of defense against

extracellular and intracellular pathogens of viral, bacterial, fungal and protozoan origin. PRRs also play an important role in linking innate immune responses to acquired immune defense functions.^{2,3}

The most prominent members of the PRR class of molecules are the Toll-like receptors (TLRs), a family of membrane proteins that recognize a variety of microbe-derived molecules.⁴ TLRs are an evolutionarily ancient receptor family. Toll was originally identified in *Drosophila* as an essential embryonic patterning molecule that was also required for innate defense against fungal infection.⁵ To date, 10 human TLRs have been identified, and function as homodimers or heterodimers. Human TLRs fall into two broad categories: those that are expressed on the cell plasma membrane and recognize structures on a variety of microbes including bacteria, fungi, protozoa and mycobacteria (e.g., TLRs 1, 2, 4, 5 and 6) and those that are found in endosomes and primarily detect viral and/or bacterial nucleic acids (e.g., TLRs 3, 7, 8 and 9). TLR10 is expressed on the cell surface, but its corresponding PAMPs have yet to be identified.⁶ Adaptor proteins, including MyD88, TIRAP/Mal, Trif and Tram, are differentially recruited by TLRs and provide a degree of specificity to TLR-induced responses (reviewed in Ref. 7).

The genes expressed in response to TLR signaling encode proteins important in many different components of immune responses. Broadly speaking, there are two major signaling pathways activated by TLRs. The first of these, common to all TLRs and mediated by MyD88, culminates in the activation of the transcription factor NF- κ B. This factor acts as a master switch for inflammation, regulating the transcription of many genes that encode proteins involved in immunity and inflammation.⁸ The second pathway is MyD88 independent. Downstream signaling is effected by TRIF and leads to the secretion of Type 1 interferons through the Interferon Regulatory Factor signaling pathways.^{9,10}

TLRs are expressed at mucosal surfaces on a variety of cell types including: neutrophils, macrophages, dendritic cells (DCs), T lymphocytes and epithelial cells.^{11,12} A number of studies have described TLR expression in the human intestine. Intestinal epithelial cells express TLRs 2, 3, 4, and 5, with colonocytes expressing more TLR3 and 5 and less TLR2 and 4 than villus enterocytes.¹³ The decreased expression of TLR4 by human intestinal cells is thought to prevent pro-inflammatory responses against endogenous commensal bacteria.^{14,15} TLR5 expression by intestinal epithelial cells is highly polarized and occurs only on the basolateral surfaces of these cells.¹⁶ Thus for intestinal epithelial cells, TLR5 only becomes activated if the integrity of the epithelium has been breached by flagellated parasites.

Various TLRs have been detected in the human female genital tract and are thought to play an important role in immune defense of these tissues.¹⁷⁻²⁰ In the first article on this topic, Fichorova et al.²¹ demonstrated that immortalized epithelial cell lines derived from human endocervical, ectocervical and vaginal tissue expressed mRNAs for TLRs 1, 2, 3, 5 and 6 but did not express TLR4 or respond to the TLR4 agonist lipopolysaccharide (LPS). These findings have been extended by Andersen et al.²² who documented that immortalized human endocervical cells respond to agonists for TLR3 and TLR9 but not for TLR4, TLR7 or TLR8 and by Herbst-Kralovetz et al.²³ who showed that both primary and immortalized cell

lines derived from the vagina, ectocervix and endocervix express several functional TLRs, with TLRs2, 3, 5 and 6 being most abundant, and TLR4 and 7–9 minimally expressed with inconsistent bioactivity. Moreover, differential expression of these TLRs has been observed along the length of the female genital tract. An immunocytochemical study demonstrated that all regions studied could express TLRs1–6 except for the vagina and ectocervix which were negative for TLR4.¹⁷ Another study showed constitutive expression of TLR1–6 mRNA in genital tract tissues with differential expression of mRNA for TLR2 which was highest in the fallopian tubes and TLR4 which declined progressively along the tract reaching its lowest levels in the ectocervix.¹⁸

It was recently reported that human Langerhans cells (LC), a population of DCs present within the stratified squamous epithelium of the vagina and ectocervix, have a selective impairment of cell surface expression of TLRs2, 4 and 5, all of which are involved in bacterial recognition.²⁴ This lack of responsiveness of genital LC and epithelial cells to LPS and other bacterial products may account for their tolerance to commensal bacterial flora present in the vaginal vault.¹⁸ TLR expression in the uterine endometrium appears to be affected by hormonal changes that occur during the menstrual cycle,²⁵ and recent studies have begun to uncover molecular events underlying steroid hormone regulation of TLR-activated intracellular signaling pathways (reviewed in Ref. 26,27). Infections per se can also alter TLR expression (reviewed in Ref. 28).

