Evaluation of protective and pathological immune response against chlamydial infection and re-infection in mice

Ph.D. Thesis

Tímea Mosolygó M.D.

Department of Medical Microbiology and Immunobiology
Faculty of Medicine
University of Szeged

Szeged
2014
1. Introduction

Chlamydiae cause infections that are common throughout the world. Although antibiotics are effective in treating chlamydial infections, the lack of obvious symptoms has the consequence that many infections remain untreated potentially leading to complications characterised by inflammatory pathologies. The immunity to these pathogens is not effective, chlamydial infections display high rates of recurrence and have long-term consequences causing a serious public health problem. Immunisation is a desirable approach for the prevention and control of chlamydial disease, but despite considerable efforts there is currently no commercially available vaccine against chlamydiae. Understanding the basis of immunity to chlamydiae will provide an indispensable knowledge for the design of a vaccine against diseases caused by chlamydiae. The present study was designated to improve our current understanding of the pathological immune response to infection and re-infection with chlamydiae, and to provide information about the host immune responses against the different chlamydial plasmid proteins.

1.1. Chlamydiaceae

Chlamydiaceae is a family of Gram negative, obligate intracellular bacteria responsible for a wide range of diseases with clinical and public health importance. Based on the 16S and 23S ribosomal gene sequences the Chlamydiaceae is divided into two genera: Chlamydyphila and Chlamydia. The genus Chlamydyphila is composed of six species: Chlamydyphila pneumoniae; Chlamydyphila psittaci; Chlamydyphila pecorum; Chlamydyphila abortus; Chlamydyphila caviae and Chlamydyphila felis. Three species belong in the Chlamydia genus: Chlamydia trachomatis; Chlamydia muridarum and Chlamydia suis.

Chlamydiae undergo a unique biphasic developmental cycle, during which the bacterium is found in two forms, the extracellular form, called elementary body, which is metabolically inert and infectious, and the intracellular form, the reticulate body, which is metabolically active, replicative but non-infectious. The duration of the developmental cycle is 48-72 hours depending on the chlamydia species. Under stress chlamydiae can enter to a dormant, non-infectious but viable state named persistence. Experimental and clinical data provide evidence for reactivation of persistent chlamydiae in vivo indicating that chlamydial recurrences were more likely due to the reactivations of persistent infections than to re-infections.

1.2. Chlamydyphila pneumoniae

C. pneumoniae is a common and important respiratory tract pathogen; it causes about 10% of community-acquired pneumonia in adults and 5% of bronchitis and sinusitis. Most of the studies regarding C. pneumoniae focus on its role as a cause of persistent infections in chronic diseases. Number of studies supported the role of C. pneumoniae infections in chronic human diseases such as chronic bronchitis, asthma, atherosclerosis, Alzheimer’s disease, reactive arthritis and lung cancer. The evidence of the participation of C. pneumoniae infections in chronic inflammatory disease comes from the fact that the primary infection elicits some protective immunity against re-infection, but provides no protection against inflammatory changes which may lead to irreversible tissue damage. Studies published recently focus on the role of C. pneumoniae infection in allergic airway inflammation. In asthma patients the airway hyperresponsiveness is mainly characterised by the infiltration of neutrophils, which is also typical for respiratory tract infection with C. pneumoniae. The mechanism, by which C. pneumoniae induces the influx of neutrophil granulocytes to the lung tissue and elicits allergic immune response in persistent infections or re-infections remains poorly understood.
1.3. Th17 cells and IL-17 cytokine family

During the last decade, researchers investigating chronic inflammatory diseases focused their attention on a newly identified subset of CD4+ lymphocytes named Th17 cells, and the members of the IL-17 cytokine family became prominent subject for investigation. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. IL-23 promotes the development and expansion of activated CD4+ T cells that produce IL-17A upon antigen-specific stimulation.

The IL-17 cytokine family consists of six members designated as IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F. Th17 cells are the main source of IL-17A and IL-17F, but CD8+ cells, γδ T cells and natural killer (NK) cells can also produce these cytokines. Both IL-17A and IL-17F induce expression of genes encoding pro-inflammatory cytokines and chemokines such as keratinocyte chemoattractant (KC), lipopolysaccharide (LPS)-induced C-X-C chemokine (LIX), and macrophage inflammatory protein-2 (MIP-2) in fibroblast, endothelial and epithelial cells. IL-17A plays a crucial role in innate immune response against pathogens by promoting granulopoiesis and recruiting neutrophils to the sites of inflammation. Numerous studies suggest pathological role of IL-17A in allergic responses and in promotion of disease progression in atopic dermatitis and asthma. The functions of IL-17B, IL-17C and IL-17D are largely elusive. IL17E, also known as IL-25 appears to be involved in Th2 immune response and in host defence against nematodes by inducing immunoglobulin E production and eosinophilia. It seems that the regulation of IL-17E is as critical as that of IL-17A in the development of allergic diseases, although IL-17E induces eosinophilia, while IL-17A recruits neutrophils to the inflammatory site.

