Modelling *Chlamydia* infection of the cardiovascular system and the gastrointestinal tract

Ph.D. Thesis

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1. Introduction

1.1. *Chlamydiaceae*

Chlamydiaceae family comprises *Chlamydia* genus with eleven species of which two are primarily human pathogens: *C. trachomatis* and *C. pneumoniae*. The other species infect animals. Chlamydiae are Gram-negative obligate intracellular bacteria. The developmental cycle of these pathogens is unique in which the organism is present in two main forms, the elementary body (EB) and the reticulate body (RB). There is a third form called atypical, aberrant or persistent body. The chlamydial developmental cycle is regulated at the transcriptional level. There are three main stages of chlamydial gene expression, including early, mid and late gene expression. In the persistent phase of the chlamydial developmental cycle altered gene expression patterns were observed.

1.1.1. *Chlamydia pneumoniae*

Chlamydia pneumoniae (*Cpn*) is a common respiratory pathogen for humans; it causes pharyngitis, sinusitis, acute or chronic bronchitis, exacerbations of chronic bronchitis and it is a significant cause of community-acquired pneumonia. Chronic-persistent infections and reinfections are frequent.

A wide spectrum of extrapulmonary diseases has been linked to *Cpn*. Numerous studies have demonstrated an association between *Cpn* infection and atherosclerosis.

1.2. Atherosclerosis

Atherosclerosis is one of the most frequent causes of death and morbidity in the world. Atherosclerosis is a chronic disease with chronic inflammation, endothelial dysfunction and lipid accumulation in the vasculature. There are several well-known atherosclerosis risk factors. The central pathogenic event that promotes atherosclerotic lesion formation is a subendothelial retention and modification of low density lipoprotein (LDL) particles and subsequent accumulation of LDL-derived lipids in the intima. High plasma LDL cholesterol concentrations, especially oxidized LDL contributes to the formation of the atherosclerotic lesion. The endothelial injury leads to platelet aggregation and release of platelet factors which trigger the proliferation of smooth muscle cells in the arterial intima.

The classical risk factors of atherosclerosis are responsible for up to 90% of cases of atherosclerosis-related morbidity. Other risk factors like infection may contribute to the disease development. Several infectious agents: viruses, bacteria, and parasites have been associated with the increased risk of atherosclerosis.

Evidence for the role of *Cpn* in the pathogenesis of atherosclerosis has been provided by seroepidemiological studies, direct detection of bacterial components in atherosclerotic lesions by histology, electron microscopy, PCR, occasional isolation of viable bacteria from atherosclerotic plaques, *in vitro* experiments and *in vivo* animal studies.

In *in vitro* experiments *Cpn* infection of the cells that are constituents of the atherosclerotic plaques was successful, e.g. *Cpn* infected smooth muscle cells and vascular endothelial cells, and stimulated the secretion of pro-inflammatory cytokines and the expression of leukocyte adhesion molecules by these cells.

In vivo studies showed an atherosclerotic lesion exacerbation following *Cpn* inoculation of hyperlipidaemic model animals of atherosclerosis.

1.3. Mouse models of atherosclerosis

There are several animal models for investigation of the pathogenesis of atherosclerosis. Animal models play an important role in experiments aiming to clarify the mechanisms involved in atherosclerosis development and in the current search for new therapeutics beyond

the lipid-lowering drugs. Normal mice do not develop atherosclerosis and it requires long-term feeding of a high-fat diet to induce atherogenesis. However, there are well-established genetically modified inbred mouse lines that allow the investigation of atherosclerosis development in mouse models. The most frequently used mouse strains are ApoE-deficient (ApoE$^{-/-}$), LDL receptor-deficient (LDLR$^{-/-}$), human apoB100 transgenic and LDLR$^{-/-}$/ApoE$^{-/-}$ mice or ApoE-Leiden transgenic mice. In ApoE$^{-/-}$ mice, atherosclerosis develops spontaneously. However, the lipid profile in these mice is distinct from that seen in most humans with atherosclerosis, i.e. apolipoprotein (apo) B48-containing LDL plasma level is high instead of apoB100 containing LDL level as in the case of humans with hypercholesterolemia. The mouse strain ApoB100only/LDLR$^{-/-}$ carries an *apoB* gene with a mutation preventing the expression of apoB48, the truncated form of apoB, similarly to humans where no apoB editing takes place in the liver. LDLR deficiency prevents the uptake of apoB100 containing LDL in tissues resulting in high plasma levels of apoB100-containing, cholesterol-rich LDL. The creating authors of this mouse strain described these mice as an authentic model of human familial hypercholesterolemia.

1.4. Effect of *Cpn* infection in animal models of atherosclerosis

In the first animal studies, *Cpn* infection accelerated atherosclerosis lesion development in rabbits on a normal diet. Most frequently mice with genetic modification resulting in alterations in lipid metabolism were used to investigate the association of *Cpn* infection with atherogenesis.