As yet little is known about the expression of TLRs in the human male genital tract. Such information would provide insight into innate immune defense mechanisms present in these tissues, as well as the role of TLRs in acquired immune defense at this site. We report here results from a comprehensive immunohistological study of TLR expression in various regions of the normal male genital tract. We also studied TLR expression in genital tissues from HIV-1 infected men with AIDS as a starting point to understand changes in TLR expression and genital tract innate immune defense that occur following HIV infection.

Materials and methods

Procurement of Tissues

This study was approved by the Institutional Review Boards of Boston University Medical School and the Brigham and Women's Hospital, Boston, MA. Genital tract tissue samples were obtained either at autopsy or as discarded surgical specimens. Different regions (testis, efferent ducts, epididymis, vas deferens, prostate, seminal vesicles and penile urethra) were removed at autopsy from 26 patients without a recorded history of HIV/AIDS. In addition, the whole penis was removed from four men at autopsy. The foreskin was intact in three of these men, and one had undergone circumcision. The primary cause of death for these patients was myocardial infarction, congestive heart failure, and/or pulmonary congestion, pneumonia and bacterial sepsis; the ages at death ranged from 26 to 83 years (mean age: 57.7 years). Autopsy specimens were collected within 30 hr postmortem (earliest 7 hr, longest 26 hr; mean 16.9 hr). Fresh surgical tissues from men without an HIV/AIDS diagnosis included 1) foreskin ($n = 6$) from men undergoing circumcision because of phimosis, and 2) prostate ($n = 24$) and seminal vesicle tissues ($n = 10$) from prostatectomies carried out to remove prostate cancer. Tissues used for this study were distal from the

malignant tissue and had a normal morphology from analysis of hematoxylin and eosin (H and E) stained sections at the light microscope level.

Tissues were obtained at autopsy from HIV⁺ men that died of AIDS (before antiretroviral drugs were readily available) aged 30–53 years old (mean 40.6 years). Tissue samples were taken along the length of the genital tract from two autopsies. Additional archived blocks of autopsy tissue including testis, rete testis and epididymal tissue were available from 18 HIV⁺ men, and prostate tissue from four HIV⁺ men. In addition, lymphoid tissues collected at autopsy from two HIV⁺ men served as positive controls for TLR expression. All HIV⁺ tissues were collected within 24 hr postmortem (earliest 4 hr, latest 24 hr, mean 14.2 hr).

An obvious caveat associated with the use of autopsy samples is the postmortem degradation of tissues that could adversely affect expression of the TLRs. The morphological integrity of all autopsy tissue was initially verified using H and E-stained sections. Despite an appreciable time interval between death and fixation of tissue, the autopsied male genital tract samples appeared at least at the light microscope level to be morphologically well preserved. Any tissues that showed evidence of autolysis were discarded from the study.

Immunocytochemistry

Genital tract tissues were fixed in 10% methanol-free unbuffered formaldehyde except for the archived samples of testes and prostate from HIV⁺ men, which had been fixed in formalin. Tissue samples were embedded in paraffin, and 5- μ m-thick sections placed on glass slides, de-waxed and then rehydrated in a graded series of ethanols. An antigen retrieval protocol was used to unmask reactive epitopes. The sections were immersed in citrate buffer (pH 6.0) in a pressure cooker which was brought up to a temperature of 125°C for 30 s. The slides were then cooled for ten minutes after which they were washed several times with distilled water and placed in Tris-buffered saline containing 0.1% Tween 20 (TBST).

Sections were incubated for 30 min with a serum-free protein blocking solution (Dako, Carpinteria, CA, USA), which was drained from the tissue sections immediately prior to the application of primary antibodies. Antibodies specific for TLRs 1–9 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA; Table I).

Rabbit primary antibodies (specific for TLRs2–5, 7 and 9) were diluted in a proprietary antibody diluent (Dako) at a concentration of 1:40. Tissue sections were incubated with the primary antibody for two hours at room temperature and then washed twice in TBST for five minutes each. Primary antibodies were detected using a proprietary secondary reagent (alkaline phosphatase EnVision Dako) that was incubated with the tissue sections for 30 min at room temperature. Slides were then washed twice in TBST for five minutes each. The antibodies were visualized by incubating the sections with a substrate for alkaline phosphatase (Fast Red Dako) that stains positive cells and tissues a bright red. Development of the staining was monitored under a light microscope. After a final wash, the sections were counterstained in aqueous hematoxylin, mounted in a glycerin-based mounting medium and cover slipped.

