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A platform for studying the transfer of *Chlamydia pneumoniae* infection between respiratory epithelium and phagocytes

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ABSTRACT

The obligate intracellular bacterium, *Chlamydia pneumoniae,* has been identified as a risk factor for several chronic inflammatory diseases in addition to respiratory tract infections. The dissemination of *C. pneumoniae* from respiratory tract to secondary sites of infection occurs via infected monocyte / macrophage line cells, in which *C. pneumoniae* can persist as an antibiotic-refractory phenotype. To allow more detailed studies on the epithelium-monocyte/macrophage transition of the infection, new in vitro bioassays are needed. To this end, a coculture system with human continuous cell lines was established. Respiratory epithelial HL cells were infected with *C. pneumoniae* and THP-1 monocytes were added into the cultures at 67 h post infection. After a 5 h co-culture, THP-1 cells were collected with a biotinylated HLA antibody and streptavidin-coated magnetic beads and *C. pneumoniae* genome copy numbers in THP-1 determined by quantitative PCR. The assay was optimized for cell densities, incubation time, THP-1 separation technique and buffer composition, and its robustness was demonstrated by a Z' value of 0.6. The mitogen-activated protein kinase (MAPK) inhibitors: SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and FR180204 (ERK inhibitor) suppressed the transfer of *C. pneumoniae* from HL to THP-1 cells, making them suitable positive controls for the assay. Based on analysis of separate steps of the process, the MAPK inhibitors suppress the bacterial entry to THP-1 cells. The transfer of *C. pneumoniae* from epithelium to phagocytes represents a crucial step in the establishment of persistent infections by this pathogen, and the presented methods enables future studies to block this process by therapeutic means.

1. Introduction

Chlamydia pneumoniae is a gram-negative, obligate intracellular bacterium, whose primary site of infection is the respiratory tract (Kuo et al., 2015). It causes various acute illnesses such as pharyngitis, sinusitis and it is a cause of 10% of community acquired pneumonia (CAP) (Kuo et al., 1995). *C. pneumoniae* is a ubiquitous bacterium and majority of adults have been infected, at least once, during their lifetime. In addition to respiratory tract infections, *C. pneumoniae* has been identified as a risk factor for several chronic inflammatory diseases such as asthma (Smith-Norowitz et al., 1971; Webley and Hahn, 2017), atherosclerosis (Campbell and Kuo, 2004; Filardo et al., 2015) and Alzheimer's disease and neurovascular complications (Little et al., 2004; Balin PhD et al., 2017; Richard, 2018).

Serological findings (Filardo et al., 2015; Saikku et al., 1988) and histological demonstration of the presence of *C. pneumoniae* in atherosclerotic plagues (Luque et al., 2012; Shor and Phillips, 2000) have triggered a spectrum of experimental studies on the role of *C. pneumoniae* in the etiology of cardiovascular diseases. Established animal models with mice and rabbits have demonstrated the induction of atherosclerotic plague development (Muhlestein, 2000; Sorrentino et al., 2015), endothelial dysfunction (Huang et al., 2012), accelerated hyperlipidemia (Blessing et al., 2001) and thus promotion of the atherosclerosis development by the infection. *C. pneumoniae* DNA has also been detected from post mortem Alzheimer's brain samples (Balin et al., 1998) and the infection has been found to induce amyloid plague

Abbreviations: AB, aberrant body; BSA, bovine serum albumin.; CAP, community acquired pneumonia.; CASMC, coronary artery smooth muscle cells.; CHX, cycloheximide.; EB, elementary body.; EDTA, ethylenediaminetetraacetic acid.; ERK, extracellular signal-regulated kinases.; FBS, fetal bovine serum.; GE, genomic equivalents.; HMEC, human arterial endothelial cells.; HSPG, heparin sulfate proteoglycans.; HtrA, high-temperature requirement A.; HUVEC, human umbilical vein endothelial cells.; IFU, inclusion forming unit.; IL, interleukin.; JNK, c-Jun N-terminal kinases.; MAPK, mitogen-activated protein kinase.; MOI, multiplicity of infection.; NF-κB, nuclear factor- κB.; OmcB, outer membrane complex protein B.; PBS, phosphate buffered saline.; PS, phosphatidylserine.; RB, reticulate body.; ROS, reactive oxygen species.; TLR, Toll-like receptor.; TNF-α, tumor necrose factor alpha

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formation in animal models for Alzheimer's disease (Little et al., 2004; Boelen et al., 2007).

C. pneumoniae has a unique biphasic lifecycle which involves extracellular, metabolically less active (Grieshaber et al., 2018), infectious form, elementary body (EB) and intracellular, metabolically active, replicative form, reticulate body (RB) (Elwell et al., 2016). To drive bacterial replication, EBs attach and internalize into the host cell by endocytosis. Within the lipid vacuole called inclusion, EBs differentiate to RBs and start to multiply. At 48 to 72 h post infection RBs asynchronously reorganize back to EBs which are released from the cell through host cell lysis or extrusion, the latter of which leaves the host cell intact. Besides the productive chlamydial replication cycle, *C. pneumoniae* can enter into a reversible dormant state. Showing analogy to persister subpopulations more recently found within other pathogenic bacteria (Balaban et al., 2019), these viable but non-replicative chlamydial cells are referred as aberrant bodies (ABs) (Panzetta et al., 2018). Persistent *C. pneumoniae* maintains the capability to modulate host cells signaling pathways, resulting in the activation of host tyrosine kinases and nuclear factor- κB (NF-κB) signaling. It produces outer membrane vesicles containing molecules such as high-temperature requirement A (HtrA) proteases targeting the host cells (Frohlich et al., 2014; Backert et al., 2018; Ong et al., 2013). The persistent state is triggered by various stress signals and it is particularly prominent within the infections of immune cells such as monocytes and macrophages (Beagley et al., 2009).

