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

Ph.D. Thesis

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Publications related to the thesis

I. Mosolygó T, Korcsik J, Balogh EP, Faludi I, Virók DP, Endrész V, Burián K. *Chlamydophila pneumoniae* re-infection triggers the production of IL-17A and IL-17E, important regulators of airway inflammation. *Inflamm Res.* 2013 May; 62(5):451-60.

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II. Mosolygó T, Spengler G, Endrész V, Laczi K, Perei K, Burián K. IL-17E production is elevated in the lungs of Balb/c mice in the later stages of *Chlamydia muridarum* infection and re-infection. *In Vivo*. 2013 Nov-Dec; 27(6):787-92.

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III. Mosolygó T, Faludi I, Balogh EP, Szabó AM, Karai A, Kerekes F, Virók DP, Endrész V, Burián K. Expression of *Chlamydia muridarum* plasmid genes and immunogenicity of pGP3 and pGP4 in different mouse strains. *Int J Med Microbiol*. 2014 May;304(3-4):476-83.

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Abbreviations

AB	aberrant body
BAL	bronchoalveolar lavage
bp	base pair
C. muridarum	*Chlamydia muridarum*
C. pneumoniae	*Chlamydophila pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
DC	dendritic cell
EB	elementary body
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
i.p.	intraperitoneal
IFN-γ	interferon-γ
IFU	inclusion forming unit
Ig	immunoglobulin
IL	interleukin
KC	keratinocyte chemoattractant
kDa	kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIX	lipopolysaccharide-induced C-X-C chemokine
LPS	lipopolysaccharide
MIG	monokine induced by IFN-γ
MIP-2	macrophage inflammatory protein-2
MW	molecular weight
NK	natural killer
ORF	open reading frame
PBS	phosphate-buffered saline
pMoPn	the plasmid of *C. muridarum*
PRR	pattern recognition receptors

RB	reticulate body
rRNA	ribosomal RNA
RT qPCR	quantitative reverse transcription polymerase chain reaction
SD	standard deviation
SFCs	spot-forming cells
SI	stimulation index
SPG	sucrose-phosphate-glutamic acid buffer
Th	T helper
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α

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.

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: *Chlamydophila* and *Chlamydia*. The genus *Chlamydophila* is composed of six species: *Chlamydophila pneumoniae*; *Chlamydophila psittaci*; *Chlamydophila pecorum*; *Chlamydophila abortus*; *Chlamydophila caviae* and *Chlamydophila felis.* Three species belong in the *Chlamydia* genus: the human pathogen *Chlamydia trachomatis*; the mouse adapted *Chlamydia muridarum* and *Chlamydia suis,* which infects swine[1].

Chlamydiae undergo a unique biphasic developmental cycle, during which the bacterium is found in two forms, the extracellular form, called elementary body (EB), which is metabolically inert and infectious, and the intracellular form, the reticulate body (RB), which is metabolically active, replicative but non-infectious. The chlamydial developmental cycle is

divided into five major steps: the infectious EB attaches to and enters the host cell resulting in the formation of an inclusion, which creates a permissive intracellular niche for replication; EB differentiates into RB; RB replicates by binary fission; increasing numbers of RBs differentiate back to EBs; the newly formed EBs are released by lysis or extrusion from the host cell allowing infection of neighbouring cells (**Fig. 1**). The duration of the developmental cycle is 48-72 hours depending on the chlamydia species[2]. Over the past years increasing number of studies suggest that considering the chlamydial developmental cycle a biphasic process may underestimate its complexity. Under stress chlamydiae can enter a dormant, non-infectious but viable state named persistence, during which they exist in an enlarged aberrant body (AB). Several different stimuli can induce persistence of chlamydiae *in vitro*: exposure to interferon-γ (IFN-γ) and antibiotics; heat shock; depletion of essential nutrients. Persistence is reversible, after the inducer is removed chlamydiae continue their productive replication[3]. 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[4].

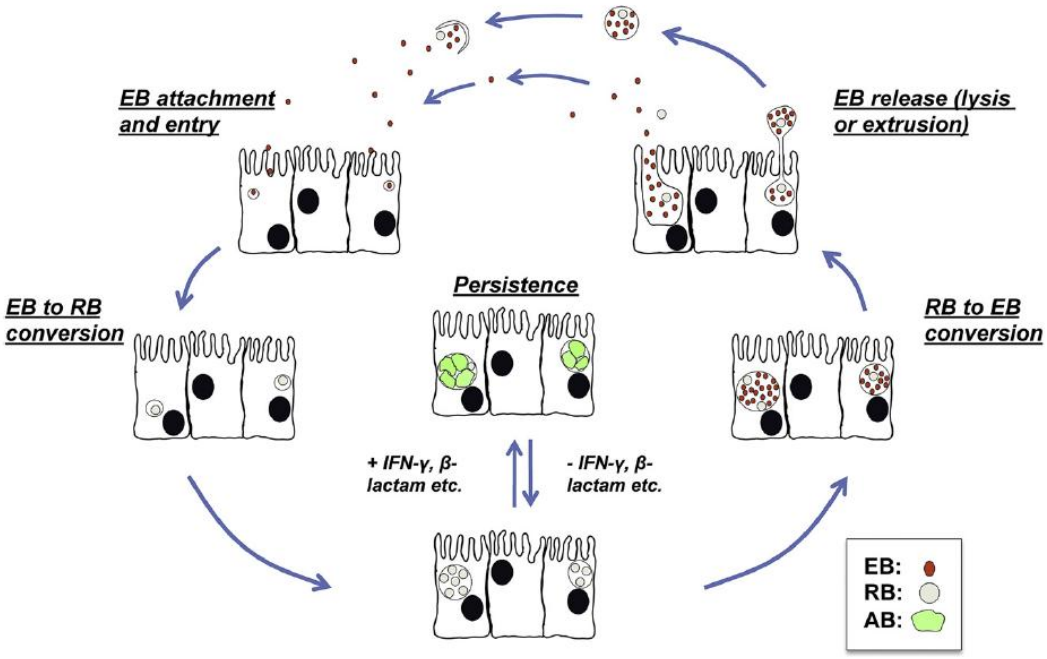

Fig. 1 General overview of the chlamydial developmental cycle[3]

Chlamydophila 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. However, due to the lack of obvious symptoms most of the infections caused by *C. pneumoniae* remain undiagnosed. Seroepidemiological studies shows that 75% of the population became infected with *C. pneumoniae* before the age of 20 and re-infection during their lifetime was very common[5]. Most of the studies regarding *C. pneumoniae* focus on its role as a cause of persistent infections in chronic diseases. The association of *C. pneumoniae* with chronic diseases was first published in 1988, when Saikku et al. revealed the role of *C. pneumoniae* in chronic coronary heart disease and acute myocardial infarction[6]. Thenceforth a 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 [7–11].

Similar to other bacteria, *C. pneumoniae* infections initiate the innate immune response of the host by recognition of the pathogen via pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one of the most important family of PRRs, which are expressed on epithelial cells, neutrophils, macrophages and dendritic cells (DCs)[12]. Moreover, the recognition of cell wall components or heat shock proteins by TLR2 appears to be essential for development of the inflammatory response to infection with *C. pneumoniae*[13]. Activation of the TLRs initiates downstream signalling, which induces transcription factors and the expression of inflammatory and immune response-related genes resulting in a localised inflammation largely mediated by polymorphonuclear and mononuclear leukocytes, which are recruited by different cytokines and chemokines[14]. Infection with *C. pneumoniae* triggers the production of a wide range of chemokines, including the monokine induced by IFN-γ (MIG); the IFN-γ-inducible protein of 10 kDa and the IFN-inducible T cell alpha chemoattractant. Besides the recruiting role of the chemokines, some of them act like defensins and possess antimicrobial activity[15]. It was earlier revealed that MIG had a dose dependent antichlamydial effect *in vitro*[16]. Thus, the MIG, which is released by non-immune cells, could directly inactivate *C. pneumoniae*, while also assisting the recruitment of neutrophils to the infected tissue.