1.4. Chlamydia trachomatis and Chlamydia muridarum

Different serovariants of *Chlamydia trachomatis* cause a wide range of diseases, including blinding trachoma (serovars A-C), urogenital tract infections leading to urethritis, cervicitis and proctitis (serovars D-K), and systemic lymphogranuloma venereum disease (serovars L1-L3). In women, infection with *Chlamydia trachomatis* causes pelvic inflammatory disease, and persistent or repeated infections lead to chronic inflammation characterised by scarring of the fallopian tubes and ovaries. Chronic pelvic inflammation has long term consequences such as ectopic pregnancy, infertility or chronic pelvic pain.

*C. muridarum* is a murine pathogen that was originally isolated from the lungs of mice. Infection of mice with *C. muridarum* provides a useful model of *C. trachomatis* infection in humans. Elucidation of the immunobiology of *C. muridarum* infection of mice helps to guide the interpretation of immunological findings in studies of human *C. trachomatis* infection. Identification of the antigens, which elicit immune responses and protection against the pathogen, is an important priority in *C. trachomatis* research.

1.5. The chlamydial cryptic plasmid

Several *Chlamydia* species harbour a highly conserved plasmid with an approximate size of 7.5 kb. The plasmid of *Chlamydia trachomatis* encodes both noncoding RNAs and eight open reading frames (ORFs), while the plasmid of *C. muridarum* (pMoPn) possesses seven ORFs, designated TCA01-07. All plasmid-borne genes are transcribed and translated. *TCA04* and *TCA05* encode the proteins pGP3 and pGP4, respectively. Studies with plasmid-deficient *C. trachomatis* and *C. muridarum* have implicated the chlamydial plasmid as a key virulence factor *in vivo*, because infection with plasmid-deficient organisms are either asymptomatic or exhibits significantly reduced pathology. In addition, the plasmid functions as a transcriptional regulator of various chromosomal genes, which may play important roles in
chlamydial pathogenicity, and pGP4 has been demonstrated to be the protein that positively regulates the transcription of plasmid-encoded pGP3 and multiple chromosomal genes during C. trachomatis infection and pGP5 is the negative regulator of the same set of chromosomal genes. pGP3, the most intensively studied plasmid protein, has been found to be secreted into the host cell cytosol during chlamydial infections. The human antibody recognition of pGP3 is dependent on the native conformation of the protein: pGP3 trimerisation is required for the recognition of pGP3 by human antibodies.

Increased knowledge of the role of the cryptic plasmid in biology and pathogenesis will enhance our understanding of chlamydial growth and development, and will be important for guiding the design of a vaccine for the prevention of chlamydial disease.

2. Aims

The present study was designed to address the following aims:

Aim 1. To determine the roles of IL-17 cytokines in pathological immune response to C. pneumoniae and C. muridarum infection and re-infection in BALB/c mice.

Aim 2. To describe the transcriptional pattern of pMoPn genes in C. muridarum-infected BALB/c and C57BL/6N mice.

Aim 3. To compare the host immune response against pGP3 and pGP4 after C. muridarum infection and re-infection in BALB/c and C57BL/6N mice.

3. Materials and methods

3.1. Propagation of chlamydial strains and culturing of chlamydiae from the lungs of mice

C. pneumoniae CWL029 and C. muridarum strain Nigg were propagated on HEp-2 and McCoy cells, respectively, and purified, as described earlier. The titre of the infectious EBs was determined by indirect immunofluorescence assay. The number of chlamydial inclusions was counted under a UV microscope, and the titre was expressed in inclusion forming unit (IFU)/ml. A mock preparation was prepared from uninfected cells processed in the same way as the infected cells. Lung homogenates from each mouse were centrifuged (10 min, 400g), serial dilutions of the supernatants were inoculated onto cell monolayers, and the titre of C. pneumoniae or C. muridarum was determined.

3.2. Experimental animals

Specific pathogen-free 6-8-week-old female BALB/c and C57BL/6N mice were obtained from INNOVO Kft. (Budapest, Hungary). The mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged, and were provided with food and water ad libitum. All experiments fully complied with the University of Szeged Guidelines for the Use of Laboratory Animals.

3.3. Model of infection and re-infection with C. pneumoniae

Before infection, BALB/c mice were mildly sedated with an intraperitoneal (i.p.) injection of 200 µl of sodium pentobarbital; they were then infected intranasally with 5×10^5 IFU of C. pneumoniae in 25 µl of sucrose-phosphate-glutamic acid (SPG) buffer. Control mice were also mildly sedated but left uninfected. After inoculation, mice were anaesthetized
and sacrificed on days 1, 2, 4, 7, 14 or 28, 7 animals at each time point. Sera were taken by cardiac puncture. The lungs were removed and homogenized. Half of the homogenized lungs was processed for quantitative reverse transcription polymerase chain reaction (RT qPCR), while the other half was suspended in 1 ml of SPG buffer for the detection of viable C. pneumoniae, and for cytokine and chemokine measurements. Spleens were destroyed with a cell strainer, and the spleen cells were kept at -80 °C until use.

In a separate experiment, BALB/c mice were infected intranasally 3 times with viable C. pneumoniae (5×10^5 IFU) or with heat-inactivated C. pneumoniae at 4-week intervals. Another group of mice were initially infected with viable C. pneumoniae and then infected twice with heat-inactivated C. pneumoniae. Groups of 7 mice were sacrificed at 2 or 4 weeks after each infection and the lungs were processed as mentioned above.

