Chemokine response induced by *Chlamydia trachomatis* in prostate derived CD45+ and CD45– cells

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Abstract

The role of innate cells and their receptors within the male genital tract remains poorly understood. Much less is known about the relative contribution of different genital tract cells such as epithelial/stromal cells and resident leucocytes. In this study, we examined innate immune responses to *Chlamydia trachomatis* by prostate epithelial/stromal cells and prostate resident leucocytes. Murine prostate primary cultures were performed and leucocyte and epithelial/stromal cells were sorted based on surface protein expression of CD45 by magnetism-activated cell sorting or fluorescence-activated cell sorting. Prostate derived CD45– and CD45+ cells were infected with *C. trachomatis* and chemokine secretion assayed by ELISA. Similar experiments were performed using prostate CD45+ and CD45– cells from myeloid differentiation factor 88 (*Myd88*^{−/−}) mice or toll-like receptor (*Tlr2*^{−/−}) and *Tlr4*^{mutant} double-deficient mice. Moreover, a TLR-signalling pathway array was used to screen changes in different genes involved in TLR-signalling pathways by real-time PCR. Prostate derived CD45– and CD45+ cells responded to chlamydial infection with the production of different chemokines. Both populations expressed genes involved in TLR signalling and required to respond to pathogen-associated molecular patterns and to *C. trachomatis* infection. Both populations required the adaptor molecule MYD88 to elicit chemokine response against *C. trachomatis*. TLR2–TLR4 was essential for chemokine production by CD45+ prostate derived cells, but in their absence, CD45– cells still produced significant levels of chemokines. We demonstrate that *C. trachomatis* is differentially recognised by prostate derived CD45+ and CD45– cells and suggest that diverse strategies are taking place in the local microenvironment of the host in response to the infection.

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Introduction

Chlamydia trachomatis is one of the most prevalent sexually transmitted pathogens throughout the world (Stamm 2008). Even though the prevalence of *Chlamydia* infection is similar in male and female, current research and screening strategies are mainly focused on females because there is well-documented evidence of disease sequelae and infertility associated with female genital tract infections (Wagenlehner *et al.* 2005). However, the prevalence of asymptomatic persistent *Chlamydia* infection in men is high in infertile couples (Eley & Pacey 2011). It is then surprising that with similar prevalence and an extensively accepted role for *Chlamydia* in the development of male urethritis, epididymitis, orchitis and prostatitis, research focusing on the pathogenic mechanisms involved in male genitourinary tract infections is still very limited (Domingue & Hellstrom 1998, Motrich *et al.* 2006, Cunningham & Beagle 2008).

The ability of mucosal surfaces, mainly epithelial cells, to detect and respond to invading pathogens such as *Chlamydia* results principally from the activation of a complex network of immune receptors with different signalling pathways where synergisms and cooperation between molecules usually occur (Akira & Takeda 2004, Delbridge & O’Riordan 2007). Expression of different pattern recognition receptors (PRRs) at mucosal surfaces is central to innate immune defences (Hall *et al.* 2002, Gribar *et al.* 2008, Saenz *et al.* 2008). Among these PRRs, toll-like receptors (TLRs) have been the most extensively studied (Akira & Takeda 2004, Delbridge & O’Riordan 2007). Some reports demonstrated the expression of several TLRs and anti-microbial peptides in epididymis, testis and prostate (Com *et al.* 2003, Mackern-Oberti *et al.* 2006). However, there are just a few functional studies where cytokine response to TLR ligands was measured in prostate derived epithelial cells (Gatti *et al.* 2006, 2009, Kundu *et al.* 2008).

Thus, Gatti *et al.* (2006) demonstrated that a prostate adenocarcinoma-derived epithelial cell line expressed TLR4 and responded to *Escherichia coli* lipopolysaccharide (LPS) inducing TNF secretion and upregulating mRNA of several chemokines. Kundu *et al.* (2008) showed that primary immortalised prostate epithelial cells express TLR4 and TLR9 and also upregulate NFκB expression on TLR4 or TLR9 ligand stimulation.

Dendritic cells and monocytes respond to *Chlamydia* infection producing a wide range of cytokines and chemokines such as IL1B, IL8, IL23 and TNF (Beagley *et al.* 2009). PRRs like TLRs and nucleotide-binding oligomerisation domain (NOD)-like receptors play a major role in immune responses to *Chlamydia* infection following ligation by pathogen-associated molecular patterns (PAMPs; Vabulas *et al.* 2001, Heine *et al.* 2003, Prebeck *et al.* 2003, Da Costa *et al.* 2004, O'Connell *et al.* 2006, Bas *et al.* 2008, Buchholz & Stephens 2008, Joyee & Yang 2008, Chen *et al.* 2010). It has been suggested that one of the most active PRR ligands from *Chlamydia* is its LPS (cLPS) and its recognition has been associated with both TLR2 and TLR4 (Heine *et al.* 2003, Prebeck *et al.* 2003, O'Connell *et al.* 2006, Buchholz & Stephens 2008, Joyee & Yang 2008). *Chlamydia* heat-shock protein 60 (cHSP60) has also been demonstrated to be able to activate immune cells in a TLR2- and TLR4-dependent fashion, although some researchers proposed that cHSP60 recognition is mainly mediated by TLR4 (Vabulas *et al.* 2001, Da Costa *et al.* 2004). Another *Chlamydia* antigen that has recently been found to strongly activate bone marrow dendritic cells is macrophage infectivity potentiator (Mip), a chlamydial lipoprotein that induces pro-inflammatory cytokines and chemokine production principally through TLR2 (Bas *et al.* 2008). On the other hand, there is general agreement related to the requirement of myeloid differentiation factor 88 (MYD88), an important adaptor molecule in most TLR-signalling pathways, in *Chlamydia*-induced activation of immune cells and in pathology development (Chen *et al.* 2010).