In the course of in vivo infections, *C. pneumoniae* first targets respiratory tract epithelium, but may later disseminate to other body sites. While direct transfer from olfactory tract to central nervous system has been proposed (Fulop et al., 2018), transfer to local phagocytes and further to peripheral blood mononuclear cells is considered the primary route for *C. pneumoniae* systemic dissemination (Moazed et al., 1998; Yang et al., 1995; Gieffers et al., 2004). The epithelium – monocyte/macrophage transition is crucial for the role of *C. pneumoniae* as an etiologic agent in chronic inflammatory diseases at various body sites. Besides acting as dissemination vehicles, monocytes and macrophages play an important role in promoting a long-lasting inflammatory state associated with persistent *C. pneumoniae* infections (Beagley et al., 2009). *C. pneumoniae* infected monocytes produce various cytokines such as interleukin (IL)-1β, IL-6, IL-8 (Lim et al., 2014), IL-10 (Mamata et al., 2007), IL-12 (Kortesoja et al., 2019) and tumor necrose factor (TNF)-α (Mamata et al., 2007) and the infection also induces reactive oxygen species (ROS) production (Mouithys-Mickalad et al., 2004) and activates NLRP3 inflammasome (Itoh et al., 2014). A major hallmark of *C. pneumoniae* persistence is its antibiotic-refractory nature. It has been reported by us and others that *C. pneumoniae* cannot be eradicated from monocytes by antibiotics (Airenne et al., 1999; Taavitsainen et al., 2020; Gieffers et al., 2001). If the transfer of *C. pneumoniae* from lung epithelium to monocytes and macrophages could be inhibited the chronic proinflammatory state could be reduced and progression of the chronic inflammatory diseases such as atherosclerosis and Alzheimer's disease may be diminished.

Despite its significance in the establishment of long-lasting infections, bioassays suitable for in vitro studies on the transfer of *C. pneumoniae* from epithelium to phagocytes have not been described. In previous studies focusing on interactions of different cell populations in the course of chlamydial infections, the interplay between vascular wall cells and monocytes has been addressed by coculture systems. A coculture between infected human arterial endothelial cells (HMECs) (Lin et al., 2000), human umbilical vein endothelial cells (HUVECs) or coronary artery smooth muscle cells (CASMCs) (Puolakkainen et al., 2003) and uninfected U937 monocytic cells have been reported to enhance the *C. pneumoniae* infection in vascular wall cells. It has also been demonstrated that infection transfers into HMECs and CASMCs from infected U937 cells. With a related pathogen *Chlamydia trachomatis* coculture setups have been established with epithelial cells and monocytes (Mpiga et al., 2006; Mpiga and Ravaoarinoro, 2006). These studies have, however, focused on cytokine production and other immunological outcomes of chlamydial infections.

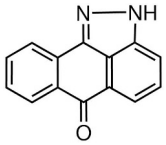
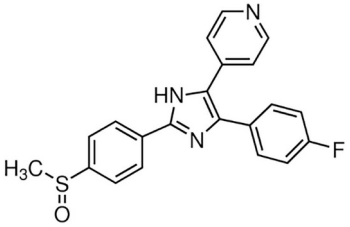
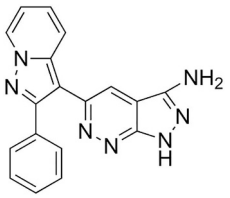
Standard techniques for detecting and quantifying *C. pneumoniae* in biological samples include immunofluorescence applying *Chlamydia*-specific anti-lipopolysaccharide (LPS) antibody and polymerase chain reaction (PCR). Chlamydial inclusions are easily visualized in cultured monolayer cells allowing chlamydial replication, but the irregular size and morphology of the inclusions in nonpermissive cells such as monocytes and macrophages limits the use of staining techniques (Poikonen et al., 2010). Furthermore, genetic transformation of *C. pneumoniae* has become feasible only very recently (Shima et al., 2018) and therefore bioassays with reporter gene inserts are generally not available. Currently the susceptibility screening of antichlamydial drug candidates is based on their impact on chlamydial inclusion counts in epithelial cells, which detects only the net chlamydial load after one productive infection cycle. This standard MIC test thus leaves the persistent infections unconsidered and does not provide information on the maturation or infectivity of the produced EBs. Due to the fact that persistent *C. pneumoniae* cannot be eradicated by traditional antibiotics, new antichlamydial compounds, as well as new methods to evaluate them, are needed. In addition to direct bacteriostatic or bacteriocidic modes of action, assessing the candidate compounds for their capability to inhibit the transfer of *C. pneumoniae* between cell populations would provide a valuable means for compound profiling and prioritizing. Here we present a robust coculture model to study the transfer of *C. pneumoniae* from lung epithelial, HL cells to THP-1 monocytes. In addition, a set of assays focusing on individual steps within the epithelium – monocyte transition is applied to study the targets of transition inhibitors. By this means we found that MAPK inhibitors reduce the internalization of *C. pneumoniae* to monocytes and decrease the transfer of infection between epithelial cells and monocytes.

2. Materials and methods

2.1. Compounds

C-Jun N-terminal kinase (JNK) inhibitor SP600125, p38 inhibitor SB203580 and extracellular signal-regulated kinase (ERK) inhibitor FR180204 (Table 1) were purchased from Tocris Bioscience (Bristol,

Table 1
MAPK-inhibitors applied in this study.