**3.4. Model of infection and re-infection with C. muridarum**

BALB/c mice were infected intranasally with 1×10^3 IFU of C. muridarum in SPG buffer; half of the mice were re-infected 28 days after the first infection. Seven mice at each time point were anaesthetized and sacrificed on each of days 1, 7, 14, 28, 29, 35, 42 and 56 after the first infection. Sera were taken by cardiac puncture. The lungs were removed and homogenized. One half of the homogenized lungs was processed for RT qPCR, while the other half was suspended in 1 ml of SPG for the detection of viable C. muridarum. Lungs of 3 mice from each group were removed, frozen and kept at -80 °C for immunofluorescent staining.

In a separate experiment BALB/c and C57BL/6N mice were infected intranasally with C. muridarum 3 times. Groups of 7 mice were sacrificed at 2 weeks after each infection. Sera were taken by cardiac puncture. Spleens were destroyed with a cell strainer for testing of cell-mediated immunity.

**3.5. mRNA extraction from the lungs of mice and RT qPCR**

Total RNA was extracted from the lung suspensions by using the TRI Reagent (Sigma). During purification, all samples were treated with DNase 1, Amplification Grade (Sigma). First-strand cDNA was synthesized by using 2 μg of total RNA with Superscript III (Invitrogen Carlsbad, CA, USA) and 20 pmol of random hexamer primers in 20 μl of reaction buffer. The cDNA product was diluted 1/30, and the qPCR was conducted with the diluted cDNA, primers (10 pmol/μl) and SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) with a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). All primers were synthesized by Integrated DNA Technologies Inc. (Montreal, Quebec, Canada). Dissociation curves were recorded after each run to ensure primer specificity. Cycle threshold (C_t) values were determined by automated threshold with Bio-Rad CFX Manager Software version 1.6. The lowest cycle number at which the various transcripts were detectable, referred to as C_t, was compared with that of β-actin in the case of different IL-17 transcripts, the difference being referred to as ΔC_t. The lowest cycle number at which the transcripts of pMoPn were detectable was compared with that of 16s rRNA of C. muridarum. The relative expression level was given as 2^{(ΔΔC_t)}, where ΔΔC_t = ΔC_t for the experimental sample minus ΔC_t for the control sample. Mice sacrificed 4 h after infection with C. pneumoniae or C. muridarum served as controls. We defined a threshold value, i.e. increases greater than 2-fold in the amount of transcripts relative to control samples were considered significant. Each sample was assayed in triplicate, and each experiment was performed at least twice.
3.6. Cytokine and chemokine measurements
The supernatants of the lung homogenates were assayed for the concentrations of IL-17A, KC, LIX and MIP-2 with different Quantikine® mouse chemokine/cytokine kits (R&D Systems, Minneapolis, MN, USA), while the quantity of IL-17E was determined with Ready-SET-Go! kit (eBioscience Inc., San Diego, CA, USA). The sensitivities of the IL-17A, KC, LIX, MIP-2 and IL-17E measurements were in the ranges 10.9–700 pg/ml, 15.6–1000 pg/ml, 15.6–1000 pg/ml, 7.8–500 pg/ml and 31.2–2000 pg/ml, respectively. The clarified supernatants were tested in duplicate in accordance with the manufacturer’s instructions.

3.7. ELISPOT assay
The spleen cells isolated from BALB/c mice 2 weeks after C. pneumoniae infection were depleted of CD4+ and CD8+ cells, respectively, by using micro-beads coated with the respective antibody [α-CD4 (L3T4) or α-CD8a (Ly-2), Miltenyi Biotec, Bergisch Gladbach, Germany] and applying the magnetic cell sorting system of Miltenyi Biotec. The outcome of the procedure was controlled by flow cytometry. The numbers of left-over CD4+ and CD8+ cells after the depletion process were <1%. Spleen cells were re-stimulated in vitro with heat-inactivated C. pneumoniae EBs at a multiplicity of infection of 0.2, or with an equivalent amount of mock preparation. To determine the number of IL-17A-producing cells, IL-17A ELISpot kit (R&D Systems) was used. Stimulated spleen cells (5×10^5) were distributed into each well in triplicate in accordance with the manufacturer’s instructions. The mean number of spots counted in triplicate wells under a dissecting microscope was used to calculate the number of spot-forming cells (SFCs) per 1 million spleen cells.

3.8. In vivo neutralization of IL-17A in mice and bronchoalveolar lavage fluid collection
Groups of 14 female BALB/c mice were treated i.p. with 100 µg/mouse of either anti-IL-17A (MAB421, R&D Systems) or an isotype control antibody (R&D Systems) 24 h before and 1 and 2 days after C. pneumoniae infection. The mice were sacrificed on day 1 or day 4 after infection and the lungs of 7 mice from each group were lavaged with 1 ml of phosphate-buffered saline (PBS). 50 µl of a 5×10^5 cells/ml cell suspension was placed into a chamber which was attached to cytocpin slides, and then centrifuged at 800 rpm for 3 min. The cells were examined morphologically and counted after staining with May-Grünwald-Giemsa solution. The lungs of 7 mice from each group were removed and the chlamydial burden was determined as mentioned above.