Goat primary antibodies, specific for TLRs1, 6 and 8, were diluted in antibody diluent (Dako) at a concentration of 1:40 and incubated with the tissue sections for two hours at room temperature. After washing twice for five minutes each in TBST, sections were then incubated with a secondary biotinylated chicken anti-goat IgG (1:300 dilution) for 20 min at room temperature. The tissue sections were then washed twice for five minutes each in TBST and incubated with streptavidin linked to alkaline phosphatase at a dilution of 1:300 for 20 min. After washing in TBST for five minutes each, the tissue sections were processed as described earlier for the rabbit primary antibodies.

Human macrophages and phorbol myristate acetate (PMA)-treated U937 cells (obtained from ATCC, Manassas, VA, USA) were used as positive controls for TLR antibody titration. Macrophages were prepared as smears that were air dried, fixed in 100% acetone for 10 min at room temperature, air dried and stored at -80°C . PMA-activated U937 cells were first pelleted then fixed in unbuffered formaldehyde and processed for embedding in wax. The macrophage cell smears were stained for the immunocytochemical detection of the TLRs in a similar fashion as the tissue samples except the antigen retrieval step was omitted. The U937 cells were sectioned at 5 microns and treated exactly as the tissue samples for immunocytochemical detection of the TLRs.

Results

Verifying Specificity and Optimizing the Titers of TLR 1–9 Antibodies

Initial testing of the antibodies against TLRs1–9 was conducted on acetone-fixed smears of macrophages and wax sections of activated U937 cells, which both express most of the TLRs. All of the anti-TLR antibodies used in this study reacted with macrophages and/or U937 cells. Lymphoid tissues from HIV⁻ and HIV⁺ men were used as a positive control to verify that the antibodies recognized TLRs in wax-embedded autopsy tissues. Lymph nodes from HIV⁺ men contain a high proportion of CD68⁺ macrophages and showed staining that was often very intense for TLRs2, 3, 4, 5 and 7 but little staining for TLRs1, 6, 8 or 9 (Fig. 1).

For negative controls, the immunocytochemical procedure was carried out in the absence of the primary antibody which was replaced with either a rabbit immunoglobulin fraction isolated from serum of healthy non-immunized rabbits for the rabbit polyclonal antibodies (TLRs2–5, 7 and 9) or normal goat serum for the antibodies raised in goats (TLRs1, 6 and 8). The negative controls were diluted to the same protein concentration as the primary antibodies. Negative controls of male genital tissue showed little non-specific staining.

TLR Expression in the Genital Tract of Normal (non-HIV-infected) Men

Testes—A total of 29 testes samples from seven patients were tested for TLR expression. Multiple tissue specimens from individual patients were examined. A majority of the samples (16 samples from six patients) showed definitive expression of TLR1 by cells resembling macrophages and/or mast cells in the interstitial tissue between the seminiferous tubules (Fig. 2a). No TLRs were detected within the seminiferous tubules.

Rete testis—A total of 19 rete testis samples were obtained from five patients. TLR expression was not detected in any of these samples.

Efferent ducts—A total of 19 efferent duct samples from eight patients were tested for TLR expression. No cells within the epithelium or connective tissue of the efferent ducts stained positive for any of the TLRs except for a single sample from one patient where TLR1⁺ cells were detected (Fig. 2b). These cells appeared to be macrophages and/or mast cells.

Epididymis—A total of 28 epididymal samples from nine patients were tested. These samples included sections from the initial segment, caput, corpus and cauda of the epididymis. For most patients, multiple samples along the length of the epididymis were analyzed. In all of the tissue regions studied, epithelial cells did not express any of the TLRs. However, in approximately half of the cases (ten samples from four patients), TLR1⁺ cells were observed in the connective tissue. When TLR1⁺ cells were observed in one region of the epididymis, i.e., initial segment, they were also observed in other regions, i.e., corpus, suggesting they were present throughout the length of the epididymis in positive cases.

A characteristic feature of the human epididymis is the presence of numerous macrophages in the lumen which phagocytose dead and dying germ cells. TLR expression was not detected on these specialized macrophages.

Vas deferens—A total of 15 vas deferens tissue samples from six patients were tested. No TLR⁺ cells were detected in these tissues, either in the epithelium or the lamina propria.

Seminal vesicles—A total of 45 seminal vesicle tissue samples from 12 patients were analyzed for TLR expression. In two patients, multiple samples along the entire length of the gland were examined. Epithelial cells did not express any of the TLRs, but in a majority of cases (11 samples from eight patients), individual TLR1⁺ cells resembling macrophages were detected within the epithelium and lamina propria, often in large numbers (Fig. 2c).