Name	Target kinase	Structure
SP600125	c-Jun N-terminal kinases (JNKs)	
SB203580	p38 mitogen activated protein kinases	
FR180204	Extracellular signal-regulated kinases (ERK)	

UK), dissolved in dimethyl sulfoxide (DMSO) as 20 mM stock solution and used as 10 μM working concentration in all the experiments. The final DMSO concentration in the assays was 0.05%.

2.2. Cell culture

Cell cultures were maintained and incubated at 37 °C, 5% CO_2 and 95% relative humidity. Cell numbers for all experiments were determined by counting the cells with hemocytometer. Human respiratory tract epithelial, human lung (HL) cells (Kuo and Grayston, 1990), obtained from professor Pekka Saikku / National Institute of Health and University of Oulu, Finland, were cultured in RPMI 1640 cell culture medium (BioWhittaker, Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (BioWhittaker), 7.5% fetal bovine serum (FBS) (BioWhittaker) and 20 mg/ml Gentamicin (Fluka, Buchs, Switzerland).

Human monocytic cell line THP-1 (ATCC TIB202) was cultured in RPMI 1640 (Dutch edition) cell culture medium (Gibco, Invitrogen, Thermo Fisher Scientific, Paisley, UK) supplemented with 2 mM L-glutamine (BioWhittaker), 10% FBS (BioWhittaker), 0.05 mM 2-mercaptoethanol (Gibco) and 20 μg/ml Gentamicin (Fluka).

2.3. C. pneumoniae infection

C. pneumoniae cardiovascular isolate CV-6 (Maass et al., 1998) was obtained from professor Matthias Maass (Paracelsus Medical University, Salzburg, Austria) and propagated in HL cells. Cells were inoculated with *C. pneumoniae* and incubated in cell culture medium (RPMI 1640 supplemented with 7.5% FBS, 2 mM L-glutamine, 0.6 μg/ml cycloheximide (CHX), 10 μg/ml gentamicin, 100 μg/ml streptomycin, 3.75 μg amphotericin B). After 72 h, the HL cells were lyzed, the cell debris was removed by centrifugation (10 min, 500 xg, 4 °C) and chlamydial EBs collected by pelleting (1 h at 21000 x g, 4 °C). The EBs were resuspended into sucrose-phosphate-glutamic acid (SPG) (Airenne et al., 1999) and stored at −80 °C. The EB stock titers, expressed as inclusion forming units (IFUs) per ml were determined by infecting the HL cells cultured on coverslips with 10-fold serial dilutions and by counting the inclusions after 72 h.

For experiments, the HL cells were infected by seeding the cells into 24-well plates at a density of 3×10^5 cells/ml and incubated overnight. The cell monolayer was inoculated with *C. pneumoniae* with a multiplicity of infection (MOI) 1 and the plate was centrifuged at 550 xg for 1 h at room temperature and then incubated at 37 °C an additional hour. The inoculum was removed and the cells were incubated 67 to 72 h in cell culture medium with or without the MAPK inhibitors.

2.4. HL - THP-1 coculture

HL cells were seeded into 24-well plates at a density of 3×10^5 cells/ml and infected with *C. pneumoniae* MOI 1 as described above. At 67 h post infection, HL cells were washed with 1 ml of sterile phosphate buffered saline (PBS) and 3×10^5 THP-1 cells were added into the wells on top of HL monolayer in THP-1 cell culture medium, and cells were incubated together for 5 h (Fig. 1). THP-1 cells were then collected by pipetting and centrifuged at 300 xg, 5 min. Supernatants were discarded and cell pellets were suspended with 100 μl of degassed buffer (1% BSA, 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS). Cells were incubated for 15 min on ice with 1:100 dilution of biotin conjugated HLA-A2 antibody (Biolegend, San Diego, US). One ml of buffer was added and the cells were centrifuged at 300 xg for 5 min. After washing the excess antibody, cells were incubated on ice another 15 min with 1:100 dilution streptavidin conjugated MojoSort nanobeads (Biolegend) in 100 μl of buffer. Then, cells were washed with 1 ml of buffer, centrifuged at 300 xg for 5 min and pellets were resuspended in 500 μl of buffer. LS column (Miltenyi Biotec, US) was placed in the magnetic field of the MidiMACS separator (Miltenyi Biotech) and it was prepared with 3 ml of buffer. Then the sample was loaded into MACS magnetic separation system and the column was washed with 4 × 3 ml of buffer. The column was released from the magnetic field and the sample was eluted from the column with 4 ml of buffer by using the plunger. After that the cells were centrifuged at 300 xg, 5 min, and resuspended in 1 ml of buffer. The collected THP-1 cells were then counted and samples were frozen at −20 °C for further DNA extraction.

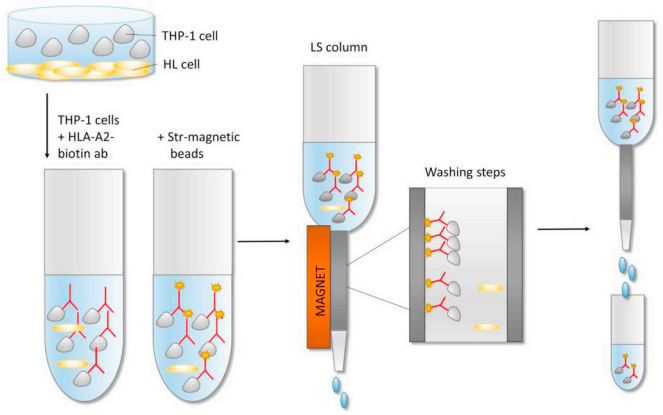


Fig. 1. A workflow for collecting THP-1 cells by magnetic separation. THP-1 cells were collected by pipetting from wells with HL monolayers and pelleted by centrifugation. THP-1 cells were labeled with a biotinylated HLA-A2 antibody and streptavidin-magnetic beads. Cells were placed into LS columns which were placed into MidiMACS magnetic separator. Remaining, unlabeled HL cells that might have come along with the THP-1 cells suspension were washed away from the sample and only THP-1 cells were collected.