3.9. Lung histopathology
Four weeks after C. muridarum infection and re-infection BALB/c mice were sacrificed. The lungs were removed in toto and immersed in frozen tissue matrix, OCT (Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands). The lungs were cut into 5 µm sections, and the sections were stained with IL-17E antibody (Acris Antibodies GmbH, Herford, Germany) as primary antibody for 45 min at room temperature, followed by staining for 30 min with FITC-labelled anti-mouse IgG antibody (Sigma). Uninfected mice were used as controls.

3.10. Western blot
Purified pGP3, pGP4 and C. muridarum EBs were boiled for 5 min in 4x Dual Color Protein Loading Buffer (Fermentas GmbH, St. Leon-Rot, Germany), and 2 µg of proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA, Heidelberg, Germany). The membranes were blocked overnight at 4 °C. Membranes were probed with a pool of uninfected mice sera or sera obtained from C. muridarum-infected
BALB/c or C57BL/6N mice. After washings, the filter was incubated with HRP-conjugated anti-mouse IgG (Sigma), and the colour was developed with diaminobenzidine tetrahydrochloride (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) with hydrogen peroxide in 10 mM Tris, pH 7.5.

3.11. Identification of proteins by mass spectrometry

The gel slices containing the polypeptides of corresponding proteins which were recognized by the sera of C. muridarum-infected BALB/c mice in the Western blot were cut out from the gel. Protein bands were diced and washed with 25 mM NH₄HCO₃ in 50% (v/v) acetonitrile/water. Disulphide bridges were reduced with dithiothreitol, and free sulfhydryl groups were alkylated with iodoacetamide. Proteins were digested with modified porcine trypsin (Promega, Madison, WI, USA) for 4 h at 37 °C. Samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The raw LC-MS/MS data were converted into a Mascot generic file with Mascot Distiller software (v2.1.1.0). The resulting peak lists were searched by using the Mascot Daemon software (v2.2.2) against the NCBI non-redundant database without species restriction (NCBI nr 20080718, 6833826 sequences). Monoisotopic masses with a peptide mass tolerance of ±0.6 Da and a fragment mass tolerance of 1 Da were submitted. Carbamidomethylation of Cys was set as a fixed modification, and acetylation of protein N-termini, Met oxidation, and pyroglutamic acid formation from peptide N-terminal Gln residues were permitted as variable modifications. Acceptance criteria were at least 2 individual peptides with a minimum peptide score of 55 per protein.

3.12. Lymphocyte proliferation assay

Single-cell suspensions from 2 spleens of triple C. muridarum-infected or uninfected BALB/c and C57BL/6N mice were pooled and re-suspended in complete growth medium. The proliferative responses of 5×10⁵ spleen cells in 3 parallel wells to 2 μg/ml of pGP3 or pGP4, purified heat-inactivated C. muridarum EBs, or the similarly treated mock preparation were detected after incubation for 3 days. The proliferation was determined by MTT assay (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer’s instructions. Stimulation indices (SIs) were calculated by dividing the optical density measured for protein-, mock- or EB-stimulated spleen cells by the optical density measured for non-stimulated spleen cells.

3.13. Statistical analysis

Statistical analysis of the data was carried out with SigmaPlot for Windows Version 11.0 software, using the Wilcoxon–Mann–Whitney two-sample test. Differences were considered statistically significant at p<0.05.

4. Results

4.1. C. pneumoniae infection induces the expression of IL-17A and IL-17F mRNA in the lungs of BALB/c mice

The infectious bacterial titre was below the level of detectability at 24 h after infection, but it had increased by day 2. The peak titre of C. pneumoniae was observed on day 7, while on day 14 after infection the titre had decreased and no viable C. pneumoniae was detected on day 28. The expression of IL-17A mRNA was increased as early as on day 2, but the highest level of expression was detected on day 7 after infection. It then decreased continuously, but the expression level was still rather high relative to the control on day 28. The expression of
IL-17F mRNA was highest on day 4 and then decreased during the observation period. The expression of IL-17C, IL-17D and IL-17E mRNA did not change during the course of infection. The mRNA expression of IL-23, the main inducer of IL-17A production, was observed on the first day, and peaked on day 2. The kinetics of IL-17A protein production correlated with the mRNA expression.

4.2. CD4+ cells are the main source of IL-17A in C. pneumoniae-infected BALB/c mice

In vitro re-stimulation of spleen cells isolated from C. pneumoniae-infected mice with heat-inactivated C. pneumoniae significantly enhanced the number of IL-17A-producing cells as compared with spleen cells re-stimulated with mock preparation. The depletion of CD8+ cells did not result in a reduction in the number of IL-17A-producing cells, whereas the depletion of CD4+ cells resulted in a significant reduction in the number of SFCs in in vitro re-stimulated spleen cells isolated from C. pneumoniae-infected mice. These results indicate that the CD4+ cells are the main source of IL-17A during C. pneumoniae infection in BALB/c mice.

