Innate immunity in the male genital tract: *Chlamydia trachomatis* induces keratinocyte-derived chemokine production in prostate, seminal vesicle and epididymis/vas deferens primary cultures

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*Chlamydia trachomatis* is an intracellular pathogen that infects mucosal epithelial cells, causing persistent infections. Although chronic inflammation is a hallmark of chlamydial disease, the proinflammatory mechanisms involved are poorly understood. Little is known about how innate immunity in the male genital tract (MGT) responds to *C. trachomatis*. Toll-like receptors (TLRs) are a family of receptors of the innate immunity that recognize different pathogen-associated molecular patterns (PAMPs) present in bacteria, viruses, yeasts and parasites. The study of TLR expression in the MGT has been poorly investigated. The aim of this work was to investigate the keratinocyte-derived chemokine (KC) response of MGT primary cultures from C57BL/6 mice to *C. trachomatis* and different PAMPs. KC production by prostate, seminal vesicle and epididymis/vas deferens cell cultures was determined by ELISA in culture supernatants. TLR2, 3, 4 and 9 agonists induced the production of KC by all MGT primary cultures assayed. In addition, we analysed the host response against *C. trachomatis* and *Chlamydia muridarum*. Chlamydial LPS (cLPS) as well as *C. trachomatis* and *C. muridarum* infection induced KC secretion by all MGT cell cultures analysed. Differences in KC levels were observed between cultures, suggesting specific sensitivity against pathogens among MGT tissues. Chemokine secretion was observed after stimulation of seminal vesicle cells with TLR agonists, cLPS and *C. trachomatis*. To our knowledge, this is the first report showing KC production by seminal vesicle cells after stimulation with TLR ligands, *C. trachomatis* or *C. muridarum* antigens. These results indicate that different receptors of the innate immunity are present in the MGT. Understanding specific immune responses, both innate and adaptive, against chlamydial infections, mounted in each tissue of the MGT, will be crucial to design new therapeutic approaches where innate and/or adaptive immunity would be targeted.

**INTRODUCTION**

It is widely known that epithelial cells are the first line of defence against pathogens and some immune properties initially thought to be exclusive to immune cells are now expanding to epithelial biology. Understanding which role epithelial cells of the male genital tract (MGT) play in pathogen immune surveillance is crucial to design new adjuvant and mucosal vaccines as well as efficient immune therapies against persistent infections (Darville & Hiltke, 2010).

Toll-like receptors (TLRs) are a conserved group of innate receptors with diverse tissue expression. These receptors recognize different pathogen-associated molecular patterns.
Escherichia coli responded to carcinoma-derived epithelial cell line expressed TLR4 and expression of TLRs in the female reproductive tract (Pioli et al. 2007; Gribar 2006). Lung epithelial cells have been shown to express a wide range of TLRs. Epithelial cells throughout the body have been shown to be infected tissue and probably results in pathogen clearance. Pathogen recognition by TLRs elicits an immune response mainly inducing proinflammatory mediators, which could eventually turn these cells into dominant players in the pathophysiology of the disease. Also, TLR2, TLR4 and MyD88, but not TLR5, were recruited to the chlamydial inclusion vicinity, suggesting an active role of these receptors in bacterial recognition and activation of PECs (Mackern-Oberti et al. 2006).

Although it is well known that C. trachomatis causes urethritis, epididymitis, orchitis and chronic prostatitis, research focused on the pathogenic mechanisms involved in MGT infections is still very limited (Jantos et al., 1998; Domingue & Hellstrom, 1998; Pal et al., 2004; Skerk et al., 2004; Motrich et al., 2006; Wagenlehner et al., 2006; Cunningham & Beagley, 2008). Data from our laboratory demonstrated that rat prostate epithelial cells (PECs) were susceptible to infection with Chlamydia muridarum, a murine pathogen closely related to C. trachomatis (Mackern-Oberti et al., 2006). Rat PECs responded to infection by upregulating proinflammatory mediators, which could eventually turn these cells into dominant players in the pathophysiology of the disease. Also, TLR2, TLR4 and MyD88, but not TLR5, were recruited to the chlamydial inclusion vicinity, suggesting an active role of these receptors in bacterial recognition and activation of PECs (Mackern-Oberti et al. 2006).