2.5. Optimizing the coculture protocol

In the course of optimization of the coculture protocol, two alternative cell densities (3×10^5 and 4×10^5 cells/well) in the 24-well plate were evaluated. The concentration of 3×10^5 cells/ml resulted in fewer detached HL cells and was thus chosen for further use.

The composition of the buffer used for the column separation was also optimized. 2 mM EDTA and 1% BSA in PBS improved the yield and viability of the THP-1 cells, after the separation, compared to pure PBS or PBS with EDTA. Degassing of the chosen buffer to remove excess oxygen and air bubbles before use also improved the results significantly. In addition to washing steps, the buffer was used to prepare the LS column before loading the sample, which improved the yield of THP-1 cells.

To separate the THP-1 monocytes from HL cells, three different methods were evaluated (Table 2). First the phagocytic activity of the monocytes was exploited by allowing a spontaneous ingestion of the magnetic beads. After incubation, cells were trypsinized and separated with MACS magnetic separation system without antibodies. After the separation, there were still epithelial cells in the positive fraction and also THP-1 cells were found in the waste. The phagocytosis of the beads by THP-1 cells was thus neither totally specific nor efficient. Furthermore, the viability of the THP-1 cells after the separation was low, 0–35%.

Thus, HLA-A2 antibody was applied for the separation. After coculture all of the cells were collected by trypsinization, incubated with the antibody and magnetic beads and were then loaded into MACS magnetic separation system. After washing the column with the buffer, there was still a large amount of epithelial cells in the samples, resulting in the need for further optimization to avoid excessive washing steps.

In the final protocol THP-1 cells were collected from the wells by pipetting and the remaining HL cells were more effectively removed by HLA-A2 antibody labeling. To allow the THP-1 suspension removal from the wells by pipetting, the incubation time of the actual coculture

Table 2
Optimizing the THP-1 separation from the coculture.

Protocol	THP-1 yield	THP-1 viability	Observations / results
Passive phagocytosis of the magnetic beads and cell collection by trypsinization	20%	≤ 35%	-Internalization of the beads not efficient- THP-1 cells in the waste -Low viability
Trypsinizing cells from the coculture, magnetic separation with HLA-A2 antibody	ND*	100%	-No THP-1 cells in the waste -HL cells in the positive fraction -Good viability after separation
Collecting THP-1 cells from the coculture by pipetting, magnetic separation with HLA-A2 antibody	75%	100%	-No THP-1 cells in the waste -No HL cells in the positive fraction

$N \geq 3$ in all the optimization steps. *not possible to determine due to the presence of HL cells in the samples.

was limited to 5 h, providing a time frame with efficient bacterial transfer without extensive adherence of the THP-1 cells to the HL monolayer.

2.6. Quantitative PCR

Total DNA from the THP-1 cells was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer's instructions and the DNA concentration in the samples was measured with NanoDrop (Thermo Fisher Scientific). *C. pneumoniae* genome copy numbers (GE) were quantified based on the bacterial OmpA gene using Step One Plus Real-Time PCR system (Thermo Fisher Scientific). The primers for *C. pneumoniae* OmpA gene were 5′-TCC GCA TTG CTC AGC C-3′ (forward) and 5′-AAA CAA TTT GCA TGA AGT CTG AGA-3′ (reverse) (Tondella et al., 2002). The reactions were performed in 96-well MicroAmp optical plates (Thermo Fisher Scientific) by adding 20 µg DNA to 10 µl of master mix. Total reaction volume was 20 µl. Conditions in thermal cycle were 95 °C for 20 s and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. In all PCR runs negative control samples without any template was included. Standard curves for GE number determination were generated by serial dilution of purified EB stocks with known titer.

2.7. Quantification of EB production and EB exit determination

HL cells were seeded into 24-well plates at a density of 3×10^5 cells/ml and infected with *C. pneumoniae* at MOI1 as described above and incubated for 67 h (passage 1). The MAPK inhibitors were added to the wells and incubated for further 5 h. Supernatants containing the released EBs were collected, EBs were pelleted by centrifugation (21,000 *g*, 1 h, 4 °C) and resuspended in cell culture medium. EBs residing inside HL monolayers were also collected by scraping the cells off from the wells and lysing them with glass beads. After that, the EBs from the supernatant and cell lysate were inoculated onto to the fresh HL monolayers (passage 2) as described above for pure EBs. At 3 h post infection cells were collected by scraping, centrifuged at 300 x*g* for 5 min and resuspended in PBS. Total cellular DNA was extracted with GeneJet Genomic DNA purification kit, and quantification of *C. pneumoniae* genome copy numbers was performed using Step One Plus Real-Time PCR system as described above.

2.8. EB infectivity assay

To study the effect of MAPK inhibitor treatment on *C. pneumoniae* EB infectivity as previously described (Hanski et al., 2016a), 3×10^5 EBs, diluted from stock solution in 100 µl THP-1 cell culture medium, were incubated with MAPK inhibitors for 2 h on ice. Then 3×10^5 THP-1 cells in 900 µl, were added into the suspension and the samples further incubated for 2 h at 37 °C. Cells were collected, the supernatant was removed, and the pellet was resuspended in PBS. Centrifugation was repeated and cells were stored at −20 °C. DNA was extracted with GeneJet Genomic DNA purification kit, and quantification of *C. pneumoniae* genome copy numbers was performed using Step One Plus Real-Time PCR system as described above.

