CHLAMYDIA TRACHOMATIS ELICITS TLR3 EXPRESSION BUT DISRUPTS THE INFLAMMATORY SIGNALING DOWN-MODULATING NFκB AND IRF3 TRANSCRIPTION FACTORS IN HUMAN SERTOLI CELLS

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***Chlamydia trachomatis*, the leading cause of bacterial sexually transmitted diseases worldwide, can disseminate and localize to the upper genital tract impairing reproductive function. Specifically, ascending *C. trachomatis* genital infection has been demonstrated to cause epididymitis or epididymo-orchitis, well-known risk factors for male infertility. *C. trachomatis* possesses the ability to infect primary human Sertoli cells, key elements for the spermatogenetic process and the immune protection of germ cells. Therefore, herein, we investigated the innate immune response in Sertoli cells following *C. trachomatis* infection, as well as its indirect effects on human spermatozoa. Specifically, we evaluated *C. trachomatis*-mediated induction of Toll-like Receptors (TLR) 2, 3 and 4 as well as of downstream intracellular signaling molecules (NFκB and IRF3) and the levels of the related inflammatory mediators (IL-1α, IL-6, IFN-α, IFN-β and IFN-γ), in an *in vitro* infection model of primary human Sertoli cells. The main result of our study shows that *C. trachomatis* induced TLR3-mediated recognition in human Sertoli cells, accompanied by the down-modulation of NFκB and IRF3-dependent signaling pathways followed by no production of pro-inflammatory cytokines. In conclusion, our findings suggest that *C. trachomatis* can disrupt the innate immune response in Sertoli cells and evade intracellular killing, potentially giving rise to a long-term infection that may exert negative effects on the male reproductive system.**

Male infertility and sub-fertility are caused by several defects that involve functional and structural alterations of the testis (1-2). Sertoli cells form the epithelium of the seminiferous tubule in the human testis and are key elements for spermatogenesis, a complex process that is finely regulated by intimate and cyclical interactions between these cells and developing germ cells (3). Sertoli cells contribute to establish a suitable testicular micro-environment for germ cell development and sperm production, building the blood-testis barrier (BTB) and creating an immune privileged environment (4,5). In fact, Sertoli cells mediate the testis ability to initiate an innate immune response against infectious agents expressing pattern recognition receptors (PRRs) and, particularly, a subset of the toll-like receptor

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(TLRs) family, such as TLR-2, TLR-3 and TLR-4 (6). In particular, uropathogenic *Escherichia coli* or their products have been demonstrated to activate TLR-2 and TLR-4 in Sertoli cells, leading to the recruitment of immune competent cells (7). TLRs are major activators of innate immunity and inflammation and detect specific motifs or pathogen-associated molecular patterns (PAMPs) (8). TLR-2 and TLR-4 are typically located on cell surface and are known to detect bacterial lipopeptides and lipopolysaccharide, whereas TLR-3 is expressed on the membrane of cytoplasmic organelles, particularly endosomes, and detects viral nucleic acids (6). After the recognition of PAMPs, TLR2 and TLR4 activate the nuclear factor kappa B (NFκB) via the recruitment of myeloid differentiation protein 88 (MyD88), leading to the synthesis of pro-inflammatory cytokines, including interleukin (IL)-1α and IL-6. TLR-3 initiates the TIR-domain-containing adaptor protein-inducing IFNβ (TRIF), that activates both transcription factors IRF-3 and NFκB, inducing the expression of interferon (IFN)-α, IFN-β and IFN-γ (6).

However, the inflammatory response, classically required for microbial clearance, is also responsible for tissue damage (9). In fact, following the infection of the testis, cytokine levels increase considerably, altering Sertoli cell activity with disruptive effects on the development of germ cells and, consequently, on male fertility (10). Many urogenital pathogens are able to infect the seminiferous epithelium and, in particular, sexually transmitted agents, such as *Chlamydia trachomatis*, were recently acknowledged as being clinically important in male infertility (11). *C. trachomatis*, the leading cause of bacterial sexually transmitted diseases (STDs) worldwide (12), is known to disseminate and localize to the upper genital tract impairing the reproductive function (13, 14), despite the numerous protective factors (15-17).