Prostate—A total of 77 samples of prostate tissue from 37 patients were studied for the expression of TLRs. In a subset of cases, TLR3 (seven samples: six surgical + one autopsy from seven patients) and TLR8 (seven samples: five surgical + two autopsy from seven patients) were expressed by epithelial cells in the prostate mucosa. TLR3 expression was limited to the apical surface of epithelial cells that protrude into the lumen of the prostate glands (Fig. 3a,b). In most of the positive samples, expression of TLR3 appeared uniform throughout the prostate. For one sample however TLR3 staining was localized to only a small number of epithelial cells of a few glands. Positive staining for TLR8 was detected as intensely staining cytoplasmic granules present mostly in the apical cytoplasm of the epithelial cells (Fig. 3c). TLR1⁺ cells that were often quite numerous were observed in both the lamina propria and epithelium (ten samples from eight patients) while TLR7⁺ intraepithelial cells ranging from few to many were also detected (15 samples from 15 patients) (Fig. 3d,e). A small number of TLR8⁺ and TLR9⁺ cells were present in the lamina propria of a few prostate tissues. All these TLR⁺ cells resembled macrophages.

Urethra—A total of 89 penile urethra samples from 25 patients were available for analysis. For each patient, multiple sites along the length of the urethra were examined. Urethral epithelial cells did not express TLRs2–8. In one case, TLR1 was observed in a small number of epithelial cells lining the urethra. In contrast, TLR9 was highly expressed by urethral epithelial cells in a majority of cases. Thirty-one urethral samples from 12 out of 16 patients tested displayed epithelial cells that contained bright red TLR9⁺ granules in the cytoplasm (Fig. 4a). These TLR9⁺ epithelial cells varied greatly in number, and there was also a dramatic difference in the abundance of positive cytoplasmic granules in the epithelial cells between individual patient urethral specimens. The TLR9⁺ epithelial cells were usually scattered throughout the epithelium and did not appear to be localized to any specific region of the urethral mucosa. However, in one patient, expression of TLR9 involved nearly the entire epithelium. The location of TLR9⁺ epithelial cells in the mucosal epithelium also differed between patient samples. In some, TLR9⁺ epithelial cells were mostly found at the base of the epithelium and contained only moderate numbers of positive cytoplasmic granules. For other samples, TLR9⁺ cells were present throughout the width of the epithelium and frequently were concentrated along the edge bordering the lumen. Moreover, in these epithelial cells, there appeared to be an increased amount of TLR9⁺ cytoplasmic granules particularly in the apical cytoplasm bordering the lumen.

Immune cells in the urethra expressed various TLRs. In approximately one-third of the samples (22 samples from seven patients), TLR1⁺ cells ranging from few to abundant were detected within the lamina propria and epithelium. For four patients, (three of which had a documented history of insertion of a Foley urethral catheter) these cells resembled granulocytes (Fig. 4b) while in two patients they resembled macrophages (Fig. 4c). The last patient displayed a differential expression of TLR1 along the length of the urethra. Tissue samples taken proximal to the urethral opening contained primarily TLR1⁺ granulocytes, whereas those located more distally contained TLR1⁺ cells that resembled macrophages. Small numbers of TLR2⁺ cells of lymphocytic morphology were detected in the urethral mucosa (53 samples from 18 out of 24 patients) and were present primarily in the lamina propria; one patient, however, had an abundance of TLR2⁺ cells in the connective tissue at the tip of the penis (Fig. 4d).

TLR3⁺ cells were observed mainly in the lamina propria (ten samples from eight out of 24 patients), although TLR3⁺ intraepithelial cells were observed in samples from two men. Abundant TLR3⁺ cells were found in the connective tissue of the tip of the penis in the patient that had numerous TLR2⁺ cells present in the same region (Fig. 4e). Few TLR4⁺ cells were detected in any of the samples studied. In contrast, abundant TLR5⁺ cells of lymphocytic morphology were commonly observed in the lamina propria (57 samples from 21 out of 25 patients). In a few cases, TLR5⁺ lymphocytes were also found within the epithelium of the fossa navicularis and penile urethra (Fig. 4f).