2.9. EB internalization assay

The *C. pneumoniae* entry to the monocytes was studied with a protocol modified from a previously published work (Hanski et al., 2016b). To evaluate whether MAPK inhibitors have an effect on internalization of *C. pneumoniae,* 3×10^5 THP-1 cells were incubated with 3×10^5 EBs (MOI1) in 100 µl of cell culture medium with MAPK inhibitors for 2 h at 37 °C. Then, to remove any uninternalized EBs, the cell pellet were resuspended in 1 ml of PBS and cells collected again by centrifuging at 300 *g*, 5 min. Total cellular DNA was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific), and quantification of *C.*

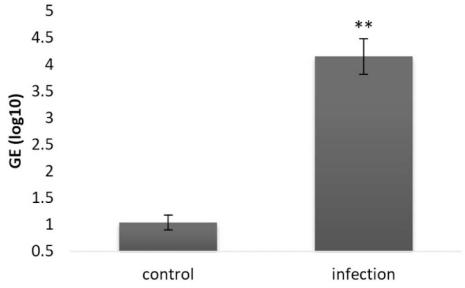


Fig. 2. The transfer of *C. pneumoniae* from epithelium to monocytes. THP-1 cells were cultured with infected and uninfected HL cells. After 5 h, THP-1 cells were collected and *C. pneumoniae* genome copy numbers inside THP-1 cells was determined by qPCR. Data are presented as log10 values ± SEM of *C. pneumoniae* genome numbers Statistical significance is determined by two-tailed Student's *t*-test and presented as marks of *P* values: < 0.05: *; < 0.01: **; < 0.001: ***. $N = 4$ independent biological replicates.

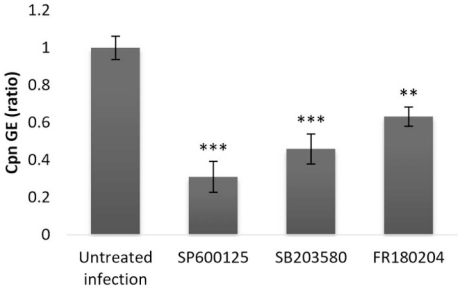


Fig. 3. MAPK inhibitors suppress the transfer of *C. pneumoniae* from HL to THP-1 cells. THP-1 cells were cultured with infected and uninfected HL cells in presence of MAPK inhibitors SP600125, SB203580 and FR180204. After 5 h, THP-1 cells were collected and *C. pneumoniae* genome copy numbers inside THP-1 cells were determined by qPCR. Data are normalized on the infection control and shown as mean ± SEM of *C. pneumoniae* genome copy numbers. Statistical significance is determined by One-way ANOVA and Dunnett's post hoc test and presented as marks of P values: < 0.05: *; < 0.01: **; < 0.001: ***. $n = 4$ independent biological replicates.

pneumoniae genome copy numbers was performed using Step One Plus Real-Time PCR system as described above.

3. Data analysis

Statistical analyses were performed using SPSS Statistics 25 software. Differences between the study groups were calculated by two-tailed student's *t*-test or by One-way ANOVA and Dunnett's post hoc test. Statistical significances are presented as *P* values, which < 0.05 were considered as significant. The formula used for Z' value calculation was $Z' = \frac{3SD\ of\ sample + 3SD\ of\ control}{|\ mean\ of\ the\ sample - mean\ of\ the\ control\ |}$. The n values of each experiment are presented in corresponding figure legend. At least 4 biological replicates (data from separate cell cultures) and two technical replicates (data derived from the same biological replicate) were applied.

4. Results

4.1. Transfer of C. pneumoniae from epithelial cells to monocytes in a coculture system

In the human body, *C. pneumoniae* transfers between epithelial cells and phagocytes (Moazed et al., 1998; Gieffers et al., 2004). To study this phenomenon in vitro, a coculture model applying human respiratory epithelial and monocytic cell lines was established. The epithelial HL cells were infected with *C. pneumoniae* and at 67 h post infection, representing a late stage of the replication cycle of *C. pneumoniae,* uninfected THP-1 monocytes were added to the wells in a ratio of 1:1. After 5 h incubation, THP-1 cells were collected and separated from HL cells by applying the biotinylated HLA-A2 antibody and streptavidin coated magnetic beads. The collected THP-1 cells were lysed, DNA extracted and the genome copy numbers of *C. pneumoniae* in THP-1 cells were determined. Fig. 2 presents a typical signal window obtained with the assay. There was a significant ($p = .001$) difference in *C. pneumoniae* genome copy numbers in THP-1 cells between the control and infection samples. In samples collected from wells with infected HL cells the average genome copy number was 28,809. In control samples collected from cultures with uninfected HL cells, minor nonspecific amplification yielded a in apparent GE numbers of 13.4, more than 2200 times smaller than in infection samples (S/B = 2209). While 15–20% day-to-day variation in *C. pneumoniae* genome copy numbers was observed in THP-1 lysates, the variation within biological and technical replicates of each experiments was only 5–10%. Typical Z' values of 0.6 were achieved, which can be considered excellent for an assay involving three biological variables, i.e. two human and one bacterial cell population.