Different possible chlamydial ligands such as chlamydial LPS (cLPS), macrophage infectivity potentiator (Mip) or Chlamydia heat-shock protein 60 (cHSP60) have been demonstrated to be recognized by TLRs on classical innate immune cells such as macrophages and dendritic cells, inducing the secretion of proinflammatory cytokines and chemokines (Erridge et al., 2004; Bas et al., 2008). It is evident that while a huge amount of information exists about Chlamydia recognition by innate immune system cells (Vabulas et al., 2001; Bulut et al., 2002; Prebeck et al., 2003; da Costa et al., 2004; Nagarajan et al., 2005; Rodriguez et al., 2005; Buchholz & Stephens, 2006, 2008; Joyee & Yang, 2008), data are lacking regarding how MGT epithelial cells, the first ones to have contact with the pathogen, deal with chlamydial infection (Pate et al., 2001; Al-Mously & Eley, 2007).

Previous studies have shown that chlamydial infection is accompanied by significant infiltration of neutrophils at the site of infection (Bai et al., 2005; Barteneva et al., 1996; Arya et al., 1984). The molecular mechanisms for recruitment of neutrophils to the MGT probably involve the local release of neutrophil chemoattractants such as keratinocyte-derived chemokine (KC) and other chemokines. In the present work, we analysed the response of murine MGT primary cultures to different TLR ligands as KC secretion. We also investigated whether MGT primary cultures respond to cLPS and are susceptible to infection by Chlamydia. We demonstrate that murine MGT primary cultures respond to TLR2, 3, 4 and 9 ligands by secreting the KC in a time-dependent manner. MGT primary cultures also respond to cLPS, they are susceptible to chlamydial infection and secrete KC after infection.

**METHODS**

**Antibodies and reagents.** The mouse anti-Chlamydia species LPS (cLPS)-FITC mAb was obtained from bioMérieux. The mouse anti-cytoketatin mAb (C-11) (recognizing cytoketatin 4, 5, 6, 8, 10, 13 and 18) was bought from Sigma-Aldrich. Endotoxin (LPS) from Escherichia coli O55:B5, N-palmitoyl-S-[2,3,5,7-tetramethyl]-propyl- (R)-cysteinyl-(lysyl)3-lysine (Pam3CSK4), a non-methylated deoxytethyl-phosphate-deoxyguanosine (CpG-ODN) and polyinosine-polycytidylic acid [poly(I:C)] were purchased from Sigma-Aldrich.
Ultrapure LPS was from E. coli K-12 (InvivoGen); cLPS was extracted as described by Hosseinzadeh et al. (2003) and further repurified by the method of Hirschfeld et al. (2001).

**Strains of mice.** C57BL/6 mice were bred and maintained under specific-pathogen-free conditions in the vivarium of the Center of Immunology and Biochemical Chemistry Research (CIBICI-CONICET) of the National University of Cordoba, Argentina. Animals were maintained in a 16 h light, 8 h dark cycle at 20 °C, with free access to food and water.