Innate immune responses can also induce the adaptive immunity and acquired immunological memory. Antigen presenting cells activate naive T lymphocytes by presenting chlamydia-specific antigens. Those T lymphocytes, which are bearing a chlamydia-specific T cell receptor undergo clonal expansion and mediate effector functions[17]. According to a classical paradigm $CD4^+$ T cells are assigned to either T helper (Th) 1 or Th2 subset based on the cytokines secreted by them. Th1 cells produce IFN-γ and are regulated by interleukin (IL)-12, while Th2 cells produce the cytokines IL-4, IL-5, and IL-13[18]. Th1 cells regulate cellular immune response and are associated with protection against intracellular pathogens; Th2 cells play crucial roles in humoral immunity, and are involved in the defence against extracellular bacteria[19]. A murine study has recently demonstrated that the influx of activated Th1 cells and the effective release of IFN-γ are critical for the defence against *C. pneumoniae*[20].

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. Moreover, in the case of persistent infection, although *C. pneumoniae* is in a dormant state, but the 60 kDa heat shock protein is expressed and the release of chlamydial antigens from infected cells may provide a prolonged antigenic stimulation, which amplifies the chronic inflammation[21]. 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*[22]. 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.

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 also became a prominent subject for investigation. Recent progress in studies revealed important roles for Th17 cells in the development of allergic and autoimmune diseases, and in protective mechanisms against bacterial and fungal

infections, functions previously believed to be mediated by Th1 or Th2 cells. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22[23]. Th17 cell differentiation from naive $CD4^+$ T cells is controlled by transforming growth factor-β, IL-6 and IL-21, however IL-1 and IL-23 are critical for survival and effector functions of Th17 cells[24]. IL-23 promotes the development and expansion of activated $CD4^+$ T cells that produce IL-17A upon antigen-specific stimulation[25].

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 (**Table 1**). 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. IL-17A and IL-17F share the strongest homology, they bind to the same receptor, which suggests that they exhibit similar biological activities. 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 fibroblasts, endothelial and epithelial cells. IL-17A plays a crucial role in innate immune response against pathogens by promoting granulocyte-macrophage colony-stimulating factor (GM-CSF) mediated granulopoiesis and recruiting neutrophils to the sites of inflammation[26,27]. Besides combating microbial infections, a most prominent function associated with IL-17A is the regulation of autoimmunity, and dysregulation of Th17 cells leads to severe disease such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and psoriasis[28–32]. In addition, numerous studies suggest pathological role of IL-17A in allergic responses and in promotion of disease progression in atopic dermatitis and asthma. Although, the exact function of Th17 cells in the development of asthma remains to be elucidated. 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 (Ig) E production and eosinophilia. IL-17E is mainly produced by Th2 cells, mast cells and eosinophils, but alveolar macrophages, endothelial and epithelial cells can also be the sources of IL-17E[33]. Several allergens can induce the expression of IL-17E mRNA in lung epithelial cells suggesting an important role of IL-17E in the pathogenesis of allergic lung disease[34]. It seems that the regulation of IL-17E is as critical as that of IL-17A in the development of allergic diseases:

however, IL-17E induces eosinophilia, while IL-17A recruits neutrophils to the inflammatory site. The mechanism, which have role in polarization of the immune response against *C. pneumoniae* towards either type of immunity is unknown.

Cytokine	**Homology with human (%)**	**Source**	**Known function**
IL-17A	63	Th17 cells; $CD8^+$ cells; γδ T cells; NK cells	induction of pro-inflammatory cytokines and chemokines; promoting neutrophil recruitment
IL-17B	88	chondrocytes; neurons	induction of proliferation of chondrocytes; promoting neutrophil recruitment
IL-17C	83	$CD4^+$ cells; DCs; macrophages; keratinocytes	promoting neutrophil recruitment
IL-17D	78	$CD4^+$ cells; B cells; skeletal muscle; adipose tissue	stimulating production of IL-6, IL-8 and GM-CSF by endothelial cells
IL-17E (IL-25)	81	$CD4^+$ and $CD8^+$ cells; epithelial and endothelial cells; eosinophils; mast cells	promoting Th2 differentiation; inducing Th2 cytokines; eosinophil recruitment; suppression of Th1 and Th17 cell responses
IL-17F	77	Th17 cells; $CD8^+$ cells; γδ T cells; epithelial cells	inducing pro-inflammatory cytokines and chemokines; promoting neutrophil recruitment

Table 1 Members of mouse IL-17 cytokine family[26,33]

Chlamydia trachomatis and *Chlamydia muridarum*

C. trachomatis is currently divided in 19 serovars, according to the antigenic characteristics of the major outer membrane proteins. Different serovariants of *C. 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)[35]. Sexually transmitted *C. trachomatis* infection has an important public-health concern, because in most cases these infections produce few or no symptoms and most of them remain undetected and without treatment they have adverse effects on reproduction. In women, infection with *C. 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[36]. Current programmes for the control of *C. trachomatis* infections are not affordable especially in developing countries and might have an inherent weakness; therefore vaccine development has been identified as essential measures for the control of infection with *C. trachomatis*.

C. muridarum (formerly the C. *trachomatis* agent of mouse pneumonitis) 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[37]. The genomes of *C. muridarum* and *C. trachomatis* serovar D display notable similarity in the content and order of genes, with the exception of a region of 50 kb from the origin of termination, deemed the plasticity zone[38]. Studies of infection with *C. muridarum* have revealed that the murine innate and adaptive immune responses to infection are closely similar to the immune responses in women infected with *C. trachomatis*[39]. 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.

Animal models have clearly disclosed that T cells, especially the IFN-γ-producing $CD4^+$ cells have a crucial role in the resolution of *C. trachomatis* infection. By contrast, B cells and antibodies are probably important for resistance to secondary infection[40]. Host genetic factors appear to be important in determining the outcome of *C. trachomatis* infections. It has been reported that the increased incidence of chlamydia-induced chronic diseases, such as scarring trachoma and tubal infertility, correlates with the carriage of certain human leukocyte antigen haplotypes and the polymorphism of genes encoding tumour necrosis factor-α (TNF-α), IL-10 and CD14[41–44]. Similarly to humans, inbred mouse strains with different haplotypes, such as C3H/HeN, C57BL/6N, BALB/c and DBA/2 mice, respond differently to respiratory

chlamydial infections[45–47]. C3H/HeN mice are more susceptible to *C. muridarum* lung infection, with more severe morbidity and higher mortality compared to C57BL/6N mice, even though both strains display similar Th1-like response[45].

The chlamydial cryptic plasmid

Bacterial plasmids bear genes considered to be non-essential for host cell survival, but confer selective advantages for survival in specific environments[48]. Several *Chlamydia* species harbour a highly conserved plasmid with an approximate size of 7.5 kb[49]. The plasmid of *C. trachomatis* encodes both noncoding RNAs and eight open reading frames (ORFs), while the plasmid of *C. muridarum* (pMoPn) possesses seven ORFs, designated *TCA01-07* (**Fig. 2**). All plasmid-borne genes are transcribed and translated. *TCA04* and *TCA05* encode the proteins pGP3 and pGP4, respectively[50]. Naturally occurring plasmid-deficient clinical isolates are extremely rare[51]. 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[52,53].