In mice, intranasal *Cpn* infection causes lower respiratory tract disease similar to that seen in humans with *Cpn* infection. The infection is followed by dissemination of bacteria and the chlamydial DNA can be detected in the circulation of mice. Most frequently, acceleration of atherosclerosis was studied after repeated *Cpn* infections which simulated chronic *Cpn* infection of humans in association with diet-induced hyperlipidaemia in C57BL/6J, in LDLR$^{-/-}$, in ApoE$^{-/-}$, in LDLR$^{-/-}$ApoE$^{-/-}$ mice or ApoE-Leiden transgenic mice and mostly found exacerbation of atherosclerotic lesions.

To investigate the immune mechanism of the effect of *Cpn* infection on atherosclerosis, different knockout mouse lines: TNF-α p55 receptor , IL-17A, TLR2, TLR4, MyD88 and inducible nitric oxide synthase (iNOS) knockout mice were used.

1.5. *Chlamydia trachomatis*

Chlamydia trachomatis (*C. trachomatis*) is the most common sexually transmitted bacterial pathogen and the causative agent of blinding trachoma. There are two different biovars of *C. trachomatis:* oculogenital (serovars A-K) and lymphogranuloma venereum (L serovars). In case of sexually transmitted serovars, primary infection occurs in the cervix or urethra. In men the *Chlamydia* infection is identified as the causative agent in 50% of non-gonorrhoeal urethritis cases. In women the infection results in inflammation of the cervix which frequently becomes ascending spreading to the uterus and the fallopian tubes. *C. trachomatis* genital infections are very frequently asymptomatic in men and women too, asymptomatic infection produces a large reservoir of unrecognized, infected individuals who are capable of transmitting the infection to their sexual partners. The acute infections and infections without symptoms and unrecognized and untreated infections lead to chronic infections which are associated with severe sequelae. Complications in man include epididymitis, prostatitis that can negatively affect fertility, and reactive arthritis and Reiter's syndrome. Severe complication in women is the pelvic inflammatory disease (PID) during which the uterus, the ovaries and the fallopian tubes can be inflamed. Tubal factor infertility, ectopic pregnancy and chronic pelvic pain can be the most severe consequences of PID.

1.6. Chlamydiae in the gastrointestinal tract

Animal pathogenic *Chlamydia* species were isolated from various animals, for example, ruminants, porcine and avian species, and they were detected in different organs as well as in faeces. In most animals, chlamydiae persist in the gastrointestinal (GI) tract and are transmitted via the faecal-oral route. Oral infection with *C. muridarum* has resulted in a long term, persistent infection of the mouse GI tract. The GI tract seems to be an ideal site, in which *Chlamydia* can persist because of a down regulated host immune response.

In humans the nature of persistent or recurrent *C. trachomatis* infection of the female genital tract has not been identified. It has been suggested that genital tract infection can be accompanied by infection of the GI tract either via oral infection, or by autoinoculation from genital secretions or during sexual activity. Several studies investigated the possibility of genital *Chlamydia* reinfection from persistent infection in the GI tract in humans.

1.7. Growth of *C. trachomatis* in different cell lines

Earlier with the purpose of diagnosis of chlamydial infections and in *in vitro* experiments to characterize the replication of chlamydiae mostly epithelial cells as the prime targets of chlamydial infection are being used. For culture of *C. trachomatis* D-L serovars most commonly non-polarized cervix-derived HeLa cells are utilized. Cell lines other than the conventional epithelial cells have been used to investigate the pathomechanism of *Chlamydia* species caused infections at extra genital sites.

In earlier studies, the growth of animal *Chlamydia* species was investigated in cell lines of a variety of origin. In intestinal mucosal epithelial Caco-2 cells, inclusions have indicated a substantial growth of porcine *C. pecorum* and *C. suis*; therefore, Caco-2 cells can be regarded as suitable hosts for animal *Chlamydia*. If *C. trachomatis* can persist in the human GI tract it is of relevance to look into the growth characteristics of *C. trachomatis* in Caco-2 cells. Caco-2 cell line could be a suitable model for investigation of *Chlamydia* infection in the human GI tract.

1.8. Defensins

Defensins are an important family of natural antimicrobial peptides acting against bacteria, fungi and enveloped viruses. Human beta (β)-defensin (hBD)-2 is present in the skin and the respiratory and gastrointestinal tracts and is expressed mainly by epithelial cells in response to inflammatory stimuli and infection, but monocytes, macrophages and dendritic cells are also capable of hBD-2 production. In the colonic mucosa, defensins, and among them hBD-2 represent the important effectors of the innate host defences not only by their microbicidal activity but by providing a link to the adaptive immune system as they attract immature dendritic cells and memory T cells. The intestinal mucosal epithelial cells have important role as physiological barrier for pathogens and function as a part of the innate immune system. In *in vitro* experiments *C. trachomatis* infection activates the production of hBD-2 and it was detected by RT-PCR in cervico-vaginal lavage of women with chlamydiasis. Secretion of hBD-2 by Caco-2 cells in response to *Chlamydia* infection would suggest the stimulation of the innate immune response in the GI tract.