4.3. In vivo neutralization of IL-17A resulted in higher bacterial burden in the lungs of C. pneumoniae-infected BALB/c mice

The in vivo anti-IL-17A treatment led to a significantly reduced IL-17A content in the BAL fluid on day 4 as compared to that in the control mice. The numbers of neutrophil cells in the anti-IL-17A-treated group on days 1 and 4 were significantly lower than those for the control. Neutralization of IL-17A did not influence the numbers of macrophages and lymphocytes in the BAL fluid. The number of viable chlamydiae in the lung suspension of the anti-IL-17A-treated group on day 4 was significantly higher than the C. pneumoniae content of the lungs of control mice. The levels of KC and MIP-2 on day 4 were significantly lower than the chemokine levels in the lungs of the control mice.

4.4. C. pneumoniae re-infection triggers the production of IL-17A and IL-17E in BALB/c mice

After the first infection, the quantity of recoverable C. pneumoniae was similar to that in the previous experiment. After the second and third infections, C. pneumoniae was not detected in the lungs of the mice at the examined time points. The re-infection of the mice with C. pneumoniae induced the production of IL-17A and the expression pattern was similar to that measured after the first infection, but the expression levels were higher, in spite of the absence of viable chlamydiae in the lungs of mice. The expression of IL-17E mRNA was not increased after a single infection, but after the second and third infections its expression increased dramatically. The expression level of IL-17E mRNA 4 weeks after the third C. pneumoniae infection was still 400 times higher than that in the control lungs.

We observed a significant increase in the expression of IL-17A mRNA in the lungs of mice infected first with viable C. pneumoniae and then with heat-treated C. pneumoniae on the second and third occasions. However, the expression of IL-17E mRNA was not detectable in the lungs of these mice. We found that the infection and re-infection of mice with heat-inactivated C. pneumoniae did not influence the expression of IL-17A and IL-17E mRNA in the lungs of the mice.
4.5. *C. muridarum* infection and re-infection induce the expression of IL-17A and IL-17E mRNA in the lungs of BALB/c mice

The course of chlamydial burden in the lungs of mice was similar to that detected during *C. pneumoniae* infection: the number of *C. muridarum* increased by day 1, peaked on day 7, and then decreased by day 28 after infection. On day 29, one day after the re-infection the bacterial titre was increased but at later time points, viable *C. muridarum* was not detected in the lungs of the re-infected mice. The expression of IL-17A mRNA displayed similar kinetics as after infection and re-infection with *C. pneumoniae*, it was highest on day 7, then decreased continuously. The fold increase in IL-17A transcripts was higher after re-infection than it was after the primary infection. Unlike that of IL-17A mRNA, the expression of IL-17E mRNA did not demonstrate a parallel change with the level of bacterial burden in the lungs of the mice. The expression started to increase on day 7, and the highest level was detected on day 28 after the first infection. On day 29 (one day after re-infection), the expression of IL-17E mRNA decreased dramatically, but after that it increased again and was highest 28 days after re-infection, when the experiment was terminated. In the lungs of the infected and the re-infected mice the kinetics of IL-17A and IL-17E protein production was similar to that of the expression of IL-17A and IL-17E mRNA, respectively.

4.6. The epithelial cells of the lung are responsible for the production of IL-17E after *C. muridarum* infection and re-infection

Production of IL-17E was observed in the lungs of the infected and re-infected mice four weeks after infection. The IL-17E-positive cells were situated especially among the epithelial cells of the bronchi, and only a few positive cells were found in the interstitium of the lungs. No fluorescence was seen in the lung sections of the uninfected mice.

4.7. The pMoPn genes displayed divergent transcriptional pattern in BALB/c mice and in C57BL/6N mice

From the first day after the infection, the BALB/c mice displayed more clinical symptoms than did the C57BL/6N mice, as indicated by ruffled fur, passivity, a lack of appetite and weight loss. In BALB/c mice, the infectious *C. muridarum* titre was increased on day 1, peaked on day 7, and then decreased continuously. On day 28 after infection, the viable *C. muridarum* titre was $3 \times 10^1$ IFU/lung, but there was no detectable *C. muridarum* on day 56 after infection. In C57BL/6N mice, the peak titre of *C. muridarum* was also detected on day 7. However, by day 28 post-infection, all of the C57BL/6N mice were culture-negative, whereas all of the BALB/c mice remained culture-positive.

Increased levels of expression of different plasmid genes were observed in the BALB/c mouse lungs on day 7 after infection. The expression of *TCA01*, *TCA02*, *TCA03*, *TCA06* and *TCA07* was 3-5-fold higher on day 7 relative to that in the control sample, and increased further to 5-7-fold on day 14. Interestingly, the increases in the expression of *TCA04* and *TCA05* in the BALB/c mice were each 3-fold on both day 7 and day 14. In C57BL/6N mice, the expression of pMoPn genes was delayed. However, on day 14 the expression levels of the plasmid genes in C57BL/6N mice were higher than those in BALB/c mice. There was no plasmid-encoded gene expression on day 28 after *C. muridarum* infection in either mouse strain.
4.8. Infection and re-infection with *C. muridarum* induced the production of pGP3- or pGP4-specific antibodies in C57BL/6N mice but not in BALB/c mice

In C57BL/6N mice, a single infection with *C. muridarum* was sufficient to induce the production of pGP3-specific antibodies. No pGP4-specific antibody production was detected in mice infected once or twice, but this antibody appeared after the 3rd infection. Contrarily, the sera of *C. muridarum*-infected and re-infected BALB/c mice did not react with the pGP3 and the pGP4. The sera of uninfected mice did not contain pGP3-, pGP4- or *C. muridarum*-specific antibodies.