Putative functions for several ORFs have been assigned on the basis of homology to known proteins in the public databases: pGP1 a DnaB like helicase; pGP7 and -8 integrase/recombinase homologues; pGP5 homologue to partitioning protein ParA[54]. The usage of deletion mutagenesis and chlamydial transformation was recently described as a new possibility for the genetic characterisation of the function of plasmid ORFs[55]. According to this, the ORFs can be grouped into two sets: essential ORFs (pGP1, -2, -6 and -8) and non-essential ORFs (pGP3, -5 and -7) for stable plasmid maintenance in tissue culture. 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[56–58].

The roles of the plasmid-encoded or regulated proteins in either chlamydial pathogenesis or protective immunity remain largely unknown, but the plasmid-mediated virulence is linked

to enhanced pro-inflammatory cytokine stimulation by the activation of TLRs in a murine model[59]. pGP3, the most intensively studied plasmid protein, has been found to be secreted into the host cell cytosol during chlamydial infections; it has been implicated as a potential TLR agonist, because purified pGP3 induced both TNF-α and IL-8 production in macrophages *in vitro*[60]. pGP3 is an immunodominant antigen in women infected with *C. trachomatis* in the urogenital tract; it is recognized by human antibodies in enzyme-linked immunosorbent assay (ELISA)[61]. Although, 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[62]. DNA immunisation with the plasmid expressing pGP3 of *C. trachomatis* was demonstrated to inhibit the spread of *C. trachomatis* infection from the lower to the upper genital tract in C3H/HeN mice[63].

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.

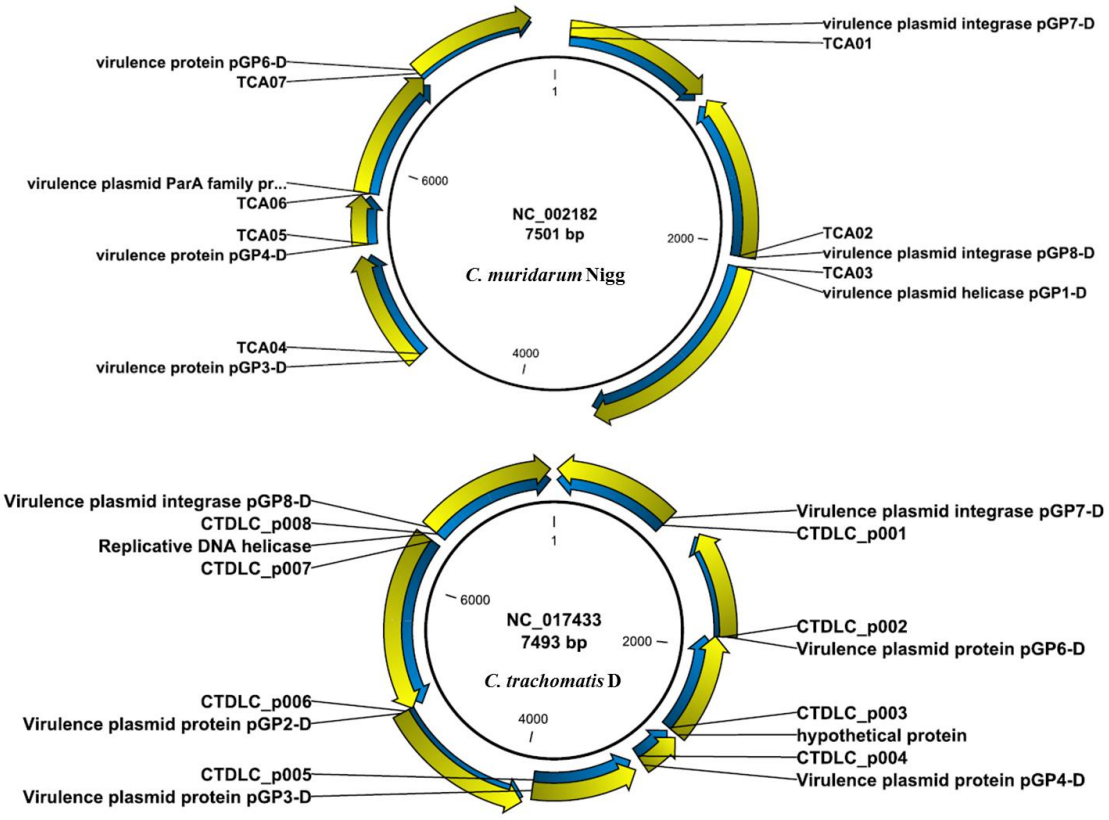

Fig. 2 Graphics of *C. muridarum* Nigg and *C. trachomatis* D plasmid created with CLC Sequence Viewer 6.4 software[64]

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

Materials and methods

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

C. pneumoniae CWL029 (purchased from the American Type Culture Collection) and *C. muridarum* strain Nigg (a kind gift from H.D. Caldwell, Hamilton, MT, USA) were propagated on HEp-2 and McCoy cells, respectively, and purified, as described earlier[65]. The purified and concentrated EBs were aliquoted and stored at -80 °C until use. The titre of the infectious EBs was determined by indirect immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto cell monolayers and, after 24 or 48 h culture, cells were fixed with acetone and stained with monoclonal anti-Chlamydia LPS antibody (AbD Serotec, Oxford, UK) and fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Sigma, Saint Louis, MO, USA). 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.

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.

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 (7.5 mg/ml); 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 mechanically. 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 in foetal bovine serum medium containing dimethyl sulfoxide 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* (concentrated EBs were kept at 90 °C for 30 min) at 4-week intervals. Another group of mice were initially infected with viable *C. pneumoniae* and then infected twice with heat-inactivated *C. pneumoniae* at 4-week intervals. Groups of 7 mice were sacrificed at 2 or 4 weeks after each infection and the lungs were processed as mentioned above.

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

Before infection, BALB/c mice were mildly sedated with an i.p. injection of 200 μl of sodium pentobarbital (7.5 mg/ml); they were then 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 three 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 at 4-week intervals. Groups of 7 mice were sacrificed at 2 weeks after each infection. Sera were taken by cardiac puncture. Spleens were dissected and homogenized by pressing through a nylon mesh into complete growth medium for testing of cell-mediated immunity.

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) to remove genomic DNA contamination. The RNA was quantified by spectrophotometric analysis and the RNA integrity was confirmed by agarose gel electrophoresis. 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^{TM} Taq ReadyMix^{TM} (Sigma) in a total volume of 20 μl, with a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primer sequences used for qPCR are given in **Table 2**, all primers were synthesized by Integrated DNA Technologies Inc. (Montreal, Quebec, Canada). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 40 cycles each of 5 sec at 95 °C, 20 sec at 60 °C and 25 sec at 72 °C. 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*, as this gene was previously shown to be an accurate normalizing gene for gene expression analysis in *C. trachomatis*[66]. The relative expression level was given as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ 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.