4.2. MAPK inhibitors suppress the transfer of C. pneumoniae from the HL epithelial cells to THP-1 cells

To find suitable positive controls for the assay, the MAPK inhibitors SP600125, SB203580 and FR180204 were studied on their potential effect on the transfer of *C. pneumoniae* between the cells in the coculture model. Applying the final concentration of 10 μM, the MAPK inhibitors were added to the culture simultaneously with THP-1 cells. After 5 h, THP-1 cells were collected and separated from HL cells. Based on *C. pneumoniae* genome copy numbers determined from THP-1 cells (Fig. 3), all MAPK inhibitors had a statistically significant inhibitory effect on the transfer of the bacteria. The JNK inhibitor SP600125 decreased the transfer by 69.0% ($p = .00001$), the p38 inhibitor SB203580, by 54.1% ($p = .0002$) and the ERK inhibitor FR180204, by 36.8% ($p = .009$), respectively.

4.3. Impact of MAPK inhibitors on C. pneumoniae EB production in epithelial cells

For step by step analysis of the events occurring within the epithelium to monocyte transition of *C. pneumoniae* and elucidation of the processes affected by the MAPK inhibitors, we next conducted a set of experiments on EB quantities, release and internalization. First, we determined the impact of 5 h exposure of the HL cell infection (starting at 67 h p.i.) to the MAPK inhibitors on EB quantities inside HL cells as well as those released in the culture supernatants. Quantity of EBs produced in the end of replication cycle inherently affects the infection transfer and changes in EB quantities are thus a possible explanation for the pharmacological effects targeting infection transfer. The 5 h incubation time was selected as comparable for the time allowing infection transfer during the coculture experiments. There was no statistically significant effect ($p = .097$) by any of the MAPK inhibitors on *C. pneumoniae* EB quantities inside HL cells. Regarding the quantities of EBs released from HL cells to culture supernatant, the P38 inhibitor SB203580 and ERK inhibitor FR180204 had no effect on the release of the EBs but JNK inhibitor SP600125 increased the quantities of *C. pneumoniae* EBs in the supernatants ($p = .004$) (Fig. 4).

4.4. Impact of MAPK inhibitors on the infectivity of C. pneumoniae EBs

Besides potential effect on EB quantities and release, compounds affecting the epithelial-monocytic transition may target the released EBs in the extracellular space prior their internalization to monocytes. Even though targeting bacterial processes is not likely for the mammalian MAPK inhibitors, this could occur via undescribed off-target

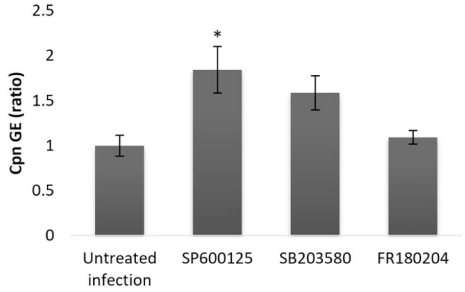


Fig. 4. Impact of MAPK inhibitors on the release of the bacteria from epithelial cells. HL cells were infected and treated for 5 h with MAPK inhibitors SP600125, SB203580 and FR180204 at the late state of the replication cycle. Released EBs were collected and *C. pneumoniae* GE copy numbers were determined by qPCR. Data are normalized on the infection control and shown as mean ± SEM of *C. pneumoniae* genome copy numbers. Statistical significance is determined by One-way ANOVA and Dunnett's post hoc test and presented as marks of P values: < 0.05: *; < 0.01: **; < 0.001: ***. $n = 6$ independent biological replicates.

effects and was thus included in the study. As expected, none of the MAPK inhibitors had an effect ($p = .82$) on the infectivity of *C. pneumoniae* EBs after 2 h exposure (data not shown).

4.5. Impact of MAPK inhibitors on the internalization of C. pneumoniae into THP-1 monocytes

Finally, the impact of the MAPK inhibitors on the internalization of *C. pneumoniae* EBs into THP-1 cells was determined by administering them to the cultures simultaneously with the bacterial inoculum and determining *C. pneumoniae* genome copy numbers inside the monocytes after 2 h incubation. As shown in Fig. 5, the JNK inhibitor SP600125 and the p38 inhibitor SB203580 decreased the *C. pneumoniae* internalization statistically significantly, by 40.0% ($p = .026$) and 37.9% ($p = .035$), respectively. The ERK inhibitor FR180204 treatment yielded 27.5% decrease in internalization, yet it does not reach the statistical significance. The effect of MAPK inhibitors on EB internalization seems to have cell type specific characteristics as comparative experiments of EB internalization into HL cells showed no difference between the MAKP inhibitor treated and not treated samples (data not shown).

5. Discussion

The propensity for systemic dissemination of *C. pneumoniae* ties this bacterium to various chronic inflammatory diseases. The transfer from the initial bacterial reservoir, the lung epithelium to local phagocytes and peripheral blood mononuclear cells initiates the dissemination. In addition, the infection in phagocytes spontaneously confers to an antibiotic-refractory form, significantly hindering the eradication of the infection. It is thus important to understand the details of epithelial-phagocyte transition of *C. pneumoniae*. By blocking it, the burden of persistent chlamydial infections and their inflammatory consequences could be reduced. Establishing the coculture assay makes it possible to evaluate future antichlamydial candidates also for their capability to block or suppress the transfer of *C. pneumoniae* between the cell populations. Furthermore, the optimization of THP-1 cell collection protocol from the coculture allowed the recovery of viable, *C. pneumoniae* infected cells, which makes it possible to follow the fate of the infection further in these cells.