In men, *C. trachomatis* accounts for up to 42% of all cases of non-gonococcal urethritis, although its real prevalence and incidence are unknown (18). This is because up to 50% of *C. trachomatis* infections are asymptomatic and, hence, undiagnosed and untreated. This may lead to severe reproductive sequelae; specifically, ascending *C. trachomatis* genital infections have been demonstrated to cause epididymitis or epididymo-orchitis, well known risk factors for male infertility (12, 19). Therefore, *C. trachomatis* has recently been involved in its etiopathogenesis. Moreover, a recent study has suggested that *C. trachomatis* causes male infertility via the infection of the seminiferous epithelium, as evidenced by altered motility and morphology of spermatozoa in a murine model of *C. muridarum* infection (20).

More recently, we reported that *C. trachomatis* serovar D is able to infect primary human Sertoli cells (21) and, herein, we investigated for the first time the immune response elicited in these cells following *C. trachomatis* infection. Specifically, we evaluated *C. trachomatis*-mediated activation of TLR2, TLR3 and TLR4 as well as of downstream intracellular signaling pathways and the levels of inflammatory mediators. Lastly, we assessed the *in vitro* effects of *C. trachomatis* infection of Sertoli cells on human spermatozoa.

MATERIALS AND METHODS

Cell culture and culture conditions

The primary human Sertoli cell line, purchased from Lonza, USA (product code MM-HSE-2305, lot number 150909091), was harvested on 8th September 2009. Human Sertoli cells were isolated from a healthy donor and checked for purity by assessing the positivity to GATA4 and SOX9 and the negativity to endotoxin, mycoplasma, HIV, HCV and HBV contamination. These cells were provided by Lonza at the 3rd passage after isolation, and all experiments were performed with the same cell line at the 2nd passage after thawing. Sertoli cells were cultured in Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F12 (1:1), with L-glutamine and HEPES (DMEM/F12, Gibco™, USA), supplemented with 10% (v/v) fetal calf serum (FCS), 100 µg/mL streptomycin sulphate and 100 Units/mL potassium penicillin at 37°C in humidified atmosphere with 5% CO2.

McCoy cell line (ATCC® CRL-1696) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco™, USA) supplemented with 10% (v/v) FCS, at 37°C in humidified atmosphere with 5% CO_2.

Propagation and titration of C. trachomatis

C. trachomatis strain D/UW-3Cx (ATCC VR-885)

was propagated in McCoy cells as previously described (22). Briefly, cells were infected with *C. trachomatis* by centrifugation at 754g for 30 min at room temperature and, then, incubated in DMEM supplemented with 10% FCS and 1μg/mL cycloheximide for 48h at 37°C in humidified atmosphere with 5% CO_2. McCoy cells were harvested by scraping and the resulting Chlamydia suspension was stored at -80°C in 4X Sucrose Phosphate (4SP) buffer (0.4M sucrose and 16mM Na_2HPO_4 at pH 7.1).

The infectious titer (Inclusion Forming Units (IFU) per mL) was determined, as previously described (23) by infecting confluent McCoy cell monolayers grown in 24 well trays with 10-fold dilutions of *C. trachomatis* suspension. Briefly, after 36 h post-infection, infected cells were fixed in 96% methanol for 10 min at -20°C and stained with fluorescein isothiocyanate-conjugated monoclonal antibody anti-*C. trachomatis* LPS (Imagen Chlamydia kit, Oxoid, UK). Titers were calculated by counting all green-stained inclusions by using a Leica DM6000B fluorescence microscope (40X magnification).

Infection of primary human Sertoli cells with C. trachomatis

Sertoli cell monolayers, grown on glass coverslips on 24-well cell culture plates, were infected with *C. trachomatis* at a MOI of 5.0 by centrifugation at 754g for 30 min. After the removal of chlamydial inoculum, infected cells were washed with phosphate buffered saline (PBS) and incubated with DMEM/F12 (Corning, USA) supplemented with 10% FCS and no cycloheximide at 37°C in humidified atmosphere with 5% CO_2. Thirty-six hours post-infection, the presence of chlamydial inclusions was visualized by confocal microscopy by utilizing a primary mouse monoclonal antibody against species-specific MOMP (Mab6ciii, The Chlamydia Biobank, UK, Cat. No. #CT602) (1:1000 dilution) combined with an anti-mouse-Alexa Fluor™ 488 conjugate secondary antibody (Invitrogen™, USA, Cat. No. A11001) (1:2000 dilution), as previously described (21).