In vivo studies have shown that *Chlamydia* infection is accompanied by a considerable infiltration of neutrophils at the site of infection (Barteneva *et al.* 1996, Bai *et al.* 2005). The molecular mechanisms for the recruitment of neutrophils to mucosal inflammation most likely involve the local release of neutrophil chemoattractants such as IL8, chemokine (C-X-C motif) ligand 2 (CXCL2; also known as macrophage inflammatory protein, MIP2) and CXCL1 (also known as keratinocyte-derived chemokine, KC). Furthermore, *in vitro* studies have also shown that during an infection of primary prostate cultures with *Chlamydia*, neutrophils are chemoattracted by CXCL1 and IL8 (Buchholz & Stephens 2006, Zaharik *et al.* 2007). Our laboratory has developed an *in vitro* model of *Chlamydia* infection of rat prostate epithelial cells. We showed that prostate

epithelial cells were susceptible to *Chlamydia* infection and responded by an upregulation of inflammatory mediators and also recruited TLR2, TLR4 and MYD88 to the chlamydial inclusion vicinity, suggesting an active role of these receptors in the recognition of the bacteria and activation of prostate epithelial cells (Mackern-Oberti *et al.* 2006).

In the present work, we show that prostate primary cultures are susceptible to infection with *C. trachomatis*. Since leucocytes (characterised by the pan-leucocyte marker CD45) were also present in these primary cultures, we set up an experimental approach in order to distinguish the response elicited after *C. trachomatis* infection by resident leucocytes or prostate cells (PCs). We demonstrate that *C. trachomatis* is differentially recognised by prostate derived CD45+ and CD45- cells. Our results suggest that diverse strategies are taking place in the local microenvironment of the host in response to the infection.

Results

C. trachomatis is able to infect primary PCs and induce the upregulation of pro-inflammatory chemokines

In previous studies, we have demonstrated that rat PC cells respond to *Chlamydia* by producing different chemokines (Mackern-Oberti *et al.* 2006, 2011). We first tested whether murine primary prostate cultures were susceptible to *C. trachomatis* infection. PCs incubated with *C. trachomatis* resulted in the formation of classical inclusions 48 h post-infection (pi), as detected by staining with an anti-*Chlamydia* antibody followed by fluorescence microscopy (Fig. 1A). Moreover, *C. trachomatis* was able to complete its biphasic developmental cycle, since supernatants from infected PCs could re-infect a highly susceptible epithelial cell line such as Hep2 cells (data not shown).

We also determined whether *C. trachomatis* infection of PCs induced the secretion of inflammatory chemokines. As can be seen in Fig. 1B–D, CXCL1, CCL2 (MCP1) and CXCL2 levels were already detectable at 24 h pi with *C. trachomatis* and was further increased at 48 h. IL1B and TNF levels were below the detection limit of the ELISA kits (data not shown). These results demonstrated that *C. trachomatis* infected PCs and induced a chemokine response.

C. trachomatis infection induces the production of CXCL1 in CD45+ and CD45- cells from prostate

In order to exclude the possibility that CXCL1 was not secreted by epithelial PCs after *C. trachomatis* infection but instead by resident leucocytes, we decided to thoroughly characterise the cell types that composed our primary prostate cultures. Since prostate basal and luminal epithelial cells express cytokeratin 5 and

Figure 1 *Chlamydia trachomatis* infects and induces the upregulation of pro-inflammatory chemokines in prostate primary cultures. Primary cultures of prostate grown on coverslips were infected with *C. trachomatis* (MOI=10). After 48 h, cells were fixed, permeabilised and chlamydial inclusions were visualised using FITC-conjugated anti-*Chlamydia* LPS-specific antibodies. Coverslips were analysed by confocal microscopy. (A) Chlamydial inclusions can be seen as intracellular green staining in fluorescence and combination of visible and fluorescence microscopy (original magnification $\times 60$). Cells were plated on 96-well dishes, supernatant was collected and CXCL1, CXCL2 and CCL2 production was assayed by ELISA at different times post-infection. CXCL1 (B), CCL2 (C) and CXCL2 (D) production by prostate culture. Data are reported as the mean $\pm$ s.d. of duplicate wells of three independent experiments. *** $P < 0.001$, ** $P < 0.01$ (stimulated versus unstimulated) by ANOVA test.

cytokeratins 8 and 18, respectively, we performed immunofluorescent staining of prostate primary cultures using an anti-pan-cytokeratin mAb. More than 80% of the cells were positive for the immune staining performed (data not shown). We also determined the percentage of cells expressing CD45, CD11b, CD11c, CD3 and CD19 by flow cytometry. As shown in Fig. 2A, $4.1 \pm 0.8\%$ of the total primary PCs cells were CD45+. Among this population, we found the presence of CD11b+ (15.5%), CD11c+ (37%), CD3+ (44%) and CD19+ (3.5%).