2. Aims

The present study was designed to address the following aims:
Aim 1: to investigate the potentially atherogenic effect of multiple *Cpn* infections in ApoB100only/LDLR$^{-/-}$ mice.

Aim 2: to investigate the growth characteristics of *C. trachomatis* in intestinal epithelial cells as potential target cell during human infection and the infection induced defensin production.

3. Materials and methods

3.1. Cell lines

Caco-2, HeLa 229, HEp-2 and McCoy cells were maintained in minimal essential medium (MEM) with Earle's salts completed with 10% FBS, 2 mmol/L L-glutamine, 1x MEM vitamins, 1x nonessential amino acids, 25 µg/mL gentamicin, and 0.5 µg/mL fungizone. The cell lines were purchased from ATCC.

3.2. Bacterial strains

Chlamydia pneumoniae, CWL029 strain and *Chlamydia trachomatis* serovar D, UW-3/CX strain from ATCC was used. *Cpn* was propagated in HEp-2 cells and *C. trachomatis* was propagated in McCoy cell line. Infective chlamydiae were quantitated by indirect immunofluorescent method. The number of infectious bacteria in the *Chlamydia* stock used for inoculation of mice or cell lines was expressed as inclusion forming units (IFU)/ml.

3.3. Mouse strains

Female ApoB100only/LDL$^{-/-}$ (B6;129S-*Ldlr^{tm1Her}Apob^{tm2Sgy}*/J) mice at 8-9 or 14-15 weeks of age and ApoE deficient (ApoE$^{-/-}$, B6.129P2-*Apoe^{tm1Unc}*/J) mice at 14-15 weeks of age were involved in our studies. The mice were kept on normal rodent regular chow or high-fat/high-cholesterol diet for 12 weeks after the first infection. All experiments were approved by the Animal Welfare Committee of the University of Szeged and conform to the Directive 2010/63/EU of the European Parliament (Permit Number: III./2187/2015.).

3.4. Infection of mice with *Cpn*

Anaesthetized mice were infected with *Cpn* intranasally three times with 2-week intervals. One week after each infection and at the end of the experiment at week 12, plasma samples were harvested. Additional groups of mice were infected and sacrificed at 1, 4 and 9 weeks, respectively, after the first infection.

3.5. Mouse tissue preparation and quantification of atherosclerosis

Twelve weeks after the first infection with *Cpn* the mice were sacrificed. After formalin-perfusion, the upper part of the heart and the descending aorta were dissected. The aorta sinus was sectioned for histological staining and descending aortas were longitudinally opened in situ, and morphometric analysis was performed to evaluate the extent of atherosclerosis. Aortic sinus samples were also collected for RNA extraction.

3.6. RNA extraction from mouse aorta and quantitative real time-PCR for *Chlamydia* 16SrRNA expression

Total RNA isolated from pooled aorta samples with RNA extraction kit was DNase treated, reverse-transcribed to cDNA which was used in qRT-PCR to detect *Cpn* 16SrRNA expression.

3.7. ELISA for detection of *Cpn*-specific antibodies

Plasma samples of mice collected one week after each infection and at the end of the experiment were tested in duplicates for *Cpn*-specific IgG, IgM and IgA by an in-house developed ELISA test as described earlier.

3.8 Serum lipoprotein analysis

Levels of total cholesterol, triglycerides, high-density lipoprotein (HDL) and LDL cholesterol were determined in plasma samples of mice through a service from the Department of Laboratory Medicine, University of Szeged, Hungary.

3.9. Infection of different cell lines with *C. trachomatis*

The cells were grown in 6-well plates (for RNA extraction and defensin detection), 96 well (for DNA quantitation), or 24-well culture plates with (for immunofluorescence staining) or without (for *Chlamydia* yield) 13 mm glass coverslips. The cells were infected with *Chlamydia* at a multiplicity of infection (MOI) of 1 or 5. For DNA quantitation, the infected cells were incubated in cycloheximide containing medium or cycloheximide-free medium. The culture plates were incubated for different time periods in CO_2 incubator at 37°C.

Assessment of the yield of infective *C. trachomatis* D in Caco-2 or HeLa cells was done by inoculation of the infected cell lysates onto McCoy cells in plates with glass coverslips. After 48 h, the cells were fixed in glass coverslips.

3.10. Immunofluorescent staining of infected cells for visualization of inclusions and quantitation of recoverable *C. trachomatis*

The staining of *Chlamydia*-infected cells on coverslips was performed via using anti-cLPS and FITC-labelled anti-mouse IgG as secondary antibody. The coverslips were treated with Evan's Blue at room temperature for 1 min. The chlamydial inclusions were photographed and counted under a fluorescent microscope.

3.11. Transmission electron microscopy (TEM)

The cells were cultured in plates and infected with *Chlamydia* at an MOI of 1. After 24 and 48 h, the infected cells were washed fixed glutaraldehyde and 1% osmium tetroxide. Samples were embedded in Embed 812 (EMS, USA) using a routine TEM embedding protocol. After staining with uranyl acetate and lead citrate, the sections were examined with a Philips CM10 electron microscope. Images were acquired by using Olympus Soft Imagine Viewer program.