**Chlamydia strain.** C. trachomatis serovar E was propagated in HEp-2 cells and purified in a sucrose Urografiin gradient as described previously by Maass & Dalhoff (1995). The C. trachomatis MoPn strain (now C. muridarum MoPn) was kindly supplied by K. H. Ramsey (Midwestern University, USA) and was propagated in HEp-2 cells. Briefly, HEp-2 cultures were grown in RPMI 1640 supplemented with 20 μg gentamicin ml⁻¹ and 5 % fetal bovine serum at 37 °C with 5 % CO₂. Cultures infected with C. trachomatis serovar E or C. muridarum were grown for 48 h in the presence of 1 μg cycloheximide ml⁻¹. Infected monolayers were detached by scraping and disrupted by sterile glass beads to lyse the host cells and cause a release of elementary bodies. Cell debris was removed by centrifugation at 500 g for 15 min. Elementary bodies were purified in a sucrose Urografiin gradient (bottom layer 50 %, w/v, sucrose solution; top layer, 30 %, w/v, Urografiin in 30 mM Tris/HCl buffer, pH 7.4) at 9000 g and 4 °C for 60 min. After one wash step with 0.2 μm-filtered PBS (pH 7.4), purified elementary bodies were stored in SPG buffer (0.2 M sucrose, 8.6 mM Na₂HPO₄, 3.8 mM KH₂PO₄, 5 mM glutamic acid, 0.2 μm-filtered, pH 7.4) at −70 °C until use. To quantify the number of elementary bodies, HEp-2 cells were infected and stained with the chlamydia-specific antibody. The number of inclusion-forming units was counted, as determined by fluorescence microscopy (Carl Zeiss Jena), 24 h after infection. For a control, non-infected HEp-2 cells were treated in the same way. Contamination with Mycoplasma was excluded regularly by PCR using specific primers (van Kuppeveld et al., 1994).

**Cell culture.** Primary prostate, seminal vesicle and epididymal-vas deferens cells were obtained following protocols described by Ilio et al. (1998). Briefly, C57BL/6 mice were sacrificed, organs were harvested and minced under sterile conditions into 1–2 mm fragments and subjected to enzymic digestion using type IV collagenase and stained with the chlamydia-specific antibody. The number of elementary bodies, HEp-2 cells were infected with C. trachomatis or C. muridarum using an m.o.i. of 10. The plates were spun at 1400 g for 60 min and placed under 5 % CO₂ in air at 37 °C for 2 h. After the incubation, cells were washed twice with HBSS, which was replaced by supplemented medium lacking gentamicin. Cultures were incubated at 37 °C for up to 3 days. In some experiments, supernatants were removed at different times after infection to determine chemokine levels.

**Chlamydia infection and direct immunofluorescence.** To determine the level of infection, cultures of prostate, seminal vesicle, epididymal-vas deferens or HEp-2 cells were grown in 24-well tissue culture plates on sterile coverslips and subsequently infected with Chlamydia elementary bodies. Following incubation for 48 h, cells were fixed with methanol and stained with anti-chlamydia LPS mAb (bioMérieux). The presence of inclusion bodies was visualized on a confocal laser scanning microscope (LSM 510; Zeiss) using LSM 510 software for image analysis.

**Detection of chemokines.** The chemokine CXCL1 or KC was detected in supernatants of stimulated or infected cultures by commercially available ELISA kits (R&D Systems). The assays were performed as described by the manufacturer.

**Statistical analysis.** Statistical analysis was performed using the LSD Fisher test and the InfoStat software (developed by the Statistics Department, National University of Córdoba). Values of P<0.05 were considered significant.

**RESULTS**

**TLR2, TLR3, TLR4 and TLR9 agonists induced the secretion of KC by MGT primary cultures**

Acute microbial infections are characterized by intense inflammation and infiltration of the mucosa predominantly by neutrophils and other inflammatory cells such as macrophages and lymphocytes. TLRs are crucial effectors in initiating this immune response by sensing extracellular milieu. Scarce reports showed that antimicrobial peptides and pathogen recognition receptors are expressed in MGT tissues. Moreover, studies where chemokine response was analysed after TLR agonist stimulation of MGT cultures have not been performed. Therefore, we performed primary cultures of the MGT following described protocols in order to evaluate the innate immune response to microbial compounds. More than 80 % of cells from prostate (Fig. 1) and seminal vesicle (data not shown) cultures stained with the anti-pan-cytokeratin antibody, a marker of epithelial cells. In contrast, primary culture of epididymis/vas deferens cells showed 70 % sperm cells (data not shown). To determine whether primary cultures of prostate, seminal vesicle and epididymis/vas deferens respond to TLR2, TLR3, TLR4 and TLR9 agonists, we tested the production of KC by TLR ligand stimulation. KC (also named CXCL1) is a proinflammatory mediator produced by immune cells and
epithelial cells from different tissues with a strong neutrophil chemoattractant activity. Primary MGT cultures were stimulated with TLR agonists and the levels of KC were evaluated at different time points. As shown in Fig. 2(a), prostate as well as seminal vesicle and epididymis/vas deferens primary cultures secreted KC after stimulation with the TLR2 agonist Pam3CSK4. The pattern of chemokine secretion differed between tissues: prostate cultures induced a higher response after TLR2 agonist activation whereas significant but lower levels of KC were measured at 24 h or 48 h after stimulation of seminal vesicle or epididymis/vas deferens cultures, respectively. These results suggest that primary cultures of prostate are more sensitive than seminal vesicle or epididymis/vas deferens cultures to TLR agonists such as peptidoglycan from bacteria.