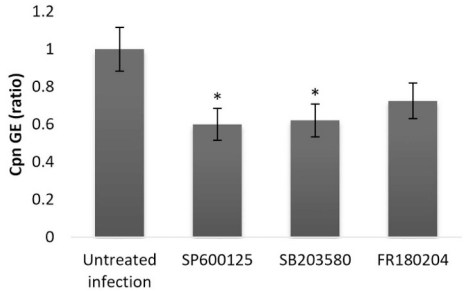


Fig. 5. MAPK inhibitors suppress the internalization of *C. pneumoniae* into THP-1 cells. THP-1 cells were incubated for 2 h with *C. pneumoniae* and MAPK inhibitors to examine the internalization of *C. pneumoniae* into THP-1 cells. The bacterial genome copy numbers were determined with qPCR. Data are normalized on the infection control and shown as mean ± SEM of *C. pneumoniae* genome numbers. Statistical significance is determined by One-way ANOVA and Dunnett's post hoc test and presented as marks of P values: < 0.05: *; < 0.01: **; < 0.001: ***. $N = 5$ independent biological replicates.

Separation of the epithelial and monocyte cell populations is an essential step for reliably quantifying the infection transfer. Detecting *C. pneumoniae* from the monocytes requires bacterial quantities reaching levels that are several orders of magnitude above the PCR detection limit. This was achieved by allowing the productive infection in the epithelial cells to proceed for 67 h prior to adding the monocytes. An alternative means for reaching high *C. pneumoniae* genome copy numbers in the monocytes is to allow longer transfer times by increasing the duration of the coculture. This approach is, however, limited by the tendency of the THP-1 monocytes to adhere on the infected HL cells. Due to this phenomenon, the coculture time was limited to 5 h, as this time frame resulted in an efficient transfer of the infection but maintained the two mammalian cell populations relatively easy to separate from each other. The sensitivity of the *C. pneumoniae* -specific PCR detection made the robust quantification of the chlamydial loads in the monocytes possible shortly after bacterial internalization.

Mitogen-activated protein kinases (MAPK) are the key signaling molecules in the transduction on the various extracellular stimuli and they regulate various physiological processes such as transcription and cell survival (Saba-El-Leil, 2016). MAPKs are activated by signals such as cytokines, environmental stress and inflammation and they are divided into different groups: extracellular signal-related kinases (ERK), Jun amino-terminal kinases (JNK) and p38 proteins, and they all can be activated by different signaling molecules depending on the stimuli. MAPKs are widely expressed, but the roles of each cascade is highly cell type dependent (Yasuda, 2015).

Activation of MAP kinase pathways is also a well-established consequence of *C. pneumoniae* infection (Krüll et al., 2004). Within minutes after contact with *C. pneumoniae* EBs, endothelial cells increase the phosphorylation of ERK1/2, p38 and JNK (Krüll et al., 2005). In macrophages, activation of MAPKs is involved with pathological changes caused by the infection, such as foam cell formation (Cheng et al., 2014) and induction of adhesion of monocytes to endothelium (Kaul and Wenman, 2001). In addition, *C. pneumoniae* induces immune response in peripheral blood mononuclear cells (PBMC) by activating p38 and p44/42 MAPK pathways (Rupp et al., 2004). It has been reported that p38 inhibitors SB203580 and SB202190 as well as JNK inhibitors SP600125 and TCSJNK60 inhibit *C. pneumoniae* inclusion counts in HL cells when they are present throughout the 72 h infection (Hanski and Vuorela, 2016). This demonstrates that besides mediating the *C. pneumoniae* induced pathologies in the host, MAPK pathways have a role in chlamydial replication and maintenance of active growth.

Due to the well-known involvement of MAPKs in *Chlamydia* – host interactions, three different MAPK inhibitors (JNK inhibitor SP600125, p38 inhibitor SB203580 and ERK inhibitor FR180204) were studied for their impact on epithelial-monocytic transition of *C. pneumoniae*. Our results demonstrate that all three studied compounds significantly suppressed the *C. pneumoniae* genome copy numbers in THP-1 cells after coculture and can thus be applied as positive controls for the assay (Fig. 3).

Compounds that block or suppress the transfer of *C. pneumoniae* between the cells can target several distinct points in chlamydial

replication cycle. They can block the exit of the EBs from epithelial cells to extracellular space, inhibit the infectivity of released bacteria or block the internalization of the EBs into the monocytes. To evaluate the impact of MAPK inhibitors on these processes, a series of experiments were carried out. Based on these data, the quantities of *C. pneumoniae* EBs inside HL cells was not affected by the 5 h exposure of the cultures to the MAPK inhibitors at late stage of infection. The differentiation of RBs back to EBs and their release from the host cells are asynchronous (Di Pietro et al., 2019). Thus the late stage of the bacterial replication cycle is characterized by a continuous RB to EB differentiation but MAPK inhibitors obviously have no role in this process.

Regarding the quantities of *C. pneumoniae* EBs released to extracellular space, p38 inhibitor SB203580 and ERK inhibitor FR180204 had no effect. In contrast, the JNK inhibitor SP600125 increased the levels of EBs in the culture supernatants (Fig. 4). In general, the EB quantities in supernatants were 20-fold lower than in cell lysates, which provides a plausible explanation why the significant increase in EB release did not translate into significantly lower EB quantities observed in the cell lysates.

Based on current knowledge, the exit of *Chlamydia* spp. bacteria from their host cells can occur by two different mechanisms: host cell lysis and extrusion (Hybiske and Stephens, 2007). In lysis, the inclusion vacuole is first erupted following the host cell plasma membrane eruption and release of the EBs to extracellular space. Lysis is mediated by cysteine proteases (Chin et al., 2012) and it has been demonstrated for several chlamydial species (Hybiske and Stephens, 2007; Rockey et al., 1996) but in fact, not experimentally demonstrated for *C. pneumoniae* in published literature.

In contrast, exit by extrusion has been experimentally demonstrated also for *C. pneumoniae* (Zuck et al., 2016). It represents a process in which the whole inclusion buds out of the host cell, generating membrane-bound vacuole packed with bacterial cells and leaving the host cell intact (Hybiske and Stephens, 2007).