3.12. *C. trachomatis* DNA quantitation

For the quantitative assessment of chlamydial replication, we followed a direct DNA quantitation method. After 0, 24, 48 and 72 h, the *Chlamydia*-infected cells were washed and MilliQ water was added. Two freeze-thaw cycles were applied to free the DNA from the cells. The lysates were used directly as templates for quantitative PCR (qPCR) with SsoFast™ EvaGreen ®Supermix. *Pyk* primers were used for the detection of *C. trachomatis* D genomes.

3.13. RNA extraction from infected cell lines

For the analysis of gene expression, total RNA was extracted from the infected cells in plates at 2, 24, 48 or 72 h after infection with GenElute Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's protocol. Concentration of RNA was determined by spectrophotometry. The extracted RNA was treated with DNase. cDNA was synthetized from DNase-treated RNA with qScript cDNA Supermix synthesis kit.

3.14. *C. trachomatis* gene expression analysis by quantitative RT-PCR

By using cDNA as template, qRT-PCR was performed with PerfeCTa SYBR Green Supermix in CFX96 Real Time C1000 Thermal Cycler. Expression of *16SrRNA* gene was used as the internal standard for counting the relative expression of *Chlamydia* genes. The relative expression of *euo*, *groEL*, *ftsK*, *omcB*, *ompA* and *pyk* transcripts of *C. trachomatis* was evaluated. The melt curve analysis was performed to prove the specificity of the amplification.

The relative gene expression levels (RQ) were given by calculating the delta-deltaCt ($\Delta\Delta$Ct) value.

3.15. ELISA for detection of hBD-2

The supernatant of the infected cells were harvested at different time points post infection. For the detection of hBD-2 production, the supernatants of the cells were tested by using hBD-2 ELISA kit. Supernatant of cells treated with *Escherichia coli* Nissle 1917 strain was used as positive control.

3.16. Statistical analysis

Data are expressed as mean ± SD. Independent-samples t-test was used with SigmaPlot for Windows Version 11.0 software. A *P* value of less than 0.05 was considered to indicate statistically significant difference.

4. Results

4.1. Infection of ApoB100only/LDLR$^{-/-}$ mice with *Cpn*

Mice infected 3 times with *Cpn* showed mild symptoms of a disease especially during the first week after the first infection. At the time of the first infection half of the mice were given a high-fat/high-cholesterol diet the other half was kept on normal rodent chow. Non-infected mice were kept under similar conditions. All infected mice produced *Cpn*-specific antibodies which were not seen in the non-infected mice. Normal and high-fat/high-cholesterol diet-fed mice produced similar level of *Cpn*-specific IgG antibodies (OD 0.36-0.4 at dilution 1:100).

4.2. Repeated *Cpn* infection aggravates atherosclerosis development in the aorta sinus and in the descending aorta of ApoB100only/LDLR$^{-/-}$ mice kept on normal diet

After three repeated *Cpn* infections in mice fed with an atherogenic diet very similar pathology was observed in the aorta sinus and in those remained uninfected. In the aorta sections of repeatedly *Cpn*-infected mice on normal diet we observed a significant increase in the length of the plaque-covered perimeter of the lumen and in the size of the plaque-occupied area in the aorta lumen compared with that in the non-infected counterpart in normal diet-fed group. On the luminal surface of the longitudinally opened descending aorta, the atherosclerosis-affected areas were measured significantly larger in the descending aorta of the infected animals than in the non-infected ones. In the high-fat/high-cholesterol diet-fed animals the infection did not enhance the lesion formation in this part of the aorta.

4.3 Viable *Cpn* was detectable in the aorta of ApoB100only/LDLR$^{-/-}$ mice

The persistence of the bacterium in the aorta was tested one and four weeks after single infection and at 9 and 12-week time points after three infections by RT-PCR. One and four weeks after single inoculation the expression of chlamydial 16SrRNA was detectable. Five weeks after the third infection one aorta sample of 2 showed metabolically active *Cpn* in the aorta and the relative expression level was similar to that after single infection at 4 weeks. At later time point no chlamydial gene expression was detected.

4.4 Infection of ApoB100only/LDLR$^{-/-}$ and ApoE$^{-/-}$ mice with *Cpn* induces similar kinetics of antibody production

With our infection protocol in ApoB100only/LDLR$^{-/-}$ and in ApoE$^{-/-}$ mice kept on a non-atherogenic diet, the humoral immune response induced by the infections was tested. The titre

of *Cpn*-specific IgG and the level of IgM and IgA antibodies did not differ significantly in the two mouse strains.