To see whether the CXCL1 levels measured in PCs supernatants were produced by either leucocytes (CD45+) or by epithelial/stromal (CD45-) cells or by both populations, cells expressing the CD45 marker were sorted by magnetism-activated cell sorting (MACS). After MACS sorting, CD45+ and CD45- cells were infected with *C. trachomatis* and then the production of CXCL1 was analysed. Both populations of cells, CD45- as well as CD45+ cells, secreted CXCL1 following *C. trachomatis* infection. Levels detected after 24 h of infection were similar for both populations, whereas at 48 h pi, CD45- cells secreted higher levels of CXCL1 (Fig. 2B). When similar experiments were performed using *Chlamydia muridarum*, again both

CD45- as CD45+ prostate derived cells secreted CXCL1 following *C. muridarum* infection (Fig. 2C). When levels of CCL2 were analysed in CD45- and CD45+ cells, the same pattern was observed (data not shown). Using fluorescence-activated cell sorting (FACS), higher purity populations were obtained and again CD45- and CD45+ cells secreted CXCL1 after *C. trachomatis* infection (Fig. 2D).

CXCL1 secretion by prostate CD45+ and CD45- cells on infection with *C. trachomatis* is MYD88 dependent

On TLR ligand recognition, the intracellular domains of all known TLRs, except TLR3, interact with the adaptor molecule MYD88 and initiate a common signalling cascade that leads to activation of transcription factors as NF κ B, AP1 and interferon regulatory factors (IRFs). To analyse the relevance of MYD88 for activation of PCs by *C. trachomatis*, we performed similar experiments described above using *Myd88*^{-/-} mice. Briefly, *Myd88*^{-/-} PCs were sorted to obtain CD45+ and CD45- cells and infected with *C. trachomatis*. Then CXCL1 production was measured by ELISA in culture supernatants. Neither CD45+ cells nor CD45- cells from *Myd88*^{-/-} mice were able to produce CXCL1 after

Figure 2 *Chlamydia trachomatis* induces the upregulation of CXCL1 in CD45+ and CD45- prostate primary cultures. Primary prostate cultures were stained with fluorophore-labelled anti-mouse CD45, anti-mouse CD11c, anti-mouse CD3, anti-mouse CD11b and anti-mouse B220-specific antibodies and analysed by FACS. (A) Histogram showing the percentage of CD45 expressing cells in prostate primary cultures (left) and chart showing leucocyte subpopulation in prostate primary cultures (right). Murine prostate CD45+ and CD45- cells from WT C57BL/6 mice were sorted by MACS or FACS, plated on 96-well dishes and infected with *C. trachomatis* (MOI=10). Supernatants were collected and CXCL1 production was assayed by ELISA at different times post-infection. CXCL1 production by CD45- and CD45+ cells sorted by MACS stimulated with *C. trachomatis* (B) or *Chlamydia muridarum* (C). CXCL1 production by CD45- and CD45+ cells sorted by FACS (D). Data are reported as the mean $\pm$ s.d. of duplicate wells of three independent experiments. *** P <0.001, ** P <0.01, * P <0.05 (stimulated versus unstimulated) by ANOVA test.

C. trachomatis infection (Fig. 3). Simultaneously, CD45- and CD45+ from wild-type and *Myd88*^{-/-} mice were stimulated with LPS for 24 h. As expected, both CD45+ and CD45- cultures from wild-type animals responded to LPS while *Myd88*^{-/-} did not. These results indicated that, under these experimental conditions, no other receptor working independent of MYD88 mediated the synthesis of CXCL1 after *C. trachomatis* infection.

TLR2-TLR4 are essential for the chemokine response of prostate CD45+ cells and involved but not essential for prostate CD45- cells on *C. trachomatis* infection

At present, there are numerous reports that indicate that TLR2 and TLR4 are critical receptors involved in immune recognition of *C. trachomatis*-derived PAMPs. In order to see whether these receptors were also involved in *C. trachomatis* recognition by both, CD45+ CD45- PCs, we compared the response of wild-type and TLR2/TLR4 double-deficient PCs. Similar experiments were performed using prostate CD45+ and CD45- cells from *Tlr2*^{-/-} and *Tlr4*^{mutant} double-deficient mice. As can be

seen in Fig. 4, wild-type CD45- cells respond to LPS stimulation by secreting CXCL1 and CCL2; however, TLR2/TLR4-deficient CD45+ cells did not.