No definitive staining for TLR6 or 8 was detected for any cell type present in the urethral mucosa. A small number of TLR7⁺ cells resembling macrophages were found primarily within the epithelium in a majority of cases (36 samples from 15 out of 18 patients) (Fig. 5a). Furthermore, cells that stained intensely for TLR9 and resembled macrophages were consistently detected along the length of the urethral epithelium of most tissue samples

analyzed (36 samples from 13 out of 16 patients) ranging in number from few to abundant (Figs 4a and 5b). Many of these TLR 9⁺ cells were located at an adluminal position. In most cases, few TLR9⁺ cells were observed in the lamina propria, but in five cases, abundant TLR9⁺ cells were detected (Fig. 5b). Interestingly, two patients had numerous TLR9⁺ cells in the corpus spongiosum (Fig. 5c).

Penile skin and foreskin—TLR expression was not detected in any of the specimens of penile skin (both the shaft and head, $n = 4$) or foreskin ($n = 9$).

Expression of TLRs in Genital Tract Tissue of HIV⁺ Men

Testes—A total of 34 samples of testicular tissue obtained from 21 patients were screened for the expression of TLRs. For several patients, multiple samples of the testis were analyzed. None of the TLRs were expressed in any of these tissues except for TLR2, which was observed on cells in the interstitium of the testis for two patients only (Fig. 6a).

Rete testes—A total of nine samples obtained from six patients were tested. No staining for any of the TLRs could be detected in any of the samples of the rete testes.

Efferent ducts—A total of 12 samples obtained from three patients were tested. No staining for any of the TLRs could be detected in any of these samples from the efferent ducts except for TLR7, where a few positive cells were observed in the lamina propria of two samples from three patients.

Epididymis—A total of seven samples from three patients were tested. No staining for TLRs1–6 was observed. In one sample from one patient, however, epithelial cells lining the caput/corpus epididymis were weakly positive for TLRs7 and 8, and strongly positive for TLR9 (Fig. 6b-d).

Vas deferens—Six samples from two patients showed no staining for TLR1–9.

Prostate—A total of 15 samples of prostate tissue from six patients were tested for TLRs. No expression of TLRs1–6 or 9 was observed. However, TLR7⁺ intraepithelial leukocytes, ranging in number from few to many, were consistently found in the glandular epithelium from four patients (Fig. 6e). One sample from one patient had bright red TLR8⁺ granules in the cytoplasm of some epithelial cells in a small number of glands.

Seminal vesicles—No seminal vesicle tissue samples were available from HIV⁺ men.

Urethra—A total of 12 samples from two patients were studied for expression of TLRs. In contrast to the abundant expression of TLRs observed in normal urethra, no TLR expression was observed in the urethral mucosa of any HIV⁺ specimens.

TLR localization in male genital tract tissues from HIV[−] and HIV⁺ men is summarized in Table II.

Discussion

This is the first comprehensive study on the expression of TLRs by cells and tissues of the human male genital tract. Previous studies have shown TLR-encoding mRNA in human prostate tissue and cell lines^{29,30} and abundant messenger RNA for TLR1–9 in the rat testes, epididymis and vas deferens. Mouse Sertoli cells also express high levels of TLR2 and 5.^{31,32} We found regional differences in the capacity of genital tissues from normal men to express selected TLRs. Except for TLR1, which was detected on immune cells in a majority of tissues, the other TLRs were not detectable on epithelial or immune cells in the testis, epididymis or seminal vesicles including the specialized macrophages in the lumen of the efferent ducts and epididymis. In the prostate and penile urethra, however, several TLRs were expressed. TLR3 and TLR8 were detected on epithelial cells in the prostate, and TLR9 was detected on epithelial cells of the penile urethra. Immune cells in the prostate and urethra from normal men expressed a wide variety of TLRs.

The TLRs3, 8 and 9 expressed by epithelial cells in the prostate and urethra are normally located in the endosomal compartments and recognize viral nucleic acids that have to be internalized to be detected.^{33–35} The epithelial cell staining pattern for TLR8 in the prostate and TLR9 in the urethra occurred as bright red positive granules located in the cytoplasm, which is consistent with their endosomal localization. In contrast, TLR3 was clearly and definitively expressed on the cell membrane of the apical cytoplasm of prostate epithelial cells that line the lumen of the glands. Cell surface expression of TLR3 reportedly occurs in the epithelium of the endocervix,¹⁷ cornea³⁶ and human fibroblast cell lines.³⁷ This would suggest that in some tissues TLR3 can be expressed on the cell surface and may be capable of responding to extracellular PAMPS. TLRs3, 8 and 9 were not constitutively expressed by the glands of the prostate or urethral epithelium because only a subset of samples expressed these TLRs. Also in some cases, staining for TLR3 and TLR8 was localized to only a few prostatic glands while in most cases TLR9 expression occurred in different regions along the length of the urethral epithelium.