4.5 The extent of atherosclerosis is similarly increased in the aorta of *Cpn*-infected and normal diet-fed ApoB100only/LDLR$^{-/-}$ and ApoE$^{-/-}$ mice

At the age of 24-25 weeks on normal chow diet in ApoE$^{-/-}$ mice more numerous advanced lesions were found than in ApoB100only/LDLR$^{-/-}$ mice. In *Cpn*-infected ApoB100only/LDLR$^{-/-}$ mice larger advanced plaques and in infected ApoE$^{-/-}$ mice more plaques with necrotic core and accumulated cholesterol crystals appeared. The lesions in the descending aorta also were larger in ApoE$^{-/-}$ mice than in ApoB100only/LDLR$^{-/-}$ mice. When the effect of *Cpn* infection was analysed, significant enhancement in the measured values was found. The plaque-covered perimeter of the lumen in the aorta sinus sections, the plaque-occupied lumen area, and the plaque size in the descending aorta increased 2.08 fold, 1.7 fold, 2.5 fold, respectively in ApoB100only/LDLR$^{-/-}$, and 2.04 fold, 1.32 fold, 2.56 fold, respectively in ApoE$^{-/-}$ mice.

4.6. Plasma lipid levels in ApoB100only/LDLR$^{-/-}$ and ApoE$^{-/-}$ mice

At each tested time point irrespective of the infection status ApoE$^{-/-}$ mice carried a higher level of total cholesterol and LDL than the ApoB100only/LDLR$^{-/-}$ mice, and the triglyceride and HDL concentration was elevated in ApoB100only/LDLR$^{-/-}$ mice compared to that in ApoE$^{-/-}$ mice. Only the third *Cpn* inoculation resulted in a significant elevation in triglyceride level in ApoE$^{-/-}$ mice. In ApoB100only/LDLR$^{-/-}$ mice no infection-related change in triglyceride level was obvious. Infection-related significant increase in LDL level was associated with the first infection in ApoB100only/LDLR$^{-/-}$ mice only. No infection-associated change in HDL level was detected.

4.7. Detection of *Chlamydia* growth by immunofluorescence staining in Caco-2 and conventional host cells

After indirect immunofluorescence staining, inclusions of *C. trachomatis* were seen in Caco-2 and HeLa cells. The detection of the inclusions suggested ongoing replication in both cell types; however, the morphology of the inclusions demonstrated different growth kinetics in the different cell types. At 48 h in Caco-2 cells the inclusions grew large but in the permissive HeLa cells expanding fluorescing areas had shown the final stage of replication cycle by this time.

4.8. Transmission electron microscopy of *Chlamydia* infected Caco-2 and conventional host cells

With TEM, the developmental stage of *C. trachomatis* D inclusions at 48 h post-infection was rather heterogeneous in Caco-2 cells; however, in HeLa cells fully developed inclusions with numerous EBs were seen.

4.9. *Chlamydia* genome accumulation in Caco-2 and conventional host cells

We followed the accumulation of *Chlamydia* genomes during a 3-day culture period in Caco-2 and in HeLa cells with a novel DNA quantitation method. Fold increase in the amount of *pyk* gene of *C. trachomatis* was calculated in comparison with the amount detected by qPCR at 0 h of infection. *C. trachomatis* genomes in Caco-2 cells propagated to similar amount by 72 h as in HeLa cells. The kinetics of replication followed a slower course in Caco-2 cells. In cycloheximide-free conditions, the yield was lower in both cells types. In Caco-2 cells, the effect of cycloheximide did not cause any major change in the course of the replication opposite to that in HeLa cells.

4.10. Production of infective *Chlamydia* progeny in Caco-2 and conventional host cells

The recoverable viable *C. trachomatis* bacteria were quantitated by inoculation of the sonicated infected cells in their media onto McCoy cells. Infective chlamydiae were recoverable showing that a full replication cycle takes place in Caco-2 cell line. The growth of *C. trachomatis* was somewhat delayed in Caco-2 cells, but at 72 h post-infection, similar amount of *C. trachomatis* was cultured from Caco-2 cells to that from HeLa cells .

4.11. Transcript patterns for selected *Chlamydia* genes during infection of Caco-2 and conventional host cells

The kinetics of the expression of selected *C. trachomatis* genes were investigated in Caco-2 cells compared to that in HeLa cells during a 3-day period. The relative gene expression levels normalized to 16S rRNA expression were calculated. At 24 h post-infection the relative expression of the early cluster gene *euo* was at much lower level in HeLa cells than in Caco-2 cells, and it increased at later time points, when the replication was already at lower rate. After 24 h, *euo* expression level moved to the opposite direction in Caco-2 cells in parallel with the continued replication. The relative expression of *pyk, ompA, ftsK* and *omcB* genes of *C. trachomatis* D followed similar trends in both examined cell lines, but the relative expression persisted at higher levels in Caco-2 cells than in HeLa cells. The highest level of *ompA* gene expression was observed at 24 h and persisted at high level for a longer time in Caco-2 than in HeLa cells. The highest level of *ftsK* gene expression was observed at 24 h after infection, at the time of frequent cytokinesis, and it remained at high level in Caco-2 cells at later time points, too. The expression of the late gene *omcB* peaked at 48 h post-infection with again a higher level in Caco-2 cells than in HeLa cells. Constantly high relative amount of *groEL* transcripts was observed in Caco-2 cells, including the earliest time point tested at 24 h. Further analysis of the ratios of gene expression values seen in Caco-2 cells versus that in HeLa cells demonstrate the delayed and prolonged replication cycle in Caco-2 cells with higher *euo* expression at early time point (24 h) and decreased *euo* expression at late time point (72 h), with higher cytokinesis related (*ftsK*) and membrane protein gene (*ompA, omcB*) expressions at later time points in Caco-2 cells. An outstanding *groEL* gene transcription, especially at 24 h post-infection in Caco-2 cells, is detectable.