When we analysed the response to *C. trachomatis* infection, we observed that prostate derived CD45+

Figure 3 *Chlamydia trachomatis* induces the upregulation of CXCL1 in CD45+ and CD45- prostate primary cultures in MYD88-dependent fashion. CD45+ and CD45- cells from prostate primary cultures from WT or *Myd88*^{-/-} mice were sorted by MACS, plated on 96-well dishes and infected with *C. trachomatis* (MOI=10) or stimulated with LPS (1 μ g/ml). Supernatants were collected and CXCL1 production was assayed by ELISA at different times post-infection. Data are reported as the mean $\pm$ s.d. of duplicate wells of two independent experiments. *** P <0.001 (stimulated versus unstimulated) by ANOVA test.

Figure 4 TLR2 and TLR4 dependence on CXCL1 response to *Chlamydia trachomatis* infection of prostate primary cultures. CD45+ and CD45- cells from prostate primary cultures from C3H/HeN WT or C3H TLR2/TLR4-deficient mice were sorted by MACS, plated on 96-well dishes and infected with *C. trachomatis* (MOI = 10). Supernatants were collected and CXCL1 production was assayed by ELISA at different times post-infection. (A) CXCL1 production by CD45- and CD45+ cells from wild-type animals and TLR2/TLR4-deficient primary cultures. (B) CCL2 production by CD45- and CD45+ cells from wild-type animals and from TLR2/TLR4-deficient primary cultures. Data are reported as the mean $\pm$ s.d. of duplicate wells of two independent experiments. *** $P < 0.001$, ** $P < 0.01$ (stimulated versus unstimulated) by ANOVA test.

and CD45- cells from wild-type mice secreted CXCL1 after *C. trachomatis* infection (24 h), while TLR2/TLR4-deficient CD45+ and CD45- cells exhibited a different pattern. Interestingly, infected CD45+ cells from double-deficient mice did not produce CXCL1 after *C. trachomatis* infection. In contrast, infected CD45- cells from double-deficient mice were partially independent of TLR2/TLR4 since they were still able to secrete CXCL1 on *C. trachomatis* exposure, showing lower levels when compared with wild-type cells. Similarly, the same pattern of response was detected when CCL2 levels were assayed; CD45+ PC from TLR2/TLR4 mice did not secrete CCL2 after *C. trachomatis* infection, while double-deficient CD45- cells were still able to produce CCL2 chemokine after infection, although secreting lower quantities (Fig. 4B).

These results showed that prostate derived CD45+ cells derived from PCs responded to *C. trachomatis* infection secreting CXCL1 and CCL2 in a TLR2- and TLR4-dependent manner, and the presence of these receptors was essential for chemokine production.

Interestingly, these two receptors were also involved in the induction of these chemokines in *C. trachomatis*-infected CD45- cells, but in their absence, CD45- cells still produced significant levels of the chemokines. The fact that both types of population require the adaptor molecule MYD88 to elicit this chemokine response against *C. trachomatis* suggests that CD45- cells employ a different set of PRR/TLRs to induce chemokine secretion after *C. trachomatis* recognition.

C. trachomatis infection of CD45- and CD45+ PC cultures induces the transcription of cytokine, chemokine and TLR-regulated genes

Taking into consideration that CD45- and CD45+ PCs respond in a different way to *C. trachomatis* exposure, we wanted to assess the ability of *C. trachomatis* infection to modulate specific components of the TLR-signalling pathway and thereby to induce the release of immune modulators, mainly cytokines and chemokines. A mouse TLR signalling pathway array was used to screen changes in different genes involved in TLR-signalling pathways by real-time PCR. Unfortunately, experiments with CD45+ cells from prostate of wild-type mice could not be performed because the obtained cell quantity was insufficient. For that reason, we worked with CD45- PCs and peritoneal CD45+ cells. CD45- and CD45+ cells were sorted with MACS as described before, cells were infected or not with *C. trachomatis* and harvested at 12 h pi. Total RNA was extracted and PCR microarray was performed following the protocol of the manufacturer.

When the relative mRNA levels of TLRs were analysed in non-infected CD45+ and CD45- cells, we observed that CD45+ cells express higher levels of *Tlr1*, *Tlr2*, *Tlr7*, *Tlr8* and *Tlr9* mRNA when compared with that expressed by CD45- cells. Detectable levels of mRNA for *Tlr1*, *Tlr2*, *Tlr3*, *Tlr4*, *Tlr5*, *Tlr6*, *Tlr7* and *Tlr9* were observed in CD45- cells. Levels of *Tlr3*, *Tlr4* and *Tlr6* were similar in both populations. *Tlr5* was the only TLR that showed higher expression in CD45- cells (Fig. 5A). Regarding mRNA expression of adaptor and signalling molecules involved in TLR-signalling pathways, we observed that both populations express mRNAs of molecules required to respond to PAMPs. Indeed, mRNA expression of *Cd14*, *Ly86* (MD1), *Ly96* (MD2), *Myd88*, *Tirap* (MAL), *Tollip*, *Btk*, *Traf6*, *Tbk1*, *Irak1*, *Irak2* and others were detected in both populations. However, for approximately half of the genes studied, higher relative expression was observed in CD45+ cells (Fig. 5B).