The factor(s) that stimulated the expression of TLRs3 and 8 in the prostate epithelium are as yet unknown. It should be noted, however, that most prostate samples that stained positive for TLR3 (six out of seven) and TLR8 (five out of seven) were obtained as surgical specimens from prostatectomies performed as treatment for prostatic cancer but showed no morphological evidence of malignancy. TLR3 and 8 can respond to ‘self’ nucleic acids,³⁴ and it is possible that soluble factors emanating from the tumor diffused out to influence TLR expression in healthy tissue proximal to the affected tissue. This could explain the up-regulated expression of both TLR3 and 8 in the mucosa of prostatectomy samples compared to prostates obtained at autopsy. Another potential factor that could affect TLR3 and 8 expression by the prostate is cytomegalovirus (CMV) infection. CMV, a member of the herpes virus family, can be sexually transmitted to persistently infect the prostate epithelium. Recent evidence suggests a relationship between CMV infection and prostatic carcinoma.³⁸

Detection of TLR9 in urethral epithelial cells from most patients provides evidence that the urethral epithelium can respond to viral infections. Moreover, although the number of

positive cells varied, it was the most prevalent of all the TLRs expressed by the epithelium lining the male genital tract. Activation of TLR9 has been found to protect the mouse vagina against herpes simplex virus type 2 (HSV-2) infection.^{39,40}

In this study, TLRs were not detected in the epithelium covering the penis or foreskin. This is in contrast to other studies which have reported that keratinocytes cultured from human skin constitutively express mRNA for TLRs1–6 and TLRs9 and 10, but not TLRs7 and 8.^{41–45} This difference could be attributable to different sensitivities of immunohistology vs. PCR assays for detection of TLRs.

There were distinct differences in the expression of TLRs by immune cells in the different tissues comprising the male genital tract. The most commonly detected TLR was TLR1 which responds to bacterial lipoprotein.⁴⁶ It has been reported that TLR1 is more widely expressed in tissues than any other TLR.⁴⁷ In the testes, TLR1⁺ cells were commonly present in the interstitium. Under normal conditions, the most prevalent immune cells in the testis are interstitial macrophages and mast cells. Although we have yet to positively identify the TLR1⁺ cells in the testicular interstitium, they morphologically resembled mast cells. Mast cells can express TLR1;⁴⁸ if the TLR1⁺ cells in the testicular interstitium prove to be mast cells, this would provide evidence that these cells perform an important defense function in the testis. Small numbers of TLR1⁺ cells were often detected in the lamina propria of the excurrent ducts. Unlike the testis, T lymphocytes are normally present in the efferent ducts and epididymis. Morphologically, the TLR1⁺ cells in these tissues were round in shape resembling mast cells or lymphocytes more than macrophages. Greater numbers of TLR1⁺ cells were detected in the accessory glands and urethral mucosa. Interestingly, although TLR1⁺ cells were present in the lamina propria in these tissues, they were also now detected within the epithelium. Moreover, these intraepithelial TLR1⁺ cells were large and irregular in shape resembling macrophages.

TLR1⁺ immune cells were prevalent along the length of the urethral mucosa and were especially abundant in samples from patients with a documented history of Foley catheter use, which can cause irritation of the mucosa and also pose as a risk factor for acquiring a urinary tract infection. In 3 of these patients, numerous TLR1⁺ granulocytes were detected in both the lamina propria and epithelium of the urethra indicating the catheter may have induced an inflammatory response.

The expression of the TLRs2, 3 and 5 was only detected on immune cells in the urethral mucosa and when present were found mostly in the lamina propria although they were occasionally located within the epithelium in a few patients. In one patient, however, numerous TLR2⁺ and TLR3⁺ cells were in the connective tissue of the head of the penis. TLR2 recognizes a broad range of microbial products from bacteria and fungi while TLR3 responds to double stranded RNA of viral origin and TLR5 to flagellin.^{33,49,50} These TLR⁺ cells had a lymphocytic morphology, and their presence indicates that the urethral mucosa is capable of mounting a localized immune response to pathogenic viruses, bacteria and fungi as well as flagellated parasites. The absence of TLR4 expression by the urethral mucosa could prevent inappropriate inflammatory responses to the normal commensal flora that

reside in the penile urethra. A similar explanation has been suggested to account for the lack of TLR4 expression by the vaginal and intestinal mucosa.^{14,17}

Most samples of the prostate and urethra contained varying numbers of TLR7⁺ cells that resembled macrophages and were primarily intraepithelial. Although no TLR9⁺ immune cells could be found in the prostate, they were commonly observed, often in large numbers, in the urethra. Urethral TLR9⁺ cells were primarily located within the epithelium, but in a few cases, abundant TLR9⁺ immune cells were also in the lamina propria and occasionally large numbers could be found within the corpus spongiosum. Common viral infections of the prostate and urethra include CMV, human papilloma virus and HSV-2. It was interesting, therefore, that in the genital tract only these two organs contained immune cells that expressed the TLRs7 and 9 involved in the detection of viral products. These cells resembled macrophages and were primarily located within the epithelium. It has been shown, however, that plasmacytoid DCs also express TLR7 and TLR9^{51,52} and respond to a broad range of pathogens to play a critical role in linking innate immunity to the adaptive immune system (reviewed by Ref. 53).