TLRs, NFκB, IRF3 and cytokine detection

Sertoli cell monolayers, grown on 6-well cell culture plates, were infected with *C. trachomatis* at a MOI of 5.0 as above described. Cell monolayers were investigated for the presence of TLR-2, TLR-3, TLR-4, NFκB and IRF3 by Western blot at different time points. Supernatants were assayed for the presence of IL-1α, IL-6, IFN-γ, IFN-α and IFN-β by ELISA.

Western blot analysis

At 5, 24 and 36 hours post-infection, Sertoli cell monolayers were lysed in RIPA lysis buffer [50mM Tris-HCl at pH 7.6, 0.5% sodium deoxycholate, 140 mM sodium chloride, 1% NP-40, 5 mM Na_2EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, supplemented with protease inhibitor mix (GE Healthcare, USA)] by incubation on ice for 15 min. Lysates were then separated on 8% polyacrylamide gel (PAGE) and immunoblotted using standard procedures. Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk in PBS-T and incubated overnight at 4°C with anti-TLR2 (clone TL2.1, cat. #MAB0067), TLR3 (clone 40C1285.6, cat. #MAB0088), TLR4 (clone 1H7, cat. # H00007099-M03), NFκB (clone 13A2F, cat. #MAB0828) and IRF3 (clone 3F10, cat. #MAB1103) mouse monoclonal primary antibodies (Abnova, USA). HRP-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, USA) was applied to stained membranes and visualized by enhanced chemiluminescence (ECL, Amsterdam).

For the evaluation of protein levels, values are expressed as band integrals normalized to the control and housekeeping gene bands.

ELISA

At 36 hours post-infection, Sertoli cell culture supernatants were collected and stored at -80°C for further downstream analysis. Supernatants were then analyzed for IL-1α, IL-6, and IFN-γ using the specific BioVendor ELISA kit (BioVendor, Czech Republic), and for IFN-α and IFN-β using the specific MyBioSource ELISA (MyBioSource, Inc., USA), according to the manufacturer's instructions. The detection limit for each cytokine is as follows: IL-1α, IL-6 and IFN-γ (1.6 pg/mL), IFN-α (15.25 pg/mL) and IFN-β (31.25 pg/mL).

In vitro exposure of human spermatozoa to C. trachomatis infected Sertoli cell supernatants

Semen samples were self-collected after 3-5 days of abstinence by 3 men attending the Laboratory of Seminology (Sperm Bank "Loredana Gandini", Department of Experimental Medicine, University of Rome "Sapienza"), for semen analysis. Study participants

had not been medically or surgically treated in the 3 months prior to the study and did not have any conditions or reproductive pathology that could interfere with the semen parameters. Written informed consent was obtained from all study participants. This study design and protocol was approved by the Umberto I University Hospital ethical committee (protocol n. 182/11) and conducted according to the principles expressed in the Declaration of Helsinki.

All samples were normozoospermic according to the World Health Organization guidelines (24). Aliquots of semen samples were added to equal volumes of *C. trachomatis* infected or uninfected human Sertoli cell culture supernatants, [previously filtered through 0.22 µm filters (Millex-GP, Millipore, USA) in order to remove all chlamydial EBs], or DMEM/F12 supplemented with FCS 10% as a control, and incubated for 1, 2 and 3 h at 37°C. Then, sperm motility and viability were investigated. Briefly, sperm motility was investigated by an automated computer analysis (CASA System; Hamilton Thorne), that included incubation at 37°C, and sperm vitality was carried out by staining with eosin Y 0.5% in saline solution (25).

At the same time, the swim-up procedure was performed to select motile spermatozoa (26). Five hundred microliters each of DMEM/F12 +10% FCS, *C. trachomatis* infected or uninfected Sertoli cell culture supernatants were layered gently onto 0.5 ml of the semen sample. The spermatozoa were allowed to migrate for 30 min at 37°C. After migration the top layer was collected, incubated at 37°C and analyzed for sperm concentration, motility, and viability at 1, 2 and 3 h.