After *C. trachomatis* infection of CD45+ and CD45- cells, some mRNAs showed upregulation. *Tlr3* was the only one, among TLRs analysed, that showed upregulation in both populations after *C. trachomatis* infection (Fig. 5C). With respect to mRNA expression of adaptor and signalling molecules involved in TLR-signalling

Figure 5 *Chlamydia trachomatis* induces changes in the regulation of many genes in CD45+ and CD45- prostate derived cells. Relative mRNA expression of TLR genes (A) and adaptors and TLR-interacting proteins (B) were measured by qPCR mouse toll-like receptor signalling pathway in CD45+ peritoneal cells and CD45- prostate cells. mRNA results are presented in relative units relative to the expression of the house-keeping gene *Hprt1*. (C) CD45+ peritoneal and CD45- prostate cells were infected for 12 h with *C. trachomatis*. Red, orange and green indicate higher, equivalent and lower expression, respectively, in comparison with the expression in non-infected CD45+ and CD45- cells.

pathways, no major increases were detected with the exception of *Tollip* and *Tbk1* (4.4- and 38-fold increase in CD45- cells respectively).

Regarding colony-stimulating factors (*Csf2* or granulocyte-macrophage colony-stimulating factor and *Csf3* or granulocyte colony-stimulating factor), we observed a strong upregulation, especially in CD45+ cells infected with *C. trachomatis*, but also CD45- cells were able to upregulate *Csf3* on infection. We also noted that both populations upregulate mRNA for the chemokines *Ccl2* (MCP1) and *Cxcl10* (IP10) with similar values for *Ccl2* in CD45+ and CD45- cells and higher upregulation for *Cxcl10* in CD45+ cells. Regarding cytokines and cytokine receptors such as *Il1a*, *Il1b*, *Il1r1*, *Il6*, *Tnf*, *Ifng*, *Ifnb1* and *Il10*, an upregulation was observed in CD45+ cells, while CD45- cells only showed a weak increase in *Il6* mRNA. Moreover, *C. trachomatis* infection of prostate CD45- induced the upregulation of several NFKB-related molecules such as *Nfkbib*, *Nfrkb* and *Rela*.

These results suggest that both populations, CD45+ cells and prostate CD45- cells, possess the machinery that is required to respond to different microorganisms such as *C. trachomatis*. At the time assayed, both populations responded up regulating mRNA for chemokines, but only CD45+ cells responded upregulating mRNA for pro-inflammatory cytokines. Our results

showed that *C. trachomatis* is differentially recognised by prostate derived CD45+ and CD45- cells and suggest that diverse strategies are taking place in the local microenvironment of the host to respond to the infection.

Discussion

The first line of defence against chlamydiae in the urogenital tract is the innate immune response of epithelial cells and resident leucocytes. However, despite the fact that many viruses and bacteria are transmitted sexually through the genital tract, the immune system of the male and female genital mucosae has received little attention (Iwasaki 2010). The chemokines and cytokines produced during the innate immune response recruit inflammatory cells and T cells needed to control the infection and to establish adaptive immune response and subsequent clearance of the bacteria (Morrison & Caldwell 2002).

Although considerable effort has been made in order to understand innate immune cells in a wide diversity of systems, the role of these cells and their receptors within the male genital tract remains poorly understood (Bhushan *et al.* 2009). Much less is known about the relative contribution of different genital tract cells such

as epithelial cells, stromal cells and resident leucocytes. In the present work, we have examined innate immune responses to *C. trachomatis* by prostate epithelial/stromal cells and prostate resident leucocytes. We showed that *Chlamydia* could productively infect murine PCs by the development of large inclusions. Moreover, our results demonstrate that PC cultures responded to chlamydial infection with the production of different pro-inflammatory chemokines like CCL2, CXCL2 and CXCL1. Since we detected the presence of low percentages of leucocytes (CD45+) in our cultures, we performed experiments sorting CD45+ and CD45- cells and demonstrated that both populations responded to the infection by secreting chemokines. Moreover, we observed that both populations expressed genes involved in TLR signalling and required to respond to PAMPs and to respond to *C. trachomatis* infection. We found that after *C. trachomatis* infection, both populations upregulate mRNA for *Ccl2*, *Cxcl10* (IP10) and the colony-stimulating factor *Csf3*. CD45- cells also upregulate NF κ B pathway genes, negative regulators of NF κ B signalling such as *Tollip* and genes involved in IRF pathways. As TANK-binding kinase 1 and *Cxcl10* mRNA upregulation occurred as well, this finding suggests the presence of some MYD88-independent responses that were not further evaluated in this study. Finally, only CD45+ cells upregulated mRNA for cytokines such as IL1A, IL1B, TNF, INF γ , IFNB1 and IL10.