TLR⁺ intraepithelial macrophages present in the prostate and urethra are strategically located to act as immunological sentinels to detect and respond to invading sexually transmitted pathogens. If these pathogens manage to breach the mucosal epithelium, however, they would encounter a complement of immune cells expressing a variety of TLRs in the lamina propria. These cells could then mount an innate immune response to limit further systemic dissemination of these pathogens.

The penile urethra is an initial site for infection by several sexually transmitted pathogens. Our data show that the urethral mucosa has the ability to recognize these pathogens because a variety of TLRs can be expressed by not only the epithelium but also by intraepithelial immune cells as well as a population of immune cells present in the lamina propria. In this respect, the prostate is also capable of detecting various pathogens. These findings indicate that the urethra and prostate may act as 'immunological gatekeepers' of the male genital tract, because they both express a selective repertoire of TLRs that would enable them to mount a vigorous innate immune response against invading organisms. This would prevent pathogens from ascending the genital tract to infect other organs which do not appear to be as well-endowed with TLRs to develop a strong innate immune response.

Whereas numerous TLRs were expressed in lymph nodes from HIV⁺ men, very little TLR expression was observed in HIV⁺ genital tract tissues. TLR2⁺ cells were detected in the interstitium of the testis in two cases, and epithelial cells in the epididymis of one patient expressed TLR7 and 8 (weakly) and TLR9 (strong). The expression of TLRs7, 8 and 9 could be related to HIV infection because all these receptors are involved in recognizing intracellular viral proteins.^{34,54-56}

During HIV-1 infection, there is a progressive decrease in spermatogenesis, and macrophages and lymphocytes infiltrate into the testicular interstitial tissue as well as the excurrent ducts and accessory glands.⁵⁷ None of these macrophages or T lymphocytes showed evidence of TLR expression, except for two patients with TLR2⁺ cells in the

testicular interstitium. There was however some localized expression of TLR8 on a few prostate glands in one patient. A more consistent finding was the detection of TLR7⁺ intraepithelial cells that morphologically resembled macrophages in all prostate tissue samples studied. The prostate can be infected by HIV,⁵⁷ and this could have upregulated TLR7 expression. Notably, there was no evidence of TLR expression in urethral tissues from HIV⁺ men.

The negative findings with HIV⁺ tissue could be because of the small sample size, and the fact that most of the samples were obtained from autopsy cases and therefore may have undergone degradation. Some studies have reported, however, that peripheral blood mononuclear cells from HIV⁺ patients can exhibit either a reduced expression of TLRs or a diminished capacity to respond to pathogens.⁵⁸⁻⁶⁰ Furthermore in HIV⁺ patients, plasmacytoid DCs have an impaired ability to respond to ligands for TLR7 and 9.⁶¹ HIV-1 disease progression also has been related to depletion and impaired function of plasmacytoid DCs.^{62,63} Further studies are needed to confirm our initial findings that HIV-1 infection can affect TLR expression in male genital tract tissues.

Additional research is needed to further understand TLR expression in the male genital tract. The immune cells that express TLRs should be identified by colocalization techniques. It would also be of importance to determine that the TLRs in the male genital tract are functionally active, and the intracellular pathways and effector molecules that are involved. Factors that upregulate TLRs in the male genital tract could be identified by associating TLR expression with the presence of sexually transmitted infections (STIs) and by stimulating primary cell lines derived from genital tract cells with PAMPS from STIs.

The results of this study indicate that TLRs may play a role in innate immune defense of the lower male genital tract, especially in the penile urethra. These results could also have implications for STI vaccine development. TLRs provide an important link between innate and acquired immunity by recruiting, mobilizing and maturing DCs and other immunocompetent phagocytes and lymphocytes at the site of infection.⁶⁴ Information about TLR expression and function in genital tract tissues could suggest new approaches to combating infections at genital mucosal tissues; appropriate TLR agonists incorporated into microbicide or mucosal vaccine formulations could enhance effective local innate immune responses against sexually transmitted pathogens.⁶⁵⁻⁶⁷

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Fig. 1. Immunohistochemistry performed on a lymph node from an HIV⁺ man showing expression of (a) TLR2, (b) TLR3, (c) TLR4, (d) TLR5, (e) TLR7 and (f) CD68 (macrophage marker) all at $\times 150$.