Target gene (Gene ID)	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
IL-17A mRNA (16171)	AAGGCAGCAGCGATCATCC	GGAACGGTTGAGGTAGTCTGAG
IL-17C mRNA (234836)	TGCTGAGGAATTATCTCACGGCCA	ACTGTGTTCCAGCTAGAGGTCCTT
IL-17D mRNA (239114)	CAAGCACATCACACACATCCCGTT	TTAGTAAGCTTGGGCCACAGGAGA
IL-17E mRNA (140806)	CAGGTGTACCATCACCTTGCCAAT	ACAACAGCATCCTCTAGCAGCACA
IL-17F mRNA (257630)	AGCAAGAAATCCTGGTCCTTCGGA	CTTGACACAGGTGCAGCCAACTTT
IL-23 p19 mRNA (83430)	CCTGCTTGACTCTGACATCTTC	TGGGCATCTGTTGGGTCTC
β-actin mRNA (11461)	TGGAATCCTGTGGCATCCATGAAA	TAAAACGCAGCTCAGTAACAGTCCG
TCA01 (1245522)	GCTTCGGAGCGCAATGACAACTAA	ACAGAAGAGTTCCCGCCAGAACAA
TCA02 (1245521)	AGAGCGTGCATGAACTTCTGAGGA	ACTATGCTGCAAGGAGGTAAGCGT
TCA03 (1245519)	TGGGAAGAGCTGTTAAGAAGGCGT	CGTATGCGAAATAGGCCTGAGCTT
TCA04 (1245520)	ACTTGGGACATCGACAACTCCTGT	CCATCAAAGATTTGGTCGCCAAGC
TCA05 (1245517)	CACCCTTAGAACTCTACCACAAGAG	TCTAGACAGAATAAGCATAATCAACGCT
TCA06 (1245518)	AGCATCGAAGGCAACCATAAAGGC	AACAGCCGTAAATTGCTGCTTGGG
TCA07 (1245523)	ACAACACGTGCTCCTTCTTGGAGA	AGCCAACGCATTATAGGCGGATGA
16s rRNA (444439667)	CATGCATATGACCGCGGCAGAAAT	ACCCTAAGTGCTGGCAACTAACGA

Table 2 Primer sequences used for qPCR

Cytokine and chemokine measurements

The supernatants of the lung homogenates were centrifuged (5 min, 12 000g) and 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.

ELISPOT assay

ELISPOT assays were performed to determine the number and phenotype of the IL-17A-producing spleen cells isolated from BALB/c mice 2 weeks after *C. pneumoniae* infection, uninfected mice served as controls. To determine the phenotype of the IL-17A-producing cells, the spleen cell suspensions 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 after direct staining of the depleted cells with α-CD4-TC and α-CD8-rPE antibodies (Caltag Laboratories, Burlingame, CA, USA). 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.

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 cytospin 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 chlamydial burden was determined as mentioned above.

Lung histopathology

Four weeks after *C. muridarum* infection and re-infection BALB/c mice were anaesthetized and then sacrificed by exsanguination through cardiac puncture. Uninfected mice were used as controls. The lungs were removed *in toto* and immersed in frozen tissue matrix, OCT (Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands). For the detection of IL-17E antigen by immunofluorescence test, lungs were cut into 5-μm sections. 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).

Cloning and over-expression of plasmid genes

723 and 309 bp DNA fragments containing *TCA04* and *TCA05* (GenBank ID 10957566:4703-5425, Locus tag *TCA04* and GenBank ID 10957566:5494-5802, Locus tag *TCA05*) were amplified by PCR, *C. muridarum* Nigg strain DNA being used as template. The PCR was performed in a GeneAmp II (Applied Biosystems, Foster City, CA, USA) thermocycler with Advantage GC cDNA polymerase (Clontech, Mountain View, CA, USA), under the amplification conditions recommended by the manufacturer. The amplicon was digested with NdeI and EcoRI and inserted into the p6HisF-11d (*icl*) pET vector[67] by

digesting it with the same enzymes and replacing the *icl* gene. For overexpression, *Escherichia coli* HB101 (Invitrogen) cells carrying this plasmid were grown and treated according to the method of Tabor and Richardson[68]. Briefly, cells containing the plasmids were grown at 32 °C on Luria-Bertani agar plates in the presence of the required antibiotics. Over-expression of these proteins was induced by shifting the temperature to 42 °C for 20 min. After induction, the temperature was shifted down to 37 °C for an additional 90 min, cells were harvested by centrifugation, and the pellet was kept at -20 °C until use.

Purification of plasmid proteins

Cell lysates from *E. coli* were prepared by suspending the frozen cells in CelLytic B-II (Sigma) with protease inhibitors. Recombinant protein was purified by using TALON CellThru Resin (Clontech) according to the manufacturer's instructions.

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 with 5% skim milk and 0.05% Tween 20 (Sigma) containing PBS. Membranes were probed with a pool of uninfected mice sera or sera obtained from *C. muridarum*-infected BALB/c or C57BL/6N mice (1:50 dilution in 5% skim milk and 0.05% Tween 20 containing PBS). 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.

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 blot assay were cut out from the gel and analysed by mass spectrometry. Briefly, protein bands were diced and washed with 25 mM NH_4HCO_3 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 (NCBInr 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.

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 the complete growth medium. The proliferative responses of 5×10^5 spleen cells in 3 parallel wells to 2 μg/ml of pGP3 or pGP4 recombinant protein, purified heat-inactivated *C. muridarum* EBs (1 μg/ml), 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.

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 $p<0.05$.

Results

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

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

In order to investigate the production of different IL-17 cytokines during *C. pneumoniae* infection, BALB/c mice were infected intranasally with *C. pneumoniae*. On days 1, 2, 4, 7, 14 or 28 after infection, mice were sacrificed and their lungs were removed for the determination of *C. pneumoniae* titres, the expression of the different IL-17 (IL-17A, C, D, E, F) and IL-23 mRNA, and the IL-17A protein content in the individual lungs.

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 (**App. I. Fig. 1a**). The expression of IL-17A mRNA was increased as early as on day 2, but the highest level of expression (26.1-fold) 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 (**Fig. 3A**). The expression of IL-17F mRNA was highest on day 4 (16.25-fold) 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 *C. pneumoniae* 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 at 62-fold (**Fig. 3B**). The kinetics of IL-17A protein production correlated with the mRNA expression: it increased from day 2, with the highest concentration on day 7 (**App. I. Fig. 1d**).

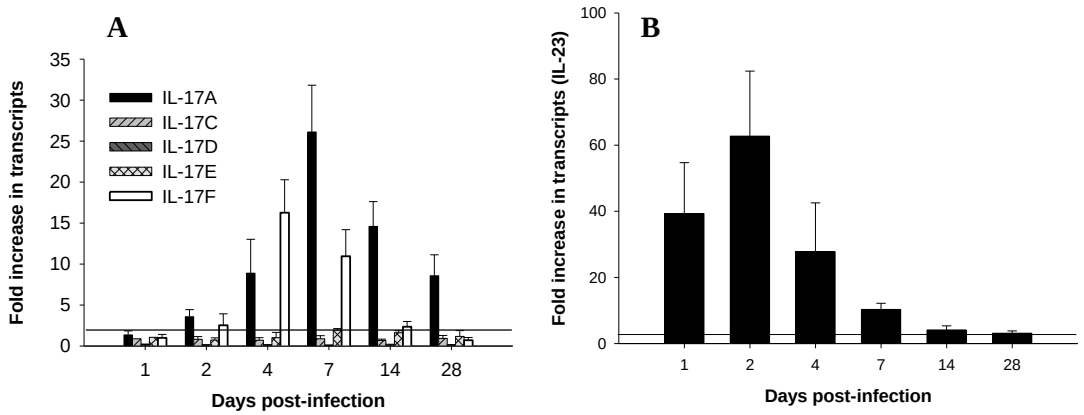

Fig. 3 Expression of different IL-17 mRNA (**A**) and IL-23 mRNA (**B**) in lung suspensions from *C. pneumoniae*-infected BALB/c mice. The line denotes a threshold value, which was set at a 2-fold increase in transcripts. Data are normalized for β-actin RNA content and plotted as fold change over the results for the control mice. Bars denote mean ± SD of the results on 7 mouse lungs.