Among inflammatory cytokines, it has been reported that IL1A is important in regulating host defences against *C. trachomatis* (Magee *et al.* 1992, Rasmussen *et al.* 1997, Buchholz & Stephens 2006). In addition, it has been suggested that IL6 may be involved in the immunopathogenesis of persistent chlamydial infection since elevated IL6 levels have been reported in cases of persistent *C. trachomatis* infection of the Fallopian tube (Mpiga *et al.* 2006). Epithelial cells from the female genital tract, such as HeLa cells, have been used to study different aspects of *C. trachomatis* infection and there are many reports showing that after HeLa infection with *C. trachomatis* IL1A, IL6, IL8 and TNF are released. However, in our experiments, the infection of prostate CD45- cells exhibited a different behaviour, mainly inducing the secretion and upregulation of chemokines but not the upregulation of inflammatory cytokines. It is possible that different epithelial cells may react in different ways to *C. trachomatis* infection, and different serovars may induce different levels of cytokines in response to infection (Rasmussen *et al.* 1997, Mpiga *et al.* 2006). In our experiments, CD45- cells were unable to secrete IL1A or upregulate mRNA for IL1A. Al-Mously & Eley (2007) using male immortalised normal human urethral epithelial cells and immortalised normal adult male prostate epithelial cells infected with *C. trachomatis* have reported results that are in agreement with our data. They have compared the response of HeLa, urethral epithelial cells and prostate

epithelial cells and found that after *C. trachomatis* infection HeLa and urethral cells responded by producing high levels of IL1A, while prostate epithelial cells secreted almost undetectable levels of this cytokine. Similar to our observations, prostate epithelial cells did not produce TNF, but produced IL6 and significant levels of IL8 (an important neutrophil chemoattractant such as CXCL1) after *C. trachomatis* infection.

The reproductive tract, especially the lower genital tract, is continuously challenged by potential pathogens present in the environment (Nasu & Narahara 2010). Therefore, robust host defence mechanisms are essential for both health and fertilisation. It is well known that infections of the male genital tract induce a robust pro-inflammatory milieu, which hampers sperm cell maturation, and thereby may contribute to an impaired male fertility (Ochsendorf 1999, Krause 2008). The functional capacity to sense pathogens in male accessory gland tissues, like prostate, is presumably important to prevent ascending infections. However, a strong inflammatory response, which reaches and damages sperm cells, could interfere with male fertility. If we consider the prostate gland as part of the upper male genital tract, the lack of inflammatory cytokine production by CD45- PCs observed in our studies is similar to responses of epithelial cells from the upper and lower female genital tract, where higher levels of IL1A were only detected in cervical secretion and lower in Fallopian tubes. These observations suggest that raised IL1A levels are only induced in the lower genital tract on infection with *C. trachomatis* to avoid damage of the male upper genital tract.

Inflammatory responses are induced by activation of the innate immune system by germ-line-encoded PRRs that include transmembrane TLR and cytoplasmically localised NOD proteins. PRRs lead to signalling events and coordinated activation of transcription factors that induce the expression of anti-microbial molecules, chemokines and co-stimulatory molecules. Recently, we have reported that TLR2, TLR3, TLR4 and TLR9 agonists were able to induce the production of CXCL1 by prostate primary cultures (Mackern-Oberti *et al.* 2011). In this study, we demonstrate that CD45+ as CD45- derived PCs express mRNA for TLRs and adapter molecules required for signalling. Using primary cultures from mice deficient in the adaptor molecule MYD88, we demonstrate that CXCL1 production in response to *C. trachomatis* was critically affected in both PC types. The fact that the CXCL1 response was completely abolished in *Myd88*^{-/-} cultures eliminates the possibility that NOD receptors or TLR3, the only TLR described as MYD88 independent at present, would be involved in the chemokine response observed. Buchholz & Stephens (2008) have demonstrated that the NOD receptor is involved during chlamydial infection. Using RNA interference to knock down NOD1 and its signalling molecule receptor RIP2, they showed that

endogenous IL8 response induced by *C. trachomatis* was dependent on NOD1, which signals via RIP2 as part of a signal system requiring multiple inputs for optimal IL8 induction. Interestingly, the only TLR that showed upregulation after infection in our experiments was TLR3; so far, there is no report in the literature that suggests a TLR3 ligand associated with *C. trachomatis*. However, this finding is functionally questionable, since no CXCL1 response was observed in our *Myd88*^{-/-} cultures.

Within the TLRs described so far, all but TLR3 are MYD88 dependent. Which components of *Chlamydia* spp and which MYD88-dependent TLRs are critical for recognition of the whole bacterium remains one of the most intriguing questions of chlamydial research (Joyee & Yang 2008). cLPS, cHSP60 and Mip have been associated as chlamydial TLR ligands (Vabulas *et al.* 2001, Heine *et al.* 2003, Da Costa *et al.* 2004, Bas *et al.* 2008). Previous studies have assigned roles for TLR2 and TLR4 receptors in recognition of *Chlamydia* (Darville *et al.* 2003, den Hartog *et al.* 2009). Moreover, some evidence has suggested a role for intracellular TLR or other PRRs that function in a MYD88-dependent manner to induce cytokines during chlamydial infection (Nagarajan *et al.* 2005). In the present work, we performed experiments with TLR2 and TLR4 double-deficient cells and observed a differential chemokine response in CD45⁻ and CD45⁺ prostate derived cells. We demonstrated that CXCL1 and CCL2 production by infected CD45⁻ cells was partially independent of TLR2/TLR4, while the chemokine response of CD45⁺ cells was mediated through TLR2/TLR4. Several studies analysing chemokine and cytokine secretion have demonstrated the importance of TLR2 and TLR4 in *Chlamydia* recognition. *Tlr2*^{-/-} mice infected genitally with *C. muridarum* had lower inflammatory cytokine responses and less oviductal pathology compared with wild-type mice. Nonetheless, *Tlr2*^{-/-} cleared infection at a rate similar to wild type (Darville *et al.* 2003). Regarding *Tlr4* knockout mice, it has been reported that they responded like wild-type mice regarding cytokine secretion and clearance of infection (den Hartog *et al.* 2009). Nagarajan *et al.* reported that *Chlamydia*-induced CXCL10 was completely independent of TLR2 and TLR4 but significantly dependent on MYD88. They performed experiments using *Tlr2*^{-/-}, *Tlr4*^{-/-} and *Myd88*^{-/-} mice and observed that CXCL10 and INFβ are produced independent of TLR2 and TLR4 but partially dependent on MYD88 during *in vivo* infection of mice with *C. muridarum* (Nagarajan *et al.* 2005). They also infected macrophages and fibroblasts *in vitro* with the same results. In contrary to the results obtained in our work, they found no differences between macrophage and fibroblast behaviour in relation to TLR2/TLR4 and MYD88 dependence. Recently, these authors also demonstrated that mouse macrophages lacking TLR3, TRIF, TLR7 or TLR9 individually or are deficient for both TLR4 and MYD88, still induce IFNβ equivalent to