Fig. 2. TLR1 expression by cells in (a) interstitium of testis $\times 100$, (b) stroma of efferent ducts $\times 175$ and (c) epithelium of seminal vesicle $\times 150$.

Fig. 3. TLR expression in prostate: (a) TLR3⁺ epithelium of glands $\times 180$, (b) TLR3 expression limited to apical membrane of epithelial cells $\times 250$, (c) TLR8⁺ granules in cytoplasm of epithelial cells $\times 200$, d) TLR1⁺ cells in epithelium $\times 160$ and (e) TLR7⁺ intraepithelial cell $\times 250$.

Fig. 4.

TLR expression in the urethral mucosa: (a) TLR9⁺ granules in cytoplasm of epithelial cells (arrows) and intensely stained TLR9⁺ intraepithelial cells $\times 200$, (b) TLR1⁺ cells that resemble granulocytes in epithelium of fossa navicularis $\times 260$, (c) TLR1⁺ cells that resemble macrophages in epithelium and lamina propria $\times 150$, (d) TLR2⁺ cells in stroma and epithelium at tip of penis $\times 160$, (e) TLR3⁺ cells in stroma and epithelium at tip of penis $\times 150$ and (f) TLR5⁺ cells in epithelium and lamina propria of the urethra $\times 120$.

Fig. 5. TLR expression by urethral mucosa: (a). TLR7⁺ intraepithelial cell $\times 260$, (b) numerous TLR9⁺ cells located in the epithelium and lamina propria $\times 150$ and (c) TLR9⁺ cells detected in the stroma of the corpus spongiosum $\times 160$.

Fig. 6. TLR expression by genital tract tissue from HIV⁺ men: (a) TLR2⁺ cells in interstitium of testes $\times 180$, (b) TLR7⁺ epithelial cells of epididymis $\times 175$, (c) TLR8⁺ epithelial cells of epididymis $\times 175$, (d) TLR9⁺ epithelial cells of epididymis $\times 175$ and (e) TLR7⁺ intraepithelial cell in prostate gland $\times 250$.

Table I**TLR Antibodies**

TLR 1	Epitope mapping near the N-terminus
TLR 2	Against an epitope corresponding to amino acids 180–354
TLR 3	Against an epitope corresponding to amino acids 653–777
TLR 4	Against an epitope corresponding to amino acids 242–321
TLR 5	Against an epitope corresponding to amino acids 154–280
TLR 6	Epitope mapping near to N-terminus
TLR 7	Against an epitope corresponding to amino acids 27–140
TLR 8	Epitope mapping to C-terminus
TLR 9	Against an epitope corresponding to amino acids 771–870

Table II

Summary of Expression of Toll-like Receptors (TLRs) in Human Male Genital Tissues

	HIV–	HIV+
Testis	<i>n</i> = 7 WBC: TLR1 (6/7)	<i>n</i> = 21 WBC: TLR2 (2/21)
Efferent ducts	<i>n</i> = 8 EC: – WBC: TLR1 (1/8)	<i>n</i> = 3 EC: – WBC: TLR7 (2/3)
Epididymis	<i>n</i> = 9 EC: – WBC: TLR1 (4/9)	<i>n</i> = 3 EC: TLRs7, 8, 9 (1/3) WBC: –
Seminal vesicles	<i>n</i> = 12 EC: – WBC: TLR1 [8/12]	NA
Prostate	<i>n</i> = 37 EC: TLR3 (7/37); TLR8 (7/37) WBC: TLR1 (8/37); TLR7 (15/37); TLR8 (3/37); TLR9 (1/37)	<i>n</i> = 6 EC: TLR8 [1/6] WBC: TLR7 (4/6)
Urethra	<i>n</i> = 25 EC: TLR1 (1/25); TLR 9 (12/16) WBC: TLR1 (7/25); TLR 2 (18/24); TLR3 (8/22); TLR 4 (1/24); TLR5 (21/25); TLR 6 [0/18]; TLR7 (15/18); TLR 8 [0/19]; TLR9 (13/16)	<i>n</i> = 2 EC: – WBC: –

n, number of cases analyzed; EC, epithelial cell; WBC, white blood cell.

No TLRs were detected in rete testis (*n* = 5), vas deferens (*n* = 6) or foreskin (*n* = 9) tissue.