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

To define the phenotype and the number of peripheral cells which release IL-17A, ELISPOT assay was carried out with the spleen cells of *C. pneumoniae*-infected mice after the depletion of CD4$^+$ or CD8$^+$ cells and *in vitro* re-stimulation with heat-inactivated *C. pneumoniae* or mock preparation. Spleen cells of uninfected mice served as controls.

C. pneumoniae infection caused a significant increase in the number of IL-17A-producing cells after *in vitro* re-stimulation of the spleen cells with heat-inactivated *C. pneumoniae*, as compared with the spleen cells of uninfected mice (data not shown). *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 (**Fig. 4**). 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.

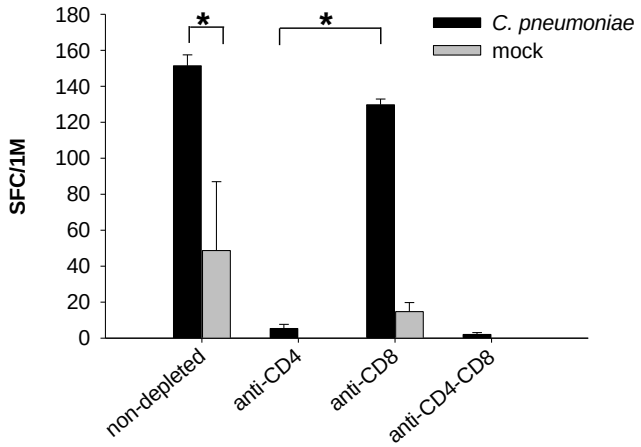

Fig. 4 The number of IL-17A-producing cells isolated from *C. pneumoniae*-infected mice and tested after depletion of $CD4^+$ and/or $CD8^+$ cells and *in vitro* re-stimulation with heat-inactivated *C. pneumoniae* or mock in ELISPOT assay. Bars indicate mean ± SD of SFCs per million spleen cells, counted in triplicate wells (*p<0.001).

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

To investigate the role of IL-17A in acute *C. pneumoniae* infection, mice were treated 24 h before, and 24 and 48 h after the infection with anti-IL-17A monoclonal antibodies or with isotype control antibodies. Mice were sacrificed on day 1 or 4 after infection. The numbers of lymphocytes, macrophages and neutrophil granulocytes in the BAL fluid of the mice were determined. From the homogenized lungs, *C. pneumoniae* was cultured and the levels of different cytokines were determined by ELISA.

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 (**App. I. Table 1**). 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 (**Fig. 5A**). The levels of KC and MIP-2 on

day 4 were significantly lower than the chemokine levels in the lungs of the control mice (**Fig. 5B**).

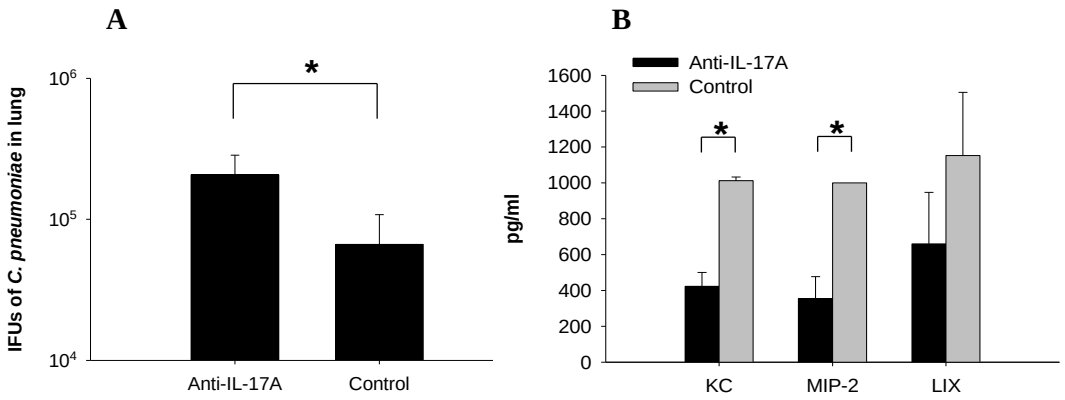

Fig. 5 The number of viable *C. pneumoniae* (**A**) and the levels of KC, MIP-2 and LIX (**B**) in the lungs of anti-IL-17A-treated and control mice on day 4 after infection. Bars denote mean ± SD of the results on 7 mouse lungs (*p<0.05).

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

In order to study the role of IL-17A and IL-17E in chronic chlamydial infection, BALB/c mice were infected intranasally with *C. pneumoniae* 3 times at 4-week intervals, and were sacrificed at 14 or 28 days after each infection, their lungs were collected for the determination of *C. pneumoniae* titres, the expression of IL-17A and IL-17E mRNA, and the IL-17A/E protein content in the individual lungs.

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 (data not shown). 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 (**Fig. 6A**).

To clarify whether viable *C. pneumoniae* is needed to induce IL-17A/E mediated pathological immune response or only the chlamydial antigens are sufficient, mice were first infected intranasally with viable or heat-inactivated *C. pneumoniae* then infected twice with heat-inactivated *C. pneumoniae* at 4-week intervals. The mice were sacrificed at 2 or 4 weeks after infection and the expression of IL-17A and IL-17E mRNA was determined.

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 (**App. I. Fig. 4b**). 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 (data not shown).

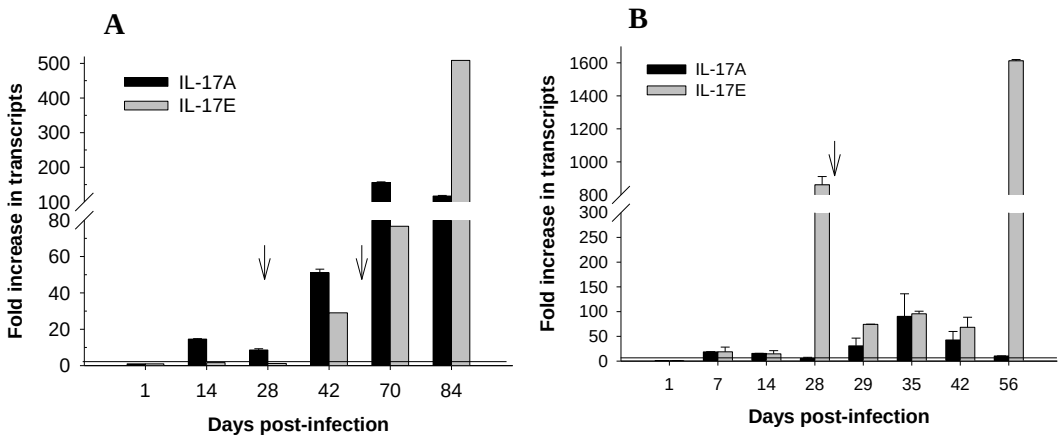

Fig. 6 The expression of IL-17A and IL-17E mRNA in the lungs of BALB/c mice infected and re-infected with *C. pneumoniae* (**A**) or *C. muridarum* (**B**). ↓ denotes the time of re-infection. The line denotes a threshold value, which was set at a 2-fold increase in transcripts.

C. muridarum infection and re-infection induce the expression of IL-17A and IL-17E mRNA in the lungs of BALB/c mice

In order to investigate the production of IL-17A and IL-17E during *C. muridarum* infection, BALB/c mice were infected intranasally with *C. muridarum*, and re-infected on day 28 after the first infection. On days 1, 7, 14, 28, 29, 35, 42 and 56 after the first infection, mice were sacrificed and their lungs were removed for the determination of bacterial burden, mRNA levels and protein contents of the IL-17A and IL-17E cytokines in individual lungs.