4.12. HBD-2 inducing capability of *C. trachomatis* in Caco-2 cells

It was investigated whether *C. trachomatis* was able to induce the production of hBD-2 antimicrobial peptide by Caco-2 cells. HBD-2 protein was detectable in high levels in the supernatant of *E. coli* Nissle-stimulated control Caco-2 cultures with a peak at 48 h post-treatment. *C. trachomatis* D-infected cells showed similar time course of hBD-2 release, however at a significantly lower level than in the case of stimulation with Nissle.

5. Discussion

5.1. Aim 1:

ApoE$^{-/-}$ mice are widely used as animal models of atherosclerosis; however, the lipoprotein metabolism of this mouse strain is different from that in humans with hypercholesterolemia. ApoE$^{-/-}$ mice accumulate in their plasma large quantities of ApoB48 containing lipoprotein of the very low-density lipoprotein (VLDL) class while humans with atherosclerosis almost always have high level of cholesterol-rich LDL containing ApoB100. Many publications investigating the relation of *Cpn* with atherosclerosis have used this model to disclose the nature of the association between infection with this pathogen and its role in the initiation and/or acceleration of atherosclerosis. It has been suggested that *Cpn* infection

exacerbate atherosclerosis in conjunction with hyperlipidaemia however ApoE deficiency might influence the immune response to this pathogen and provides increased resistance to vascular infection. We aimed at examining the influence of repeated *Cpn* infection on the formation of atherosclerotic plaques in ApoB100only/LDLR$^{-/-}$ mouse strain another model for lipoprotein abnormalities which can be regarded as the most faithful model of human familial hypercholesterolemia.

ApoB100only/LDLR$^{-/-}$ mouse strain was created by genetic modification, so that majority of their plasma cholesterol is in the LDL class with ApoB100 and develops atherosclerosis on low-fat, chow diet. First, we wanted to establish that ApoB100only/LDLR$^{-/-}$ mice can serve as a model for investigating the role of *Cpn* in atherosclerosis. Therefore groups of mice were fed with a normal or high-fat/high-cholesterol diet and were repeatedly infected with *Cpn* or left uninfected, and development of atherosclerosis was followed. As our experiments showed, in ApoB100only/LDLR$^{-/-}$ mice which were fed with normal diet repeated three *Cpn* infections resulted in an enhanced atherosclerosis development in the aortic sinus and the descending aorta. This effect was not observed in high-fat/high-cholesterol diet-fed infected animals. It seems that the effect of hyperlipidaemia in ApoB100only/LDLR$^{-/-}$ mice can be aggravated by *Cpn* infection but *Cpn* does not exacerbate atherosclerosis further in the presence of high-fat/high-cholesterol diet. Nevertheless, the bacterium influenced the course of atherosclerosis development indicating that ApoB100only/LDLR$^{-/-}$ mice are suitable for further research. Our results are consistent with findings of earlier studies describing atherosclerosis-accelerating effect of *Cpn* infection in ApoE$^{-/-}$ mice eating regular chow diet. However, atherosclerosis was also exacerbated in ApoE$^{-/-}$ mice kept on high fat diet by single or 3 repeated *Cpn* infections.

We hypothesized that based on the genetic difference between ApoB100only/LDLR$^{-/-}$ and ApoE$^{-/-}$ mice, features that characterize *Cpn* infection or atherosclerosis may also differ, and therefore we compared the effects of the bacterium in these mouse strains. In our infection model the successful infection was demonstrated by detecting *Cpn*-specific IgG, IgA and IgM antibodies in the mice. When IgG antibody level was compared in ApoB100only/LDLR$^{-/-}$ and ApoE$^{-/-}$ mice, no significant difference was observed. Earlier studies reported that ApoE$^{-/-}$ mice produced more *Cpn*-specific antibodies than wild-type mice which was attributed to ApoE deficiency. Our results do not support this suggestion considering that in ApoB100only/LDLR$^{-/-}$ mice repeated *Cpn* infection led to high level of antibody production without the contribution of ApoE deficiency.

We were able to detect metabolically active *Cpn* in the aorta samples for nine weeks after the first infection. Previous studies demonstrated the presence of *Cpn* in the aorta of repeatedly infected ApoE$^{-/-}$ mice by isolating the bacterium early two weeks after infection. Furthermore, bacterial DNA was amplified by PCR at later time points. As reviewed in the literature several lines of evidence point to the ability of *Cpn* to establish persistent infection *in vivo*. Our results provide additional information about persisting chlamydiae as 16SrRNA gene transcripts in the aorta suggest metabolically active bacteria not only persisting DNA or antigen late after repeated infections. Long-term presence of viable chlamydia in the aorta tissues of some infected mice might contribute to the atherogenic effect of the infection.