4.9. Sera of *C. muridarum*-infected BALB/c mice recognized only the trimeric form of pGP3

Although the sera of *C. muridarum*-infected and re-infected BALB/c mice did not react with the 28 kDa pGP3, but they reacted with an additional protein with 80-85 kDa. As it was reported earlier that the pGP3 plasmid protein exists in trimeric or dimeric form, the gel samples containing the band recognized by the infected mouse sera were subjected to further analysis. The result of the LC-MS/MS showed that the protein which was reacted with the sera of *C. muridarum*-infected BALB/c mice was the trimeric form of pGP3. The sera of *C. muridarum*-infected C57BL/6N mice did not show any reactivity with the trimeric form of pGP3.

4.10. Re-infection with *C. muridarum* induced the production of pGP3-specific cellular immune response in both mouse strains

Spleen cells isolated from triple *C. muridarum*-infected C57BL/6N mice reacted with pGP3 after *in vitro* re-stimulation. In addition, spleen cells of C57BL/6N mice also responded with proliferation to pGP4. The spleen cells of *C. muridarum*-infected BALB/c mice did not show reactivity to the recombinant pGP4 protein after *in vitro* re-stimulation, but they recognized and responded with proliferation to the pGP3 protein. Lymphocytes of both mouse strains showed proliferation after re-stimulation with heat-inactivated *C. muridarum* EBs.

5. Discussion

Members of the IL-17 cytokine family can aggravate the pathology of autoimmune and allergic diseases, but they have a beneficial role during infection caused by different pathogens. We have demonstrated here that *C. pneumoniae* induced the expression of IL-17A and IL-17F mRNA in BALB/c mice, whereas the expression of IL-17C, IL-17D and IL-17E mRNA did not change after a single infection with the pathogen. The expression of IL-23 mRNA, preceded the production of IL-17A.

The involvement of IL-17A in protective immunity against intracellular pathogens such as *C. pneumoniae* is rather controversial. In the event of mycobacterial infection, IL-17A exerts an impact on inflammation and the formation of granulomas, but it is not required for overall protection. IL-17A is important in protective immunity at an early stage of infection with *Listeria monocytogenes* in the liver. IL-17A promotes neutrophilic inflammation through the induction of KC, LIX and MIP-2 production by a variety of target cells. We found that the quantities of KC and MIP-2 in the anti-IL-17A antibody-treated mice were reduced 4 days after *C. pneumoniae* infection. The *in vivo* neutralization of IL-17A resulted in an increased pathogen burden at an early stage of infection suggesting that the decreased release of KC and MIP-2 in response to anti-IL-17A treatment was associated with decreased lung neutrophil
These results showed that the epithelial cells, mast cells and other haematopoietic immune cells were potent contributors to the immune responses following infections by intracellular pathogens, such as L. monocytogenes, Mycobacterium tuberculosis and M. bovis. Moreover, other cell types, e.g. CD8+ T cells and NK cells have been demonstrated to be IL-17A-producing cells.

Immunopathological mechanisms of chlamydial infections have been widely studied by using animal models of repeated infections. Multiple episodes of re-infections with C. trachomatis and C. pneumoniae infection in BALB/c mice is the CD4+ cells. Although, most of the recent studies focused on IL-17A produced by CD4+ Th 17 cells, γδ T cells are potent contributors to the immune responses to the late stages of pulmonary C. muridarum infection of mice with C. pneumoniae, which belongs not in the Chlamydia genus – can also induce the production of IL-17A and IL-17E in BALB/c mice. The kinetics of IL-17A mRNA expression in our experiment was similar to that observed by Zhou et al. after a single infection with C. muridarum. Concordant with our earlier results regarded to the re-infection of mice with C. pneumoniae, the quantity of IL-17E increased four weeks after C. muridarum-infection and re-infection. It is noteworthy that the re-infection of mice with C. muridarum resulted in acutely decreased levels of expression and production of IL-17E. We speculate that the strong Th1 cytokine IFN-γ can inhibit the expression of IL-17E during the early stages of C. muridarum infection. It was reported earlier that the production of IL-17E by T cells, mast cells and other haematopoietic immune cells is not essential for the development of Th2-type eosinophilic airway inflammation, suggesting that the IL-17E produced by non-immune cells such as airway epithelial cells, is crucial for its development. We found that the epithelial cells of the lung are responsible for the production of IL-17E in the later stages of pulmonary C. muridarum infection.
The cryptic plasmid of chlamydiae is considered to be a virulence factor, because plasmid-free variants have been found to be less invasive and to cause pathologies of relatively low severity. Loss of the plasmid from *C. muridarum* impacts two virulence-associated phenotypes, infectivity and TLR2 activation, and also the ability of chlamydiae to accumulate glycogen.