Statistical analysis

All values are expressed as mean ± standard deviation (SD) of at least 3 replicates from two independent experiments. Comparisons of means were performed by using a two-tailed Student *t*-test for independent samples. The quantification of protein levels was performed in ImageJ software (version 1.52j) and all statistical calculations and graphs were produced in Excel (Microsoft, USA). A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

C. trachomatis induces TLR-3 expression in human Sertoli cells

Initially, we demonstrated that *C. trachomatis* infection of primary human Sertoli cells resulted in the development of chlamydial inclusions, as shown in Fig. 1. Then, we investigated whether *C. trachomatis* serovar D induced the expression of TLR2 and TLR4, since several studies reported that TLR2 and TLR4 were critical receptors involved in the immune recognition of *C. trachomatis* (27,28). Neither TLR2 or TLR4 were expressed in human Sertoli cells following *C. trachomatis* infection (Fig. 2 A and B). Then, we evaluated whether TLR3 was stimulated during *C. trachomatis* infection of primary human Sertoli cells, since more recent studies reported that *Chlamydia* elicits TLR3 expression (29-31). A significant increase in the protein levels of TLR-3 was observed in primary human Sertoli cells following *C. trachomatis* infection when compared to uninfected cells (Fig. 2 C and D, $P<0.05$).

C. trachomatis fails to stably induce TLR-3 dependent signaling pathways

Since we observed a significant induction of TLR3 following *C. trachomatis* infection of primary human Sertoli cells, we investigated whether *C. trachomatis* promoted the downstream expression of NFκB and/or IRF3 transcription factors, the next steps in the TLR3-mediated signaling pathway (6).

Primary human Sertoli cells were infected with *C. trachomatis* and the expression of NFκB and IRF3 was analyzed at different time points post-infection (5, 24 and 36 h). As shown in Fig. 3, *C. trachomatis* modulated the expression of both NFκB and IRF3 in a time-dependent fashion. The NF-κB was only detected at 5 hours post-infection ($P<0.05$) with a dramatic down-modulation of protein levels at later time points.

IRF3 protein level was at its highest at 24 hours post-infection ($P<0.05$) but decreased at the last point, 36 hours post-infection (Fig. 3 A and B) ($P<0.05$). Interestingly, human Sertoli cells constitutively expressed NFκB and IRF3 proteins and maintained their expression in culture.

Lastly, since it is known that IRF3 and NFκB induce the expression of proinflammatory cytokines, such as interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-1α and IL-6 (6), their levels were evaluated, by ELISA, in human Sertoli cells infected with *C. trachomatis*. However, *C. trachomatis* did not elicit the secretion of

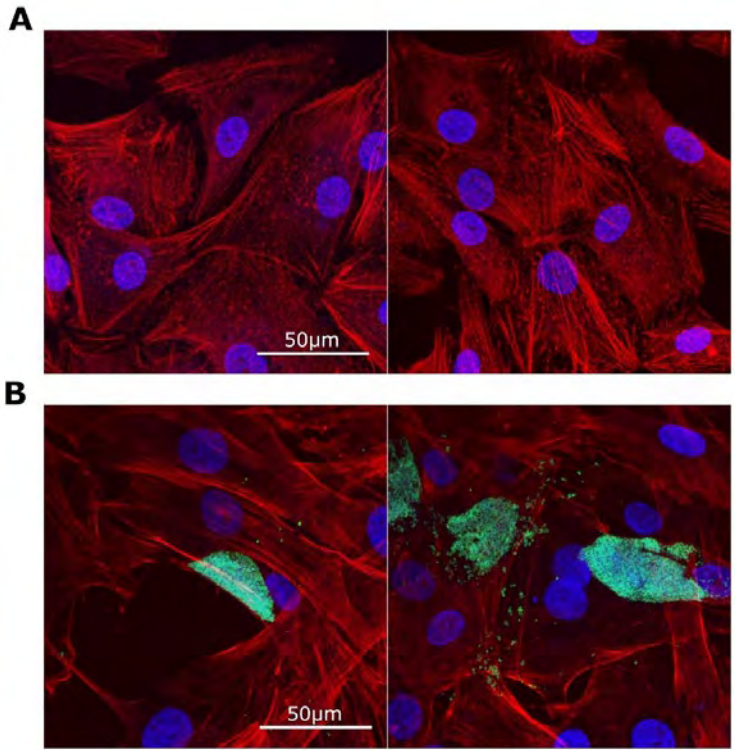


Fig. 1. *Confocal micrographs of C. trachomatis uninfected (**A**) and infected (**B**) primary human Sertoli cells. Cell monolayers, infected by C. trachomatis were stained after 36 hpi as reported in Materials and Methods (all experiments were performed in duplicate, magnification 100X).*