wild-type controls, leading to the hypothesis that TLR-independent cytosolic pathogen receptor pathways are crucial for INFβ response (Prantner *et al.* 2010). In addition, Ouburg *et al.* (2009) using *Tlr9* KO mice have reported that although no significant differences in the course of initial infections were observed between KO mice and wild-type mice, the *Tlr9* KO mice were more protected against re-infection, suggesting a detrimental role for TLR9 in *C. trachomatis* infections. Taken together, it is possible that other PRRs in addition to TLR2/TLR4 are involved in the chemokine production described here.

The physiological function of these different behaviours in the prostate microenvironment may serve as a link between immune cells and epithelial/stromal cells. In this setting, epithelial cells, which first establish contact with the pathogen, may use different PRRs than local immune cells in order to efficiently recruit circulating leucocytes, modulate the immune response and eliminate the infection. Expression, function and synergism of individual innate receptors on particular cells would be crucial for understanding local innate immunity against *Chlamydia*. In this regard, our findings show that *C. trachomatis* is differentially recognised by prostate derived CD45⁺ and CD45⁻ cells. Studying the specific PRR(s) that mediate *Chlamydia* recognition in male genital tract cells will be helpful for designing new therapeutic strategies for chronic prostatitis.

Materials and Methods

Antibodies and reagents

The mouse anti-*Chlamydia* spp LPS-FITC monoclonal antibody (mAb) was obtained from BioMerieux (Marcy l'Etoile, France) and monoclonal anti-pan-cytokeratin was obtained from Sigma-Aldrich (EEUU, clone C-11, recognising several cytokeratins (4, 5, 6, 8, 10, 13 and 18)). The allophycocyanin-labelled mAb specific for CD11b (553312), PE-labelled anti-CD45 mAb (553081), FITC-labelled anti-GR1 mAb (553126) and CD16/32-specific mAb (553142) to block Fc receptors were purchased from BD Pharmingen (San Diego, CA, USA).

Strains of mice

C57BL/6 mice were purchased from Harlan Winkelmann (Borchen, Germany). Breeding pairs of *Myd88*-deficient mice were provided by Dr S Akira and were bred in the animal facility at the Institute of Medical Microbiology, Immunology and Hygiene. C3H/HeJ mice, expressing a non-functional TLR4 and lacking TLR2, and C3H/HeN mice were bred in the animal facility at the Institute of Medical Microbiology, Immunology and Hygiene (Munich, Germany). All mice were kept under specific pathogen-free conditions.

Chlamydia strain

The *C. trachomatis* serovar L2 was propagated in HEp2 cells and purified in a sucrose Urografin gradient as described

previously by Maass & Gieffers (1997). The *C. trachomatis* MoPn strain (now *C. muridarum* MoPn) was supplied by K H Ramsey (Midwestern University, USA) and was propagated in HEp2 cells. Briefly, HEp2 cultures were grown in RPMI 1640 supplemented with 20 µg gentamicin/ml and 5% fetal bovine serum at 37 °C – 5% CO₂. Cultures infected with *C. trachomatis* serovar L2 elementary bodies were grown for 48 h in the presence of 1 µg/ml cycloheximide. Infected monolayers were detached by scraping, disrupted by sterile glass beads to lyse the host cells and release elementary bodies. Cell debris were removed by centrifugation at 500 g for 15 min. Elementary bodies were purified in a sucrose Urografin gradient (bottom layer 50% (w/v) sucrose solution; top layer, 30% (v/v) Urografin 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 (0.22 M sucrose, 8.6 mM Na₂HPO₄, 3.8 mM KH₂PO₄, 5 mM glutamic acid and 0.2 µm filtered, pH 7.4) at –70 °C until use. To quantify the number of elementary bodies, HEp2 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 GmbH, Göttingen, Germany), 24 h after infection. For control, non-infected HEp2 cells were treated in the same way. Contamination with mycoplasma was excluded regularly by PCR using specific primers.