Our experimental findings confirmed the results of Jiang et al. that the chlamydia burden is higher in BALB/c mice than in C57BL/6N mice. In C57BL/6N mice, the expression of the plasmid genes was not increased in the early phase of the infection, but by day 14 it was more pronounced than in the BALB/c strain. The expression levels of the plasmid genes in the BALB/c mice rather followed the kinetics of the pathogen burden. A further interesting finding was that the levels of expression of *TCA04* and *TCA05*, which encode pGP3 and pGP4, respectively, were uniformly elevated 3-fold in the BALB/c mice on day 7 and day 14 suggesting that these 2 genes are closely related, or interdependent. It was very recently reported by Song et al. that pGP4 is the gene that regulates the transcription of plasmid-encoded pGP3 and multiple chromosomal genes during *Chlamydia trachomatis* infection. Moreover, a sequence of 30 or more nucleotides in the pGP3 gene was required for the optimal expression of pGP4.

Our Western blot experiment showed that the sera of *C. muridarum*-infected C57BL/6N mice reacted with the monomeric form of pGP3 (28k Da). Moreover, the sera of the mice infected 3 times with *C. muridarum* reacted with the recombinant pGP4 protein. In contrast, the sera of multiply *C. muridarum*-infected BALB/c mice did not recognize the monomeric form of pGP3 in Western blot assays, but the sera did react with a protein band with higher molecular weight (80-85 kDa). Since the pGP3 plasmid protein has been reported to exist in trimeric form, the sample corresponding to the recognized 80-85 kDa protein was subjected to further analysis. The results of the LC-MS/MS analysis clearly demonstrated that the protein recognized by the sera of the *C. muridarum*-infected BALB/c mice was the trimeric form of pGP3. Li et al. demonstrated that the trimeric form of pGP3 is secreted into the host cell cytosol, and their results indicated that human antibodies recognized trimeric, but not monomeric pGP3, suggesting that pGP3 is presented to the human immune system as the trimer during *C. trachomatis* urogenital infection. However, others have found that seropositive human sera react with the monomeric form of pGP3 in the Western blot assay. Moreover, we detected strong monomeric pGP3-specific antibody production after a single inoculation with *C. muridarum* in C57BL/6N mice. Our results lead us to suppose that the genetic background of the host can determine whether the monomeric or the trimeric pGP3 is recognized.

The finding that the sera of triply *C. muridarum*-infected mice recognized the pGP4 protein suggests that re-infection leads to the increased pGP4-specific antibody production which was detected in our Western blot assay after the third infection. Moreover, an increasing pGP4-specific humoral immune response was observed not only in the pooled sera of mice, but in each individual mouse serum, suggesting that the processes of presentation and recognition of the pGP4 epitopes are similar in each C57BL/6N mouse.

Furthermore, we described for the first time that the infection of mice with *C. muridarum* can elicit a cellular immune response to plasmid proteins. We observed that the SIs of spleen cells collected from multiply *C. muridarum*-infected C57BL/6N mice were significantly higher after *in vitro* re-stimulation with pGP3 or pGP4 than the SIs of the lymphocytes of uninfected mice. The spleen cells of *C. muridarum*-infected BALB/c mice did not respond with proliferation to the recombinant pGP4 protein, but they were able to respond with
proliferation to the pGP3 antigen after *in vitro* re-stimulation for 3 days. Further experiments are needed to clarify the roles of pGP3 and pGP4 in the initiation of the cellular and humoral immune responses of the host in different mouse strains.

**The following of our results are considered novel**

- *C. pneumoniae* infection induces the expression of IL-17A and IL-17F mRNA in the lungs of BALB/c mice
- *C. pneumoniae* re-infection triggers the production of IL-17A and IL-17E in BALB/c mice
- *C. muridarum* infection and re-infection induce the expression of IL-17A and IL-17E mRNA in the lungs of BALB/c mice
- The epithelial cells of the lung are responsible for the production of IL-17E after *C. muridarum* infection and re-infection in BALB/c mice
- The pMoPn genes display divergent transcriptional pattern in BALB/c mice and in C57BL/6N mice
- Infection with *C. muridarum* induces the production of pGP3-specific antibodies in both mouse strains, but the recognition of pGP3 is dependent on the native conformation of the protein
- Re-infection with *C. muridarum* induces the pGP4-specific humoral and cellular immune responses in C57BL/6N mice but not in BALB/c mice
- Re-infection with *C. muridarum* induces the production of pGP3-specific cellular immune response in both mouse strains

**Summary**

Chlamydiae are obligate intracellular bacteria that cause infections which are common throughout the world. *Chlamydophila pneumoniae* is an important respiratory tract pathogen; it causes community-acquired pneumonia, bronchitis and sinusitis. Different serovariants of *Chlamydia trachomatis* cause a wide range of diseases, including blinding trachoma, urogenital tract infections leading to urethritis, cervicitis and proctitis, and systemic lymphogranuloma venereum disease. Although antibiotics are effective in treating chlamydial infections, the lack of obvious symptoms has the consequence that many infections remain untreated potentially leading to complications characterised by inflammatory pathologies. The immunity to these pathogens is not effective, chlamydial infections display high rates of recurrence and have long-term consequences causing a serious public health problem. Understanding the immunological basis of immunity to chlamydiae will provide an indispensable knowledge for the design of a vaccine against diseases caused by these pathogens.