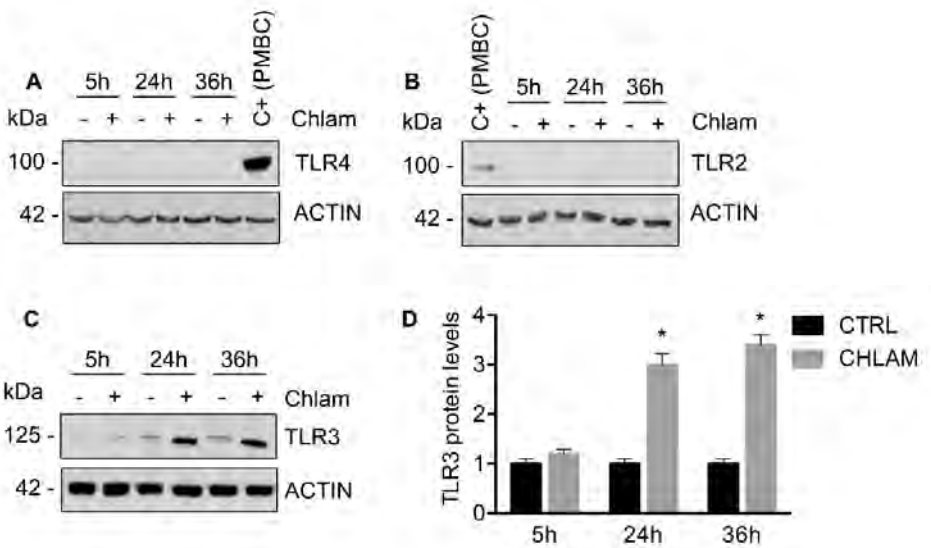


Fig. 2. *C. trachomatis activates TLR-3 in primary human Sertoli cells. Representative western blot of endogenous TLR4 (A) and TLR2 (B) levels in primary human Sertoli cells after C. trachomatis infection at indicated time points. Peripheral blood mononuclear cells (PBMC) were used as positive control and actin was used as loading control. Representative western blot (C) and densitometric analysis (D) of endogenous TLR3 levels in primary human Sertoli cells after C. trachomatis infection at indicated time points. Actin was used as loading control. The bars in (D) represent the mean values and the standard errors of the means of at least 3 replicates from two independent experiments for each group. CHLAM, C. trachomatis; CTRL, uninfected control. *P < 0.05.*