When KC production triggered by a TLR3 agonist was evaluated, similar results were found. Prostate as well as seminal vesicle and epididymis/vas deferens primary cultures secreted KC after stimulation with the TLR3 agonist poly(I:C). Prostate and epididymis/vas deferens primary cultures produced a higher KC response when compared to seminal vesicle cultures, suggesting that prostate and epididymis/vas deferens cells would be the best responders to double-stranded viral RNA (Fig. 2b).

The TLR4 agonist *E. coli* LPS also induced KC secretion by MGT cultures (Fig. 2c). Prostate primary cultures induced the greatest response after LPS stimulation, but seminal vesicle and epididymis/vas deferens cultures also induced a significant secretion of KC. When ultrapure LPS was used to stimulate MGT cultures, KC secretion was again observed, confirming that the KC response was due to LPS (Fig. 2d). These results suggest that primary cultures of prostate, seminal vesicle and epididymis/vas deferens are highly sensitive to Gram-negative pathogens.

Finally, when stimulation with the TLR9 agonist CpG was analysed, epididymis/vas deferens cultures exhibited a high KC response. In contrast, prostate and seminal vesicle cultures stimulated with CpG produced low KC secretion (Fig. 2e). These results suggest that epididymis cells may be more sensitive to bacterial DNA.

Altogether these results showed that MGT cells are able to respond to TLR agonists with prostate culture cells more sensitive to the different PAMPs reported to be present in Gram-positive or -negative bacteria, viruses or bacteria DNA. Moreover, seminal vesicle cells were less responsive to TLR agonists.

**Chlamydial endotoxin stimulation induced secretion of KC by primary cultures of prostate, seminal vesicle and epididymis/vas deferens**

Several recent studies have suggested that chlamydial pathogenesis may be caused by a consistent generation of proinflammatory mediators secondary to bacterial recognition, epithelial cells being the major target of infection. cLPS is one of the most studied chamydial components. We asked whether cLPS stimulation would induce KC production by MGT primary cultures. As can be seen in Fig. 3, the addition of cLPS to the cultures induced the secretion of KC by prostate, seminal vesicle and epididymis/vas deferens cells. Similarly to values observed after stimulation with TLR2/4 agonists (the proposed TLRs associated with cLPS), KC production by seminal vesicle cultures was lower than that observed in prostate or epididymis/vas deferens culture supernatants. Epididymis/vas deferens cultures stimulated with cLPS secreted similar levels of KC to prostate cultures. It has been reported that macrophages or dendritic cells stimulated with cLPS produce lower levels of TNFα than after stimulation with *E. coli* LPS (Heine et al., 2003; Prebeck et al., 2003; Erridge et al., 2004). Our results are in agreement with these findings, since KC values observed after cLPS stimulation were also lower than those after *E. coli* LPS stimulation.

![Fig. 1. Cytokeratin expression by prostate primary cultures.](image-url)
These results suggest that MGT cells can sense *C. trachomatis* bacteria recognizing cLPS and secreting a neutrophil chemoattractant chemokine.