Several *Chlamydia* species harbour a cryptic plasmid, but the roles of the plasmid-encoded or regulated proteins in either chlamydial pathogenesis or protective immunity remain largely unknown. Growing evidence indicates that the immune responses mediated by different IL-17 cytokines play a critical role in the protective mechanisms against bacterial and fungal infections, and in the development of allergic and autoimmune diseases.

The present study was designated to improve our current understanding of the pathological immune response to infection and re-infection with chlamydiae, and to provide information about the host immune responses against the different chlamydial plasmid proteins.
We have demonstrated that infection with C. pneumoniae induced the expression of IL-17A and IL-17F mRNA in BALB/c mice, whereas the expression of IL-17C, IL-17D and IL-17E mRNA did not change after a single infection with the pathogen. The in vivo neutralization of IL-17A significantly reduced the number of neutrophil granulocytes and increased the pathogen burden in the lungs of C. pneumoniae-infected mice. We have revealed that the main source of the IL-17A after C. pneumoniae infection in BALB/c mice is the CD4+ cells.

Our mouse model of repeated infections revealed that re-infection increased the expression of IL-17A and IL-17E mRNA in the lungs of mice relative to that in mice infected only once. Infection with C. muridarum can also induce the production of IL-17A and IL-17E in BALB/c mice. The production of both cytokines was still elevated when viable chlamydiae were not present in the lungs, suggesting a role of IL-17A and IL-17E in the chronic inflammatory process.

Infection of BALB/c and C57BL/6N mice with C. muridarum revealed that BALB/c mice are more susceptible to C. muridarum infection than C57BL/6N mice. The chlamydial plasmid genes displayed divergent transcriptional pattern in BALB/c and in C57BL/6N mice, and the immune response to pGP3 and pGP4 plasmid proteins was also different in the two mouse strains. The sera of C. muridarum-infected C57BL/6N mice reacted with the monomeric form of pGP3 in Western blot assay. In contrast, the sera of multiple-infected BALB/c mice did not recognize the monomeric form of pGP3, but the sera did react with an additional protein band which was proved to be the trimeric form of pGP3. These results suggest that recognition of pGP3 is dependent on the native conformation of the protein, and the genetic background of the host can determine whether the monomeric or the trimeric form is recognized. Triple infection with C. muridarum elicited pGP4-specific humoral immune response in C57BL/6N, but not in BALB/c mice. Spleen cells isolated from C. muridarum-infected C57BL/6N mice reacted with proliferation after in vitro re-stimulation with pGP3 and pGP4. The spleen cells of C. muridarum-infected BALB/c mice did not show reactivity to the recombinant pGP4 protein after in vitro re-stimulation, but they responded with proliferation to the pGP3 protein.

Acknowledgements
I would like to express my sincere and ever-lasting gratitude to my supervisor Dr. Katalin Burián for introducing me to scientific research. I would like to thank her for continuous support, never-failing interest and her encouragement.

I greatly acknowledge Professor Yvette Mándi, Head of the Doctoral School of Interdisciplinary Medicine and former Head of the Department of Medical Microbiology and Immunobiology for providing working facilities.

I would like to thank to Dr. Valéria Endrész for supporting me in both professional and human aspects.

I thank to Mrs Lévai and Mrs Müller for their excellent technical assistance and advice.

I wish to thank to my fellow Ph.D. students for creating a great atmosphere at work and nice times during coffee breaks.

Colleagues and staff members at the Department of Medical Microbiology and Immunobiology are gratefully thanked for creating a supportive and pleasant environment.

I deeply thank my Family for the continuous encouragement, even though they have no idea what I am doing at work.
I express my deepest thanks to my true and only Love who have always supported, encouraged and believed in me.

I dedicate my thesis to my Mother and my Grandmother.

This work was supported by OTKA National Research Fund Grant PD 100442, and Grant TÁMOP-4.2.2.A-11-1-KONV-2012-0035

Publications related to the thesis


Impact factor: 1.964


Impact factor: 1.219


Impact factor: 4.537

Total impact factor: 7.72

Publications not directly related to the thesis


Impact factor: 0.646

Cumulative impact factor: 8.37

Conference abstracts

2013: Timea Mosolygó, Gabriella Spengler, Emese Petra Balogh, Valéria Endrész, K Laczi, Katalin Perei, Katalin Burián. IL-17E production is elevated in the lungs of BALB/c mice in the later stages of *Chlamydia muridarum* infection and reinfection; *ACTA MICROBIOL IMM H* 60:(Suppl.1) pp. 189-190. (2013)


Katalin Burián, Timea Mosolygó, Emese Petra Balogh, Adrienn Karai, Fanni Kerekes, D Virók. Transcription analysis of Chlamydia trachomatis D and Herpes simplex-infected HeLa cells; ACTA MICROBIOL IMM H 58: p. 131. (2011)