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 (**App. II. Fig. 1**).

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 (**Fig 6B**). 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 (**App. II. Fig. 2b**).

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

To reveal which cells are responsible for the production of IL-17E in the later stage of *C. muridarum* infection and re-infection, the lungs of infected, re-infected and uninfected mice were sectioned and stained with monoclonal antibody to IL-17E as primary, and FITC-labelled anti-mouse IgG as a secondary antibody.

No fluorescence was seen in the lung sections of the uninfected mice (**Fig. 7A**). 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 (**Fig. 7B** and **C**).

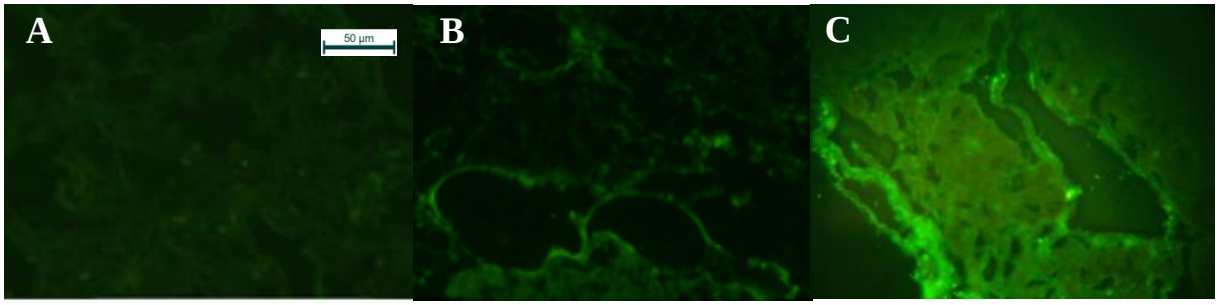

Fig. 7 The production of IL-17E in the lungs of uninfected (**A**), *C. muridarum*-infected (**B**) and re-infected mice (**C**). The lung sections were stained with IL-17E-specific monoclonal antibody and with FITC-labelled anti-mouse IgG.

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

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

In order to compare the susceptibility of BALB/c mice and C57BL/6N mice to *C. muridarum* infection, mice were infected intranasally with 1×10^3 IFU of *C. muridarum* and sacrificed 1, 7, 14, 28 or 56 days after infection, and the chlamydial burden was determined in the lungs of 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 3×10^1 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 (**App. III. Fig. 1**).

To compare the transcriptional pattern of pMoPn genes in BALB/c and C57BL/6N mice during *C. muridarum* infection, RT qPCR was carried out with total RNA isolated from the lungs of infected mice.

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 (**Fig. 8A**). 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 (7-47-fold) than those in BALB/c mice (**Fig. 8B**). There was no plasmid-encoded gene expression on day 28 after *C. muridarum* infection in either mouse strain.

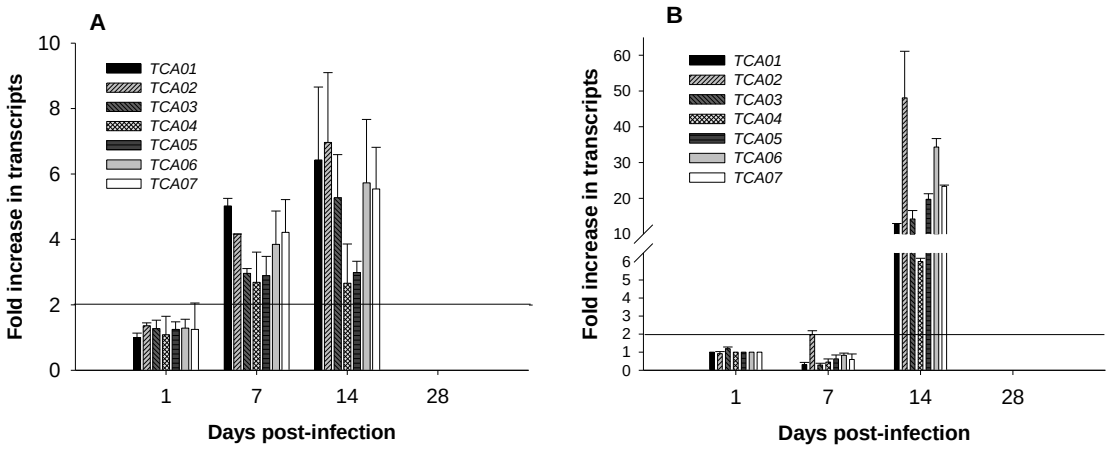

Fig. 8 Expression levels of pMoPn genes in the lungs of BALB/c mice (**A**) and C57BL/6N mice (**B**). The line denotes a threshold value, which was set at a 2-fold increase in transcripts.

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

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

To clarify whether pGP3 and pGP4 are able to induce the production of specific antibodies or not in *C. muridarum*-infected mice, BALB/c and C57BL/6N mice were infected with *C. muridarum* 3 times at 4-week intervals. Mice were sacrificed 14 days after infection, sera were taken, and Western blot analysis was performed. Recombinant, purified pGP3 or pGP4 protein or *C. muridarum* EBs served as antigens, and sera collected from uninfected mice were used as controls.

In C57BL/6N mice, a single infection with *C. muridarum* was sufficient to induce the production of pGP3-specific antibodies (**Fig. 9A panel b-d**). No pGP4-specific antibody production was detected in mice infected once or twice, but this antibody appeared after the 3rd infection (**Fig. 9A panel d**). Contrarily, the sera of *C. muridarum*-infected and re-infected BALB/c mice did not react with the pGP3 and the pGP4 (**Fig. 9B panel b-d**). The sera of uninfected mice did not contain pGP3-, pGP4- or *C. muridarum*-specific antibodies (**Fig. 9A,B panel a**). Sera of *C. muridarum*-infected mice reacted with the lysate of concentrated *C. muridarum* EBs.