In female ApoE$^{-/-}$ mice we have disclosed more advanced atherosclerosis than in ApoB100only/LDLR$^{-/-}$ mice at the same age at the end of the 12-week observation period. Nevertheless, in ApoE$^{-/-}$ mice, similarly to ApoB100only/LDLR$^{-/-}$ mice, the infection resulted in an enhanced lesion formation without the need of feeding the mice with high-fat diet.

It has been described that *Cpn* can cause hepatic fatty acid imbalance dysregulation of lipid metabolic genes in the liver, altered macrophage cholesterol homeostasis however; most of the studies in mice did not detect major changes in plasma lipid profile after *Cpn* infection. Increase in triglyceride concentration after repeated infection of ApoE$^{-/-}$ mice similarly to our

results has been noted previously. Early increase of LDL level in ApoB100only/LDLR$^{-/-}$ mice after primary infection may be due to the primary infection caused inflammation. Our findings are concordant with results of other studies suggesting a significant association between chronic infection with *Cpn* and increased risk of coronary heart disease in patients with familial hypercholesterolemia.

5.2. Aim 2:

In this study, we investigated the morphological and molecular features of *Chlamydia trachomatis* infection in Caco-2 human intestinal cells. We selected this cell line for our investigation because it shows the characteristics of enterocytes, and in recent publications, the hypothesis of chlamydial persistence in the GI tract and the infected GI tract behaving as a source of genital tract reinfection has been proposed. Furthermore, *C. trachomatis* has been implicated in GI tract pathologies. As hBD-2 is produced by epithelial cells in the GI tract in response to infection as part of the innate defence, we tested the defensin inducing capability of *C. trachomatis* in Caco-2 cells.

C. trachomatis D inclusions in Caco-2 cells visualized by immunofluorescence staining and TEM suggested similar but prolonged replication cycle of this *Chlamydia* strain compared to that in HeLa cells. The slower accumulation of chlamydiae was also detectable by finding lower amount of genomes and infective chlamydiae at 48 h post-infection in Caco-2 cells. However, by 72 h post–infection, the values had reflected similar level of chlamydial reproduction in the two cell lines. These results allow us to conclude that for this human genital *Chlamydia* strain, Caco-2 cells provide a growth-conducive environment. In case the growth medium of the host cells is supplemented with cycloheximide, *Chlamydia* replication cycle occurs in optimal conditions, where the host cell protein synthesis has less influence on the bacterial growth. When cycloheximide was omitted from the medium, chlamydial genome accumulation reached a lower level and suffered early decline in HeLa cells, but this phenomenon was not as pronounced in Caco-2 cells. Gene expression analyses were done in the absence of cycloheximide, when the natural cellular environment would prevail.

It has been described that chlamydial developmental cycle is regulated at transcriptional level. The change in the transcription profile has been demonstrated in *in vitro* models, where different stimuli (IFN-γ, penicillin) induced a persistent phase of chlamydiae. Certain cell types proved to be non-permissive for normal *Chlamydia* growth, and the transcription pattern suggested a persistent form of infection in these cells. Since the growth kinetics in Caco-2 cells was found different from that in the optimal *in vitro* host cell line, we aimed to examine the expression of *C. trachomatis* genes in Caco-2 cells and compared it to that in HeLa cells. The expression level of selected chlamydial genes representing early (*euo*), mid-cycle (*ompA, groEL, pyk*) and late stage (*omcB*) replication cycles, and cell divisions related *ftsK* gene were analysed and was normalized to the expression level of *16S rRNA* gene. As the *euo* gene products were described as suppressors of the late genes, decreased expression of this gene could explain the higher expression level of *omcB* gene encoding the membrane protein of EBs in Caco-2 cells and of the cytokinesis related *ftsK* gene, even at later time points during the infection. Based on *ftsK* transcript level, it seems that cytokinesis dropped earlier in HeLa cells than in Caco-2 cells. The declining expression of the ATP synthesis related *pyk* gene in HeLa cells after 48 h paralleled with the decrease in the amount of the chlamydial chromosome detected in this cell line. Longer high level of *pyk* expression coexisted with a more extended genome accumulation in Caco-2 cells. The *groEL* gene of *C. trachomatis*, a heat shock protein 60 (GroEL-1) encoding gene was highly upregulated throughout the observation period in Caco-2 cells. These results suggest that replication of *C. trachomatis* in Caco-2 cells evoked a certain stress response of the bacteria.

It has been suggested that in the male and female reproductive tracts, small antibacterial molecules may control inflammatory, innate and adaptive immune responses. HBD-2 has been suggested to contribute to host defence by recruiting neutrophil leukocytes and by attraction of immature dendritic cells and memory T cells to the site of microbial invasion. Expression of hBDs has been reported in women with *C. trachomatis* positive cervicovaginal samples, albeit at a significantly lower level than in non-infected controls. In our experiments with Caco-2 cell line, *C. trachomatis* infection induced the production of hBD-2 at a moderate level compared to the effect of a strong inducer *E. coli* Nissle strain. In a murine model of *C. muridarum* infection, long carriage and a lack of inflammatory response in the large intestine was seen; however, the infection of the genital tract was cleared after a short period and following inflammation at the infection site. The low level of hBD-2 production by the *Chlamydia* infected Caco-2 cell line might be a sign of suppressed innate immune response, and potentially, a subsequently suppressed adaptive response as well.