**C. trachomatis** and **C. muridarum** induced KC secretion by primary cultures of prostate, seminal vesicle and epididymis/vas deferens

We have previously demonstrated that rat prostate primary cultures were susceptible to *C. muridarum* infection, but limited reports are available demonstrating that urethral, prostate or epididymis/vas deferens cells can be infected by *C. trachomatis*. To our knowledge, there is no evidence so far that seminal vesicle cells are susceptible to infection with *C. trachomatis*. To analyse whether MGT cells were susceptible to chlamydial infection, we performed classical infection protocols and evaluated the presence of *C. trachomatis* by immunofluorescence. Infection of murine prostate (Fig. 4) and seminal vesicle cultures resulted in the formation of typical chlamydial inclusions (data not shown). Moreover, we also analysed KC levels in MGT cultures after *C. trachomatis* infection. As can be seen in Fig. 5(a), the addition of viable bacteria induced the secretion of KC by MGT cells. Prostate cultures responded to *C. trachomatis* infection in a time-dependent fashion. Prostate and seminal vesicle cultures produced higher levels of KC in response to bacterial infection. Epididymis/vas deferens cultures also responded to *C. trachomatis*, producing KC but less efficiently. Levels of KC secreted after *C. trachomatis* infection in all MGT cultures were considerably lower than those measured after 48 h of cLPS stimulation of the cultures (Fig. 3). In order to analyse the response of MGT cultures using a murine strain of *Chlamydia*, we performed experiments incubating MGT cultures with *C. muridarum* and analysed KC secretion after 48 h of stimulation. As can be seen in Fig. 5(b), *C. muridarum* was also able to stimulate the secretion of KC in MGT cultures. Interestingly, the response of epididymis/...
vas deferens cells was higher than that observed after *C. trachomatis* stimulation.

These results suggest that murine MGT cells are able to respond to microbial ligands such as cLPS and also are susceptible to *C. trachomatis* and *C. muridarum* infection. They upregulate mediators which could chemoattract innate cells to the site of infection contributing to the clearance of the pathogen.

**DISCUSSION**

Although considerable effort has been made to understand TLR responses in a wide diversity of systems, the role of TLR-related molecules in the MGT remains poorly understood. Expression, function and synergism of individual TLR molecules would be crucial for understanding local innate immunity against specific pathogens. The reproductive tract, especially the lower genital tract, is continuously challenged by potential pathogens present in the environment (Krause & Bohring, 2003; Krause, 2008). Therefore, robust host defence mechanisms are essential for both health and fertilization. It is well known that bacterial, viral and yeast infections of the MGT hamper sperm cell maturation and contribute to an impaired male fertility, principally associated with a strong proinflammatory milieu. Infections also induce the production of reactive oxygen species, particularly by macrophages, causing oxidative damage to spermatozoa (Fraczek & Kurpisz, 2007; Eley et al., 2005). The role of pathogen recognition receptors in the male reproductive tract has also been poorly studied. In particular, most scientific studies have been related to detection of antimicrobial peptides and TLR expression by immunohistochemical techniques without evaluating functional activity (Palladino et al., 2007).

In recent years, a number of studies have reported the presence of different TLRs in the female reproductive tract (Andersen et al., 2006). Individual TLRs appear to have specific expression patterns in the endometrium, cervix, ectocervix and fallopian tube, suggesting important differences in the response to pathogens by each region of the female genital tract (Pioli et al., 2004). By comparison, little is known about the significance of TLR expression in the male reproductive tract. In addition, most studies have evaluated TLR expression (Palladino et al., 2007). By contrast, cytokine response to TLR ligands by primary cultures of MGT organs still has not been fully studied.

Nishimura & Naito (2005) showed the presence of TLR1–TLR10 mRNA in human testes by real-time PCR. In addition, Palladino et al. (2007) showed TLR1–9 mRNA and protein in testes, epididymis and vas deferens. Expression of TLR2–6 mRNA in mouse Sertoli cells and subsequent activation of the NF-κB pathway following stimulation with TLR2 and TLR5 agonists were demonstrated by Riccioli et al. (2006). Bhushan et al. (2008) demonstrated that all testicular cell types expressed mRNAs of one to six TLRs, and, in particular, TLR3 transcription was evident in all testicular cells, suggesting a strong capacity for the recognition of double-stranded viral RNA in the testes.