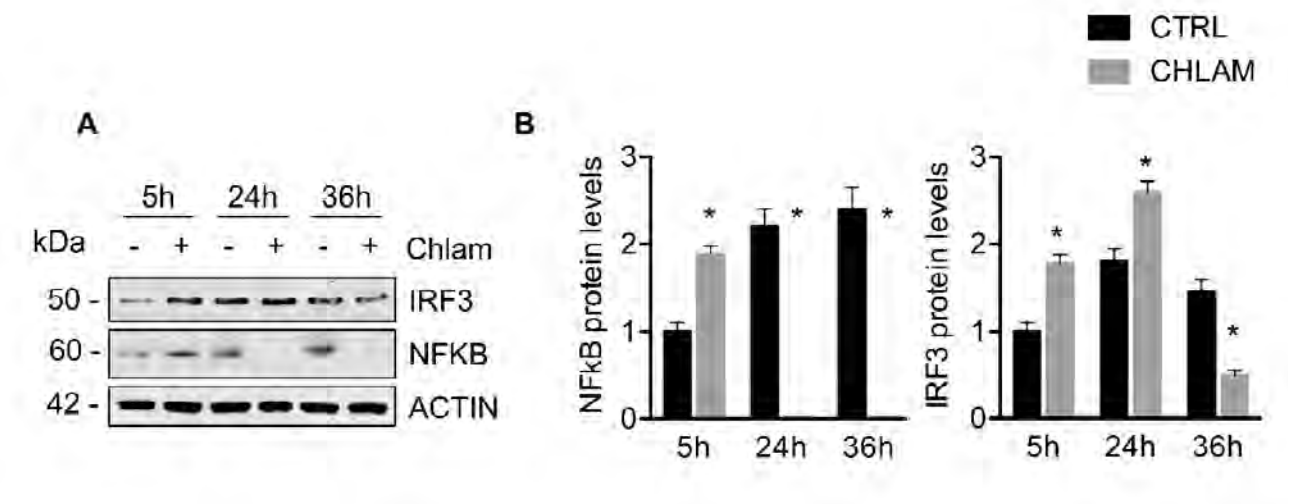
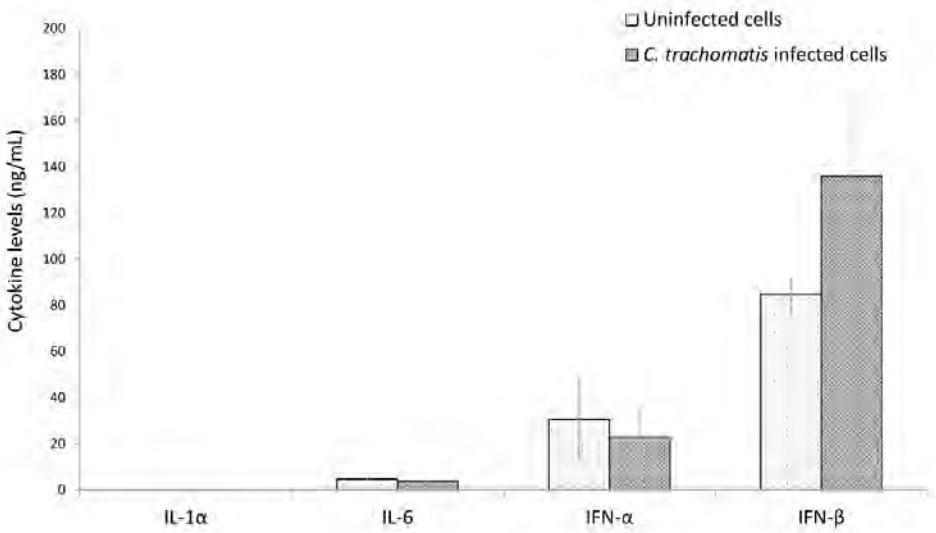


Fig. 3. *C. trachomatis disrupts TLR3-dependent signalling pathways. Representative western blot (**A**) and densitometric analysis (**B**) of endogenous NFκB and IRF3 levels in primary human Sertoli cells after C. trachomatis infection at indicated time points. Actin was used as loading control. The bars represent the mean values and the standard errors of the means of at least 3 replicates from two independent experiments for each group. CHLAM, C. trachomatis; CTRL, uninfected control. *P < 0.05.*



	IL-1α (ng/mL)	IL-6 (ng/mL)	IFN-α (ng/mL)	IFN-β (ng/mL)	IFN-γ (ng/mL)
Uninfected cells	0.0049±0.001	4.6±0.5	30.6±18.5	84.8±8.2	ND
***C. trachomatis* infected cells**	0.0038±0.0002	3.6±0.8	22.9±13	136±38.8	ND

Values are expressed as mean ± Standard Deviation; ND, not detected

Fig. 4. *IL-1α, IL-6, IFN-α, IFN-β and IFN-γ levels in C. trachomatis infected and uninfected primary human Sertoli cells. All values are expressed as mean ± standard deviation (SD) of at least 3 replicates from two independent experiments. ND, not determined.*