In conclusion, Caco-2 cell line representing the epithelial cell lining in the large intestine seems to be a sufficiently permissive host cell for *C. trachomatis*, which primarily infects the genital tract, thus allowing these bacteria to survive in the intestine without provoking an inflammatory response. These results seem to support the theory that the gastrointestinal tract could serve as a site of extra genital survival of chlamydiae, and this site could potentially serve as a source of reinfection in the genital tract, especially in women.

6. Summary

Chlamydiae are ubiquitous obligate intracellular bacteria causing infection in the human eyes, urogenital and respiratory tract.

Chlamydia pneumoniae a common respiratory pathogen has been implicated in the pathomechanism of several chronic non-infectious diseases. Association between atherosclerosis and *Cpn* infection has been investigated most thoroughly. Hyperlipidaemia model animals have been used to elucidate the role of *Cpn* infection in atherosclerosis. The aims of this study were to investigate the proatherogenic effect of multiple *Cpn* infections in ApoB100only/LDLR$^{-/-}$ mice which based on the lipid profile can be regarded as the most suitable mouse model of human hypercholesterolemia, and to compare the lesion development to that in a major atherosclerosis model ApoE$^{-/-}$ mice. Aorta samples of ApoB100only/LDLR$^{-/-}$ mice infected three times with *Cpn* were subjected to morphometric analyses. RT-PCR was used for searching for viable *Cpn* in the ascending aorta. Morphometric evaluation disclosed that *Cpn* infections exacerbated atherosclerosis development in the aortic root and descending aorta of the mice fed with normal diet but further increase in response to *Cpn* infection was not observed in the mice kept on high fat/high cholesterol diet. Chlamydial 16SrRNA expression showed the presence of viable *Cpn* in the aorta of infected animals. A similar rate of acceleration of atherosclerosis was observed when the infection protocol was applied in ApoB100only/LDLR$^{-/-}$ and in ApoE$^{-/-}$ mice. In conclusion, similarly to ApoE$^{-/-}$ mice, ApoB100only/LDLR$^{-/-}$ mice with more human relevant serum lipoprotein composition, develop increased atherosclerosis after *Cpn* infections, thus this mouse strain can be used as a model of infection-related atherosclerosis enhancement and provide further evidence for the proatherogenic influence of *Cpn* in mice.

C. trachomatis causes infections of the eyes, urogenital and respiratory tracts. It is the most frequently identified sexually transmitted pathogen. Asymptomatic, repeat and chronic infections with *C. trachomatis* are common in the urogenital tract potentially causing severe reproductive pathology. Animal models of infection and epidemiological studies suggested the gastrointestinal tract as a reservoir of chlamydiae and as a source of repeat urogenital infections. Thus, we investigated the growth characteristics of *C. trachomatis* urogenital serovar D in human intestinal epithelial Caco-2 cells and the infection induced defensin production.

Immunofluorescence staining and transmission electron microscopy showed the presence of chlamydial inclusions in the cells. Chlamydial DNA and viable *C. trachomatis* were recovered from Caco-2 cells in similar quantity compared to that detected in the usual *in vitro* host cell of this bacterium. The kinetics of expression of selected *C. trachomatis* genes in Caco-2 cells indicated prolonged replication with persisting high expression level of late genes and of heat shock protein gene *groEL*. Replication of *C. trachomatis* induced moderate level of β-defensin-2 production by Caco-2 cells, which might contribute to avoidance of immune recognition in the intestine. According to our results, Caco-2 cells support *C. trachomatis* replication, suggesting that the gastrointestinal tract is a site of residence for these bacteria.

The following of our results are considered novel

- In ApoB100only/LDLR$^{-/-}$ mouse strain as another mouse model for lipoprotein abnormalities, repeated *Cpn* infection has exacerbating effect on the formation of atherosclerotic plaques.
- The enhanced atherosclerosis development in *Cpn* infected ApoB100only/LDLR$^{-/-}$ mice was observed in normal diet-fed animals but not on high fat/high cholesterol diet.
- Metabolically active *Cpn* was detected in the aorta samples with RT-PCR method.
- Caco-2 cells provide a growth-conducive environment for the human genital *C. trachomatis* strain.
- Growth characteristics of *C. trachomatis* D in Caco-2 cells suggested prolonged replication cycle of this *Chlamydia* strain compared to that in HeLa conventional host cells.
- The *groEL* gene of *C. trachomatis*, a heat shock protein 60 (GroEL-1) encoding gene was highly upregulated throughout the observation period in Caco-2 cells which might be a sign of stressful growth environment.
- In Caco-2 cell line *C. trachomatis* infection induced the production of hBD-2 at a moderate level which might be an immune evasion mechanism for the bacteria.

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