To allow the pathogen to orchestrate the cellular events necessary for extrusion, chlamydial effector proteins secreted through type III secretion system (T3SS) recruit actin to polymerize and form a coat to facilitate the extrusion (Chin et al., 2012). Also, MAPKs have a role in actin polymerization (Coombes and Mahony, 2002) which forms a potential link between the JNK inhibitor and chlamydial exit. Yet laying beyond the scope of the current study, the role of JNK in *C. pneumoniae* exit from epithelial cells warrants further investigation.

The recent findings on chlamydial exit by extrusion has shed light also on the extracellular survival and the entry mechanisms of these obligate intracellular pathogens to phagocytic cells. The extrusion vacuole protects the bacteria from immune system effector molecules and the extrusion-bound chlamydial cells can survive in the extracellular space much longer than free EBs (Zuck et al., 2017). It has also been demonstrated that the whole extruded inclusion can be phagocytized by macrophages, facilitating a secondary infection and migration inside the body.

In the coculture model, unsheltered EBs or extrusion vacuoles containing EBs are released from the epithelial cells to extracellular space and may be affected by the studied compounds. Methods for isolating the extrusions from the extracellular space have been previously described (Zuck et al., 2017) and can be applied to study the impact of compounds of interest on their stability or infectivity. However, to determine the EB amount accurately and set a defined infection multiplicity for the internalization assay, we used EBs from a prediluted stock, instead of extracted extrusions. These experiments revealed no impact of any of the studied MAPK inhibitors on the infectivity of EBs.

Within the step-by-step evaluation of the epithelium – monocyte transition, the internalization of EBs into THP-1 cells was suppressed by the JNK inhibitor SP600125 and p38 inhibitor SB203580 (Fig. 5). These findings represent previously undescribed activities of MAPK signaling pathways on chlamydial replication cycle yet they are not surprising owing to well established connection of MAPK signaling with actin polymerization. Relying on endocytosis for entering mammalian cells, actin dynamics is essential for chlamydial entry (Coombes and Mahony, 2002). Blocking another MAPK, MEK1/2, which operates upstream from ERK, has decreased the *C. pneumoniae* internalization into Hep2 epithelial cells (Mölleken et al., 2013).

Internalization of *C. pneumoniae* EBs into the host cells involves several different proteins from the bacterium and the host (Elwell et al., 2016). Initiated by the low-affinity interaction of EB adhesion protein, outer membrane complex protein B (OmcB) with heparin sulfate proteoglycans (HSPGs) (Moelleken and Hegemann, 2008), the endocytosis is mediated by the interaction of chlamydial surface proteins with cellular receptors. The receptor type differs between the cell line and chlamydial strain and various receptors can be involved in attachment of bacteria (Elwell et al., 2016). Cell surface receptors e.g. mannose 6-phosphate (Puolakkainen et al., 2005), epidermal growth factor receptor (EGFR) (Mölleken et al., 2013), insulin-like growth factor 2 receptor (Puolakkainen et al., 2005), estrogen receptor complex (Krüll et al., 2005) and apolipoprotein E4 (Gérard et al., 2008) have been related to binding of *C. pneumoniae* to host cells. EGFR remains, however, the only cellular receptor whose direct contact with *C. pneumoniae* adhesins has been demonstrated (Mölleken et al., 2013).

In phagocytes, innate immune sensors Toll like receptors (TLR) 2, 3 and 4, and Nod-like receptors recognize *C. pneumoniae* and trigger proinflammatory reactions (Abdul-Sater et al., 2010). TLRs trigger a MYD88 mediated MAPK pathway phosphorylation, leading to activation of JNK, p38 and ERK kinases in these cells (Krüll et al., 2004). Despite the well-established role of TLRs in promoting the inflammatory responses, it still remains unknown which receptors mediates the internalization of *Chlamydia* into immune cells (Lausen et al., 2019).

As described above, macrophages are able, in addition to free EBs, to internalize the whole extrusion vacuole. The extrusions are taken up by phagocytic cells by actin dependent phagocytosis. Owing to their surface-exposed phosphatidylserine (PS), extrusions resemble extracellular apoptotic bodies which has been suggested to facilitate their recognition and internalization by phagocytes. The monocytic THP-1 cells are capable of phagocytosis (Schwende et al., 1996; Tsuchiya et al., 1980), and the bacterial internalization into them in the coculture model may involve both unsheltered and extrusion-packed EBs. This may also explain the greater suppression of epithelium – monocyte transition by the MAPK inhibitors than what is expected by the step-by-step analysis of individual phases of the process. This feature also demonstrates the added value of the coculture assay compared to analyses of separate steps. Allowing the direct interactions of the bacterium and its host cell populations brings in an additional layer of complexity and contributes to the biological relevance of the results.

6. Conclusions

We report here a new method for studying the epithelial monocytic transition of *C. pneumoniae*, an obligate intracellular pathogen. *C. pneumoniae* transfer spontaneously into monocytes from epithelial cells also in vitro and the bacterial transition can be reproducibly quantified by qPCR.

This coculture platform is useful for studying the mechanisms of different phases of epithelium-monocyte transition as well as evaluating new treatment options for *C. pneumoniae* infections.

MAPK inhibitors can be applied as positive control in these coculture studies as they are blocking the transfer of *C. pneumoniae* possible by inhibiting the internalization of the bacteria. Our findings concerning the ability of JNK inhibitors to increase the exit of EBs from THP-1 cells is interesting and may be related to a different outcome of activation of MAPKs between the cell types but needs further investigation.

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Declaration of Competing Interest

None.

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