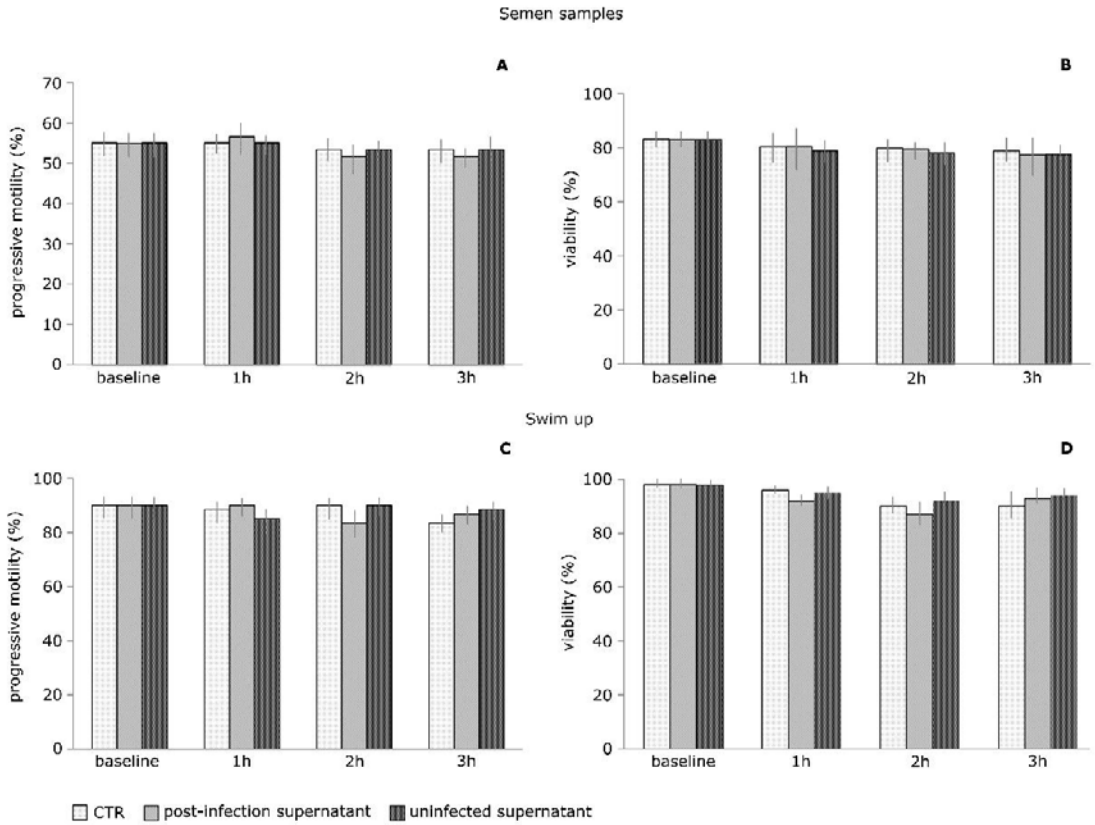


Fig. 5. *Effects of C. trachomatis infection of Sertoli cells on semen parameters. Percentage of progressive motility and viability of semen samples (**A, B**) and swim up preparations (**C, D**) at baseline and at 1, 2 and 3-hour post-incubation with C. trachomatis infected or uninfected primary human Sertoli cell supernatants or DMEM/F12 supplemented with FCS 10% (CTR). The bars represent the mean values and the standard errors of the means of at least 3 replicates from two independent experiments for each group.*

IFN-α, IFN-β, IFN-γ, IL-1α and IL-6 in human Sertoli cells in a significative way, as shown in Fig. 4.

C. trachomatis does not affect semen parameters

The effects of *C. trachomatis* infection of human Sertoli cells on sperm viability and progressive motility were assayed by challenging Chlamydia-infected Sertoli cell supernatant with human spermatozoa from three normozoospermic semen samples at different time-points (0, 1, 2 and 3 h) (Fig. 5 A and B). The swim-up technique was used to investigate its effects on the ability of motile spermatozoa to migrate against gravity in the media (Fig. 5 C and D).

C. trachomatis infection of human Sertoli cells did not affect the viability or the progressive motility of spermatozoa from either the semen samples or the motile spermatozoa selected by swim-up ($P > 0.05$). It also had no effect on their migratory ability, since the number of migrated spermatozoa exposed to either *C. trachomatis* infected or uninfected human Sertoli cell culture supernatants remained unvaried (data not shown).

DISCUSSION

Sertoli cells are responsible for building the BTB and creating an immune-privileged niche

that protects developing germ cells from the host immune system as well as from infectious threats. Indeed, Sertoli cells are well known to play a key role in the innate immune defenses of the testis via the TLR-mediated recognition of pathogens (6).

The main results of our study are: (*i*) the induction of TLR3 production by *C. trachomatis* infection of Sertoli cells; (*ii*) no expression of TLR2 and TLR4 in human Sertoli cells following *C. trachomatis* infection; (*iii*) the down-modulation of NFκB and IRF-3 transcription factors in infected human Sertoli cells, and lack of an increased synthesis of proinflammatory cytokines, such as IFN-α, IFN-β, IFN-γ, IL-1α and IL-6; (*iv*) no harmful effects on human spermatozoa following *C. trachomatis* infection of Sertoli cells.