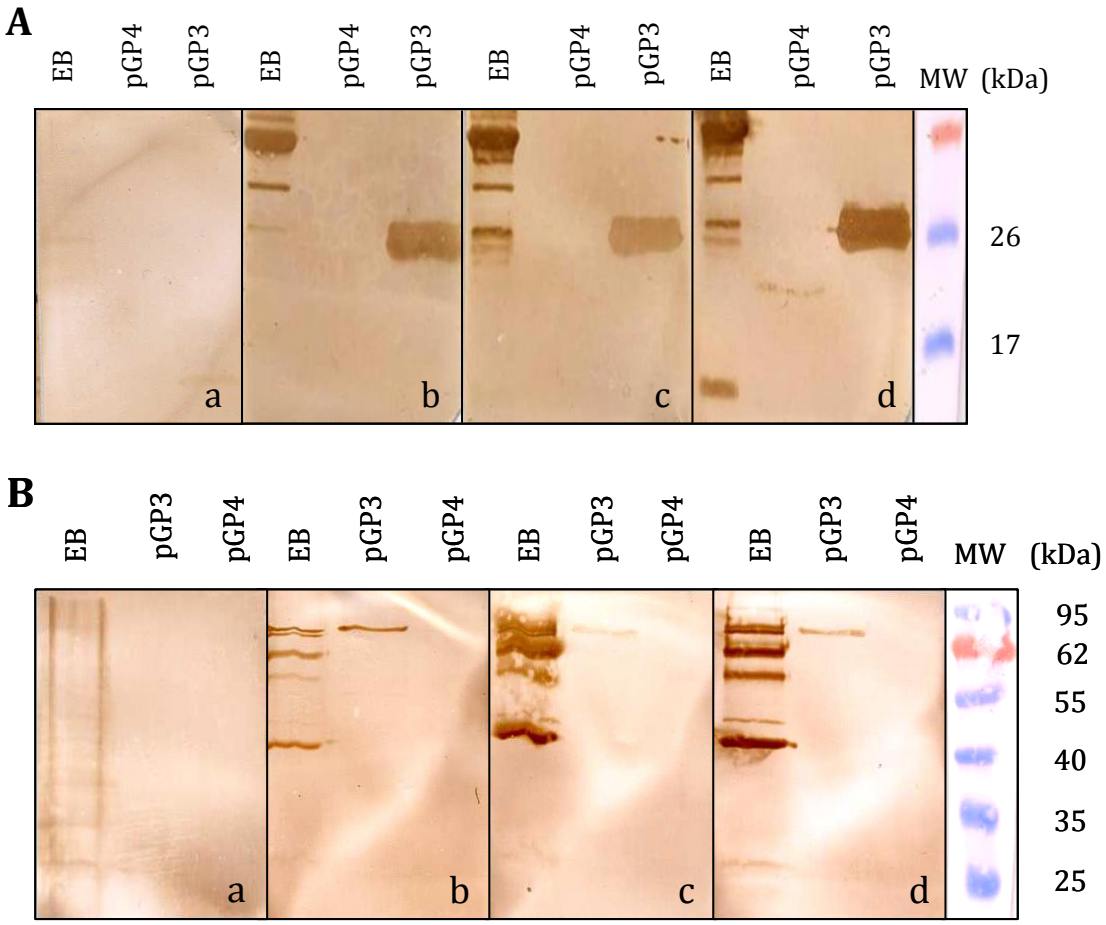

Fig. 9 Production of pGP3- and pGP4-specific antibodies in C57BL/6N mice (**A**) and in BALB/c mice (**B**) infected 1-3 times with *C. muridarum* as tested in Western blot assay. Purified *C. muridarum* EBs or pGP3 or pGP4 were loaded onto a denaturing polyacrylamide gel. After electrophoresis, the gel was blotted onto a PVDF membrane for Western blot detection with pooled sera of mice infected 1 (**panel b**), 2 (**panel c**) or 3 times (**panel d**) with *C. muridarum*. A serum sample pooled from uninfected mice was used as a negative control (**panel a**).

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 (**Fig. 9B panel b-d**). As it was reported earlier that the pGP3 plasmid protein exists in trimeric or dimeric form[69], 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 (84

kDa) form of pGP3 (**App. III. Fig. 4B**). The sera of *C. muridarum*-infected C57BL/6N mice did not show any reactivity with the trimeric form of pGP3 (**App. III. Fig. 3B**).

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

For comparison and quantitation of pGP3- and pGP4-specific T cell reactivity following *C. muridarum* infection in the two mouse strains, spleen cells isolated from *C. muridarum* re-infected mice were *in vitro* re-stimulated with recombinant pGP3 or pGP4 protein, purified heat-inactivated *C. muridarum* EBs, or the similarly treated mock preparation; the proliferative responses of the re-stimulated spleen cells were detected after incubation for 3 days. Uninfected mice served as controls.

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 (**Fig. 10A**). 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 (**Fig. 10B**). Lymphocytes of both mouse strains showed proliferation after re-stimulation with heat-inactivated *C. muridarum* EBs.

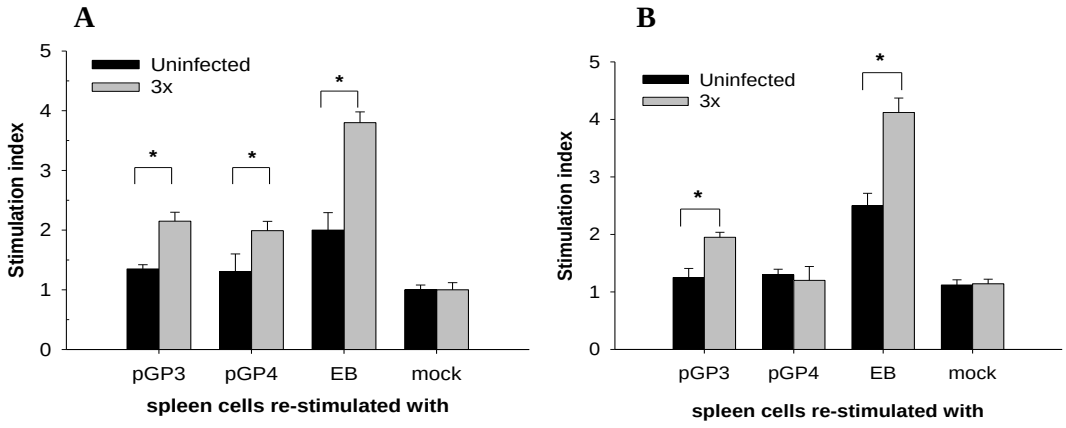

Fig. 10 pGP3- and pGP4-specific cellular immune response in triply *C. muridarum*-infected C57BL/6N mice (**A**) and BALB/c mice (**B**), as tested in MTT test. Spleen cells of mice were re-stimulated *in vitro* with recombinant pGP3 or pGP4 protein; heat-inactivated *C. muridarum* EBs or mock antigens. Uninfected mice served as controls. The data are the means ± SD of the SIs of triplicate assays (*p<0.05).

Discussion

Aim 1. 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[33]. We have demonstrated here that *C. pneumoniae* induced the expression of IL-17A and IL-17F mRNA in BALB/c mice, with peak levels on day 7 and day 4, respectively, 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, which expands and stabilizes Th17 cells to produce IL-17A and IL-17F, 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[70,71]. Differences in the role of IL-17A have been observed for different *Salmonella* species: IL-17A is not required for the protection against *S. enterica*, but the depletion of Th17 cells in the intestines dramatically increases the frequency of bacteraemia in the case of *S.* Typhimurium infection in monkeys infected with simian immunodeficiency virus[72,73]. IL-17A is important in protective immunity at an early stage of infection with *Listeria monocytogenes* in the liver because IL-17A-deficient mice exhibited a reduced protective response[74]. The over-expression of IL-17A after a challenge with *Klebsiella pneumoniae* resulted in the local induction of TNF-α, IL-1β and MIP-2, augmented polymorphonuclear leukocyte recruitment, and enhanced bacterial clearance and survival[75].

IL-17A promotes neutrophilic inflammation through the induction of KC, LIX and MIP-2 production by a variety of target cells[27]. 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. Moreover, the neutralization of IL-17A significantly reduced the number of neutrophil granulocytes in the BAL fluid relative to that for the isotype antibody-treated mice. The *in vivo* neutralization of IL-17A resulted in an increased pathogen burden at an early stage of infection; the amount of recoverable *C. pneumoniae* was increased 3-fold as compared with the isotype-treated controls, suggesting that the decreased release of KC and MIP-2 in response to anti-IL-17A treatment was associated with decreased lung neutrophil recruitment

and attenuated bacterial clearance. Based on these results we assumed that IL-17A exerts an indirect antimicrobial effect during *C. pneumoniae* infection. A similar antibacterial effect of IL-17A was seen in the case of extracellular *K. pneumoniae*[75]. It has recently been reported that IL-17A does not exert a direct antimicrobial effect, because it does not affect the growth of either *C. muridarum* or *C. trachomatis* L2 in the L-929 cell line[76].