In the present study, we examined the KC response to TLR agonists. We demonstrated that TLR agonists Pam3CSK4, poly(I:C), *E. coli* endotoxin and CpG stimulated prostate, seminal vesicle and epididymis/vas deferens primary cultures to secrete the chemokine KC. The pattern of KC secretion by the three primary cultures assayed was different, suggesting specific sensitivity to TLR ligands or distinct TLR protein expression in different parts of the tract.

**Fig. 3.** Response of MGT primary cultures to cLPS. Primary cultures of prostate (PC), seminal vesicle (SVC) and epididymis/vas deferens (EVC) (10×10⁶ cells) were plated in 96-well dishes and stimulated with cLPS (1 μg ml⁻¹) for 48 h. Supernatant was collected and KC production was assayed by ELISA. Data are reported as the mean ± SD of duplicate wells of three independent experiments. *P<0.05, ***P<0.001, versus unstimulated cells (LSD Fisher test).

**Fig. 4.** *Chlamydia trachomatis* infects and replicates in prostate primary cultures. *Chlamydia trachomatis*. After 48 h, cells were fixed and permeabilized, and chlamydial inclusions were visualized using FITC-conjugated specific antibodies. Coverslips were analysed by confocal microscopy. Chlamydial inclusions can be seen as intracellular green staining. (a) Fluorescence microscopy. (b) Combination of visible and fluorescence microscopy. Original magnification ×60.
Leukocytes (CD45+ by immunofluorescence) we detected small amounts of TLR2, TLR3, TLR4 and TLR9 since these cells were also present. KC levels were recently detected at 24 h of infection. In the present report, we analysed cLPS as a possible PAMP to be sensed by our male accessory gland cultures. As expected, prostate, as well as seminal vesicle and epididymal-vas deferens primary cultures, also induced KC in response to cLPS challenge, suggesting that MGT cells can sense *Chlamydia trachomatis*, recognizing its endotoxin. Not only was cLPS able to stimulate MGT cells, but also the bacteria were able to form chlamydial inclusions in MGT primary cultures, inducing the secretion of KC. In our primary cell culture studies, epithelial and stromal cells might be present, but also in epididymis/vas deferens primary cultures, sperm cells are present. KC levels were recently detected at 24 h of stimulation and augmented with increasing length of cLPS, *Chlamydia trachomatis* or *C. muridarum* stimulation. Our results are in agreement with data shown by Al-Mously & Eley (2007) where *Chlamydia trachomatis* induced IL-6 production after 24 h post-infection in immortalized normal adult prostate epithelial cells. Both stromal and epithelial cells have been shown to secrete an array of cytokines such as TNFα, IL-1β, TGFβ, IL-6 and IL-8 (Tabibzadeh et al., 1989; Hunt, 1994; Brunham & Rey-Ladino, 2005). These observations suggest that stromal–epithelial interactions play an important role in regulating the local immune environment in response to chlamydial infection of the male reproductive tract.

These studies provide the foundation needed to examine interactions between *Chlamydia trachomatis* and MGT tissues to more fully characterize immune responses to infection in the genital tract.
In summary, our findings suggest that murine MGT cells are able to respond to microbial ligands such as cLPS and are also susceptible to Chlamydia trachomatis and Chlamydia muridarum infection, upregulating mediators which in turn could attract innate immune cells to the site of infection and finally contribute to pathogen clearance.

Finally, understanding specific immune responses, both innate and adaptive, against chlamydial infections, mounted in each tissue of the MGT, will be crucial to design new therapeutic approaches where innate and/or adaptive immunity could be targeted. Development of new models of infections as well as the full characterization of MGT immune responses might help in disease prevention. Identifying the main cellular source of proinflammatory mediators that cause disease sequelae in males remains a principal priority in chlamydial research.

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