Cell culture

Primary PCs were obtained following protocols described by Ilio *et al.* (1998). Briefly, C57BL/6 mice were killed, organ harvested and minced under sterile conditions into 1–2 mm fragments and subjected to enzymatic digestion using type IV collagenase (200 U/ml) and trypsin (0.25%) in DMEM/F12 at 37 °C. After incubating for 1 h, cells were dispersed and washed subsequently through a 200, 70 and 40 µm nylon cell strainer. Complete DMEM/F12 medium was supplemented with 10% defined FBS and analysed for cell number and viability.

Infection protocol

Primary PCs were seeded into 24- or 96-well plates at a density of 2–5 × 10⁵ cells/well. Cultures were washed with HBSS and infected with *C. trachomatis* or *C. muridarum* using a multiplicity of infection (MOI) of 10. The plates were spun at 1400 g for 60 min and placed at 5% CO₂ in air at 37 °C for 2 h. After the incubation, cells were washed twice with HBSS and 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 cytokine and chemokine levels.

C. trachomatis infection and direct immunofluorescence

To determine the level of infection, cultures of prostate or HEp2 cells were grown in 24-well tissue culture plates on sterile coverslips and subsequently infected with *C. trachomatis*

elementary bodies. Following incubation for 48 h, cells were fixed with methanol and stained with anti-*Chlamydia* LPS mAb. The presence of inclusion bodies was visualised on a confocal laser scanning microscope LSM510 (Zeiss, Thornwood, NY, USA) using the LSM 510 software for image analysis.

Detection of chemokines and cytokines

The chemokines and cytokines CXCL1 (KC), CCL2, CXCL2, IL1B and TNF were determined in supernatants of stimulated or infected cultures by commercially available ELISAs (R&D Systems, Minneapolis, MN, USA). The assays were performed as described by the manufacturer.

Confocal microscopy

Primary PCs were plated onto 12 mm circular coverslips in 24-well plates (5 × 10⁵ cells/well) and cultured overnight. Coverslips were washed twice in PBS and then fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. Following fixation, coverslips were washed twice in PBS and cells were then permeabilised by treatment with 0.5% saponin, blocked with 1% BSA and incubated with mAb directed against pan-cytokeratin (30 min, 4 °C). After three-wash steps, the cells were stained with Alexa Fluor 546 goat anti-mouse IgG secondary antibody. The cells were washed again and analysed by confocal microscopy.

Isolation of leucocytes and FACS analysis

Leucocytes from primary prostate cultures were sorted based on surface protein expression of CD45 by FACS or by MACS. For FACS cell sorting, prostate leucocytes were stained using FITC conjugate anti-mouse CD45 (30-F11). Cell sorting was performed at the National University of Cordoba, Faculty of Chemist Sciences, with a FACS Aria II cell sorter (BD Bioscience, San Diego, CA, USA). The purity of the sorted populations was more than 99% for CD45+ cells and >95% for CD45– cells. For MACS cell sorting, cell suspensions were stained using anti-CD45 microbeads. CD45– cells were purified by negative selection using a MiniMACS separator as recommend by the manufacturer (Miltenyi Biotec, Auburn, CA, USA), while prostate CD45+ resident cells were obtained by positive selection. Resident leucocyte composition was evaluated by FACS. The following Abs were used to identify different populations: FITC-conjugated anti-mouse CD45 (30-F11), APC-conjugated anti-mouse CD3e (17A2), PE-conjugated anti-mouse GR1 (RB6-8C5), FITC-conjugated anti-mouse CD11b (M1/70), APC-conjugated anti-mouse CD11c (HL3) and PE-conjugated anti-mouse CD19 (ID3) after Fc-block (anti-CD16/CD32). Cells were stained according to standard protocols. Flow cytometry were performed on a BD FACS CANTOII (BD Bioscience) and data were analysed with FlowJo software 6.4 (Tree Star, Inc., Ashland, OR, USA).

PCR array

Real-time PCR array for TLR pathway was performed using mouse TLR signalling pathway, RT² Profiler PCR array,

SABiosciences (Frederick, MD, USA) according to the manufacturer's protocol. The PCR array combines the quantitative performance of SYBR Green-based real-time PCR with the multiple gene profiling capabilities of microarray. After performing thermal cycling, real-time amplification data were gathered by using ABI Prism 7500 cycler. Gene expression was normalised to internal controls (housekeeping genes) to determine the fold change in gene expression between test and control samples by $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). Purified CD45⁻ PCs (from C57BL/6 mice) were isolated by FACS-negative selection while CD45⁺ cells (from C57BL/6 mice) were obtained from the peritoneal compartment by FACS-positive selection. Sorted cells were washed, re-suspended in DMEM/F12 with 10% defined FBS and 20 µg gentamicin/ml at 37 °C and infected with *C. trachomatis* at a MOI of 10. After incubating for 12 h, first-strand cDNA was synthesised from 1 µg RNA using an RT² first-strand kit (SABiosciences) and PCR arrays were performed according to the manufacturer's protocols (SABiosciences).

Statistical analysis

Statistical analysis was performed using the *t*-test and ANOVA GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Values of $P < 0.05$ were considered significant.

Declaration of interest

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

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