As revealed by ELISPOT, the main source of the IL-17A after *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 following infections by intracellular pathogens, such as *L. monocytogenes*, *Mycobacterium tuberculosis* and *M. bovis*[74,77,78]. Moreover, other cell types, e.g. $CD8^+$ T cells and NK cells have been demonstrated to be IL-17A-producing cells[79]. In our experiments the depletion of $CD8^+$ cells did not influence the number of IL-17A-producing cells in the ELISPOT assay.

Immunopathological mechanisms of chlamydial infections have been widely studied by using animal models of repeated infections. Multiple episodes of re-infections with *C. trachomatis* elicit some protective immunity, but the limited growth of chlamydia induces a severe inflammation that may lead to irreversible tissue changes[21]. Moreover, a primary *C. pneumoniae* infection conferred a partial resistance to re-infection in a mouse model, but provided no protection against inflammatory changes, as an equally strong inflammatory response was observed after re-infection[80]. Our mouse model of repeated infections revealed that the 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. The production of both cytokine was still elevated when viable *C. pneumoniae* was not present in the lungs, suggesting a role of IL-17A and IL-17E in the chronic inflammatory process. Our results suggest that in recurrent chlamydial infections the synthesis and release of chlamydial antigens from repeatedly infected mucosal epithelial cells or alveolar macrophages may provide a prolonged antigenic stimulation, which strongly amplifies chronic inflammation. This is an interesting finding in the light of the reported putative role of respiratory pathogens such as *Chlamydia* and *Mycoplasma* in the activation of asthma[81]. On the basis of our results, viable pathogen is needed for the expression of IL-17E mRNA, because there was no increase in IL-17E mRNA expression after the infection and re-infection of the mice with heat-inactivated *C. pneumoniae*, not even when the mice were inoculated with viable *C. pneumoniae* first and

subsequently treated twice with killed *C. pneumoniae*. The pattern of expression of IL-17A mRNA was different from that of IL-17E mRNA. We observed an increased expression of IL-17A mRNA in the lungs of mice infected first with viable *C. pneumoniae* and then twice with heat-treated *C. pneumoniae*.

We demonstrated that infection with another member of the *Chlamydiaceae* family – with *C. muridarum*, which belongs not in the *Chlamydophila*, but 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*[82]. 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[83]. 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.

Aim 2. 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[53]. 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[84]. The kinetics of the expression of plasmid genes has not been examined earlier in different mouse strains.

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[85]. 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[57]. Moreover, a sequence of 30 or more nucleotides in the pGP3 gene was required for the optimal expression of pGP4[86].

Aim 3. 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[60]. However, others have found that seropositive human sera react with the monomeric form of pGP3 in the Western blot assay[87]. 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.

Összefoglalás

A *Chlamydiaceae* család tagjai obligát intracelluláris baktériumok, melyek által okozott betegségek az egész világon elterjedtek. A *Chlamydophila pneumoniae* egy jelentős légúti patogén, a közösségben szerzett tüdőgyulladás, a bronchitis és a sinusitis gyakori kórokozója. A *Chlamydia trachomatis* szerovariánsok különböző betegségeket okoznak, többek között a trachomát, urogenitális traktus fertőzéseit (urethritis, cervicitis, proctitis) és a szisztémás lymphogranuloma venereumot. Az antibiotikumok hatásosak ugyan a chlamydia fertőzések kezelésében, de a jellegzetes tünetek gyakori hiánya miatt a fertőzések legtöbbször kezeletlenek maradnak, mely komoly gyulladásos szövődmények kialakulásához vezethet. A chlamydiák ellen kialakult immunológiai védelem nem maradandó, ezért gyakoriak a visszatérő fertőzések, melyeknek a hosszú távú következményei jelentős közegészségügyi problémát jelentenek. Ennek megoldására a leghatékonyabb módszer egy vakcina kifejlesztése lenne, melyhez nélkülözhetetlen a chlamydiák ellen kialakult immunválasz részletes megismerése.

Néhány chlamydia faj hordoz egy plazmidot, mely fontos virulencia faktor, azonban a plazmidon kódolt fehérjék szerepe a fertőzések pathomechanizmusában ill. a chlamydiák által kiváltott immunválaszban még nem teljesen tisztázott. Az immunrendszer baktériumok és gombák ellen irányuló védelmében egyre nagyobb jelentőséget tulajdonítanak a különböző IL-17 citokinek által mediált immunfolyamatoknak, melyek fontos szerepet játszanak az allergiás és az autoimmun betegségek kialakulásában is.

A kutatásunk célja a chlamydia fertőzések által indukált gyulladásos immunfolyamatokról megszerzett ismereteink további bővítése, továbbá a plazmidon kódolt fehérjékre kialakult immunválasz vizsgálata a fertőzés során.

Kísérleteink során kimutattuk, hogy a *C. pneumoniae*-vel való fertőzés indukálja a BALB/c egerekben az IL-17A és IL-17F mRNS kifejeződését, miközben az IL-17C, IL-17D és IL-17E mRNS szintje egyszeri fertőzés hatására nem változik. Az IL-17A *in vivo* neutralizációja szignifikánsan csökkenti a neutrophil granulocyták számát és növeli a visszatenyészthető chlamydia mennyiségét a *C. pneumoniae*-vel fertőzött egerek tüdejében. Bebizonyítottuk, hogy BALB/c egerek *C. pneumoniae* fertőzése során a $CD4^+$ sejtek a felelősek az IL-17A termeléséért.

Az ismételt chlamydia fertőzések gyulladásra gyakorolt hatásának vizsgálata során kimutattunk, hogy az IL-17A és az IL-17E mRNS mennyisége a reinfekciók hatására jelentősen megemelkedik az egerek tüdejében az első fertőzés után mérthez képest. A fertőzés késői szakaszában, amikor már élő kórokozót nem tudtunk kimutatni az egerek tüdejéből, mindkét citokin szintje jelentősen megemelkedett, mely felveti annak lehetőségét, hogy az IL-17A és IL-17E szerepet játszik a krónikus gyulladásos folyamatokban.

A *C. trachomatis* modelljeként használt egérpatogén *C. muridarum*-mal végzett kísérleteink alapján elmondhatjuk, hogy a BALB/c egerek sokkal érzékenyebbek *C. muridarum*-mal szemben, mint a C57BL/6N egerek. A plazmid gének kifejeződési mintázata eltérő a két egértörzsben és emellett két plazmidfehérjére, a pGP3-ra és a pGP4-re kialakult immunválasz is különbözik. Western blot során a *C. muridarum*-mal fertőzött C57BL/6N egerek széruma felismeri a monomer pGP3 fehérjét. Ezzel szemben a többször *C. muridarum*-mal fertőzött BALB/c egerek széruma nem reagál a pGP3 monomerjével, de felismer egy másik fehérjét, melyről további vizsgálat során bebizonyosodott, hogy a pGP3 trimer formája. Ezek az eredmények arra utalnak, hogy a pGP3 felismerése függ a fehérje natív konformációjától és a gazdaszervezet genetikai hátterétől. A többszöri *C. muridarum* fertőzés csak a C57BL/6N egerekben indukálja a pGP4 specifikus immunválasz kialakulását. Az *in vitro* restimulációt követően a *C. muridarum*-mal fertőzött C57BL/6N egerek lépsejtjei reagáltak mind a pGP3, mind pGP4 fehérjékre, míg a *C. muridarum*-mal fertőzött BALB/c egerek lépsejtjei nem ismerték fel a pGP4-et, de a pGP3 fehérjére proliferációval válaszoltak.

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Appendix