Overall, this peculiar innate immune response by human Sertoli cells in the male genital tract after the exposure to *C. trachomatis* is surprising, since we expected to observe increased expression of TLR2 and TLR4, as evidenced in epithelial cells from the female genital tract (27-31). In our study, instead, human Sertoli cells detected *C. trachomatis* through TLR3-mediated pathways, classically observed for viral infections. This shows interesting differences with the Chlamydia-mediated response in the female genital tract, where both TLR2 and TLR3 have been shown to be important mediators of the innate immunity against *C. trachomatis*, although with different pathophysiological roles. TLR2 was involved in the early production of inflammatory mediators (i.e. IL-1α, IL-6) and the development of chronic inflammatory pathology (27-31), whereas the activation of TLR3 resulted in the control of bacterial replication via IFNβ, as well as in the prevention of severe reproductive sequelae (29,31).

In our study, *C. trachomatis*, despite triggering the expression of TLR3 in human Sertoli cells, hindered the activation of the downstream signaling pathways, as evidenced by a short-term expression of the transcription factor NFκB, followed by its suppression, and a decreased expression of IRF3. As a result, IFN-α, IFN-β, IFN-γ, IL-1α and IL-6 were not produced in infected human Sertoli cells, demonstrating the absence of a specific innate immune response to *C. trachomatis*. This suggests the compelling hypothesis that *C. trachomatis* could create a suitable micro-environment for its survival in these cells, resulting, most likely, in the persistence of the infection. In fact, *C. trachomatis* has been shown to persist for a long time in epithelial cells by generating non-infectious forms that are usually demonstrated by the reduced production of chlamydial infectious progeny (32). In this regard, in a previous study, we observed a decrease in *C. trachomatis* infectious progeny in human Sertoli cells and this finding, alongside the absence of a specific innate immune response to *C. trachomatis* found in this study, suggests the possibility of a novel persistence model in human Sertoli cells (21), although further studies are warranted.

To date, it has been demonstrated that persistent forms of *C. trachomatis* arise under different stressful conditions, including treatment with IFN-γ or the growth in monocyte-macrophages (14). These persistent forms are known to evade the host immune system and induce a chronic inflammatory state, leading to the tissue damage responsible for infertility (33). In our study, the suppression of the inflammatory response mediated by *C. trachomatis* infection, as evidenced by the lack of detection of IFN-α, IFN-β, IFN-γ, IL-1α and IL-6 in infected cells, might hint, at first, to the absence of testicular tissue damage. However, other mechanisms might be involved in the etiopathogenesis of male infertility mediated by *C. trachomatis*, like modifications in the expression of paracrine and endocrine factors or alterations in the levels of other inflammatory cytokines or in cell structure. Indeed, a potential negative effect of *C. trachomatis* on human Sertoli cells has been described in our previous study, as evidenced by the alteration of human Sertoli cell cytoskeleton following *C. trachomatis* infection, most likely affecting the integrity of the BTB and impairing germ cell development (21).

In addition, *C. trachomatis* infection of human Sertoli cells might indirectly exert detrimental effects on the host cell function by releasing soluble factors, including HSP60, LPS and ROS. In this regard, we assayed sperm viability and motility from semen samples exposed to the supernatants from *C. trachomatis*-infected human Sertoli cells, observing

no alterations (P>0.05). However, we cannot exclude that *C. trachomatis* infection of human Sertoli cells might have a negative impact on the earlier phases of spermatogenesis, since we used mature spermatozoa.

It should be noted that there is extensive controversial literature regarding the relationship between *C. trachomatis* genital infection and sperm parameters, with several studies demonstrating that *C. trachomatis* genital infection had no effects on sperm parameters, whereas others showing an association between *C. trachomatis* DNA in semen and poor semen motility (34).

The main strength of our study is the investigation of the immunological pathways initiated by *C. trachomatis* infection of human Sertoli cells, that might better reflect a human testicular micro-environment.

In conclusion, our results showed that *C. trachomatis* can modulate the innate immune response in human Sertoli cells and evade the host immune-mediate killing, potentially giving rise to a long-term infection that may exert a negative effect on the testicular tissue, impairing its regulation of the phases of the spermatogenetic process.

Overall, our data open the way for more complex studies, such as those utilizing specifically engineered organoids of the seminiferous epithelium in order to improve our knowledge of *C. trachomatis* effects on the multiple regulatory pathways involved in the spermatogenetic process, clarifying the role of *C. trachomatis* in male infertility.

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