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THE ANTI-CHLAMYDIAL ACTIVITY OF DEFENSIN-LIKE PROTEINS

Ph.D. Thesis

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2. Abbreviation list

AMPs	antimicrobial peptides
ATCC	American Tissue Culture Corporation
C. pneumoniae	*Chlamydophila pneumoniae*
C. psittaci	*Chlamydophila psittaci Chlamydia*
C. trachomatis	*trachomatis* complementary
cDNA	deoxyribonucleic acid
Cys	cysteine
DTT	dithiothreitol
EB	elementary body
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycans
Gln	glutamine
HEPES	(2-hydroxyethyl)- 1 -piperazineethanesulfonic acid
HRP	horseradish peroxidase
IF	immunofluorescence
IFN	interferon
IFU	inclusion forming unit
IL	interleukin
IP- 10	interferon-inducible protein- 10 interferon-
I-TAC LC-	inducible T-cell alpha chemoattractant liquid
MSMS	chromatography-tandem mass spectrometry
LPS	lipopolysaccharide
MIF	microimmunofluorescence
MFB	mouse fibroblast
MIG	monokine induced by interferon gamma
MOMP	major outer membrane protein

mRNA	messenger ribonucleic acid
MW	molecular weight nodule-
NCR	specific cysteine rich
NK	natural killer
OmcB	outer membrane complex B
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
PE	phycoerythrin
RB	reticular body
RNA SD	ribonucleic acid
SDS-PAGE	standard deviation
SPG	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TNF	sucrose-phosphate-glutamic acid buffer
	tumour necrosis factor

3. Introduction

3.1. Chlamydiaceae

The family Chlamydiaceae consists of two clinically important genera, *Chlamydia* and *Chlamydophila,* with three species responsible for human disease: *Chlamydia trachomatis (C. trachomatis), Chlamydophila psittaci (C. psittaci),* and *Chlamydophila pneumoniae (C. pneumoniae).* Other species have been placed into the two genera, but they are uncommon human pathogens.

Chlamydiaceae are obligate intracellular parasites that were once considered viruses because they are small enough to pass through 0.45 µm filters. However, the organisms have the

regions are found in *C. psittaci* MOMP; in contrast, the *C. pneumoniae* MOMP is homogeneous, and only a single serovar has been described. A second, highly conserved outer membrane protein, OMP 2, is shared by all members of the family Chlamydiaceae. This cysteine-rich protein is responsible for the extensive disulfide cross-links that provide the stability in the EBs (Penttila et al., 2000; Watson et al., 1994).

The replication of Chlamydiaceae occurs within susceptible host cells (Figure 1). The cycle is initiated when the small (300 to 400 nm), infectious EBs become attached to the microvilli of susceptible cells, followed by active penetration into the host cell. After they are internalized, the bacteria remain within cytoplasmic phagosomes, where the replicative cycle proceeds. If the outer membrane of the EBs is intact, fusion of cellular lysosomes with the EB-containing phagosome is inhibited, thus preventing intracellular killing. If the outer membrane is damaged or the bacteria are inactivated by heat or coated with antibodies, phagolysosomal fusion occurs with subsequent bacterial killing. Within 6 to 8 hours after entering the cell, the EBs reorganize into the larger (800 to 1000 nm), metabolically active RBs. The Chlamydiaceae are energy parasites, because they use the host cell's adenosine triphosphate for their energy requirements. Some strains may also depend on the host to provide specific amino acids. The RBs replicate by binary fission, similar to other bacteria; histological stains can readily detect the phagosome with accumulated RBs, called an inclusion. Approximately 18 to 24 h after infection, RBs begin to reorganize into smaller EBs, and between 48 and 72 h, the cell ruptures and the infectious bacteria are released (Murray et al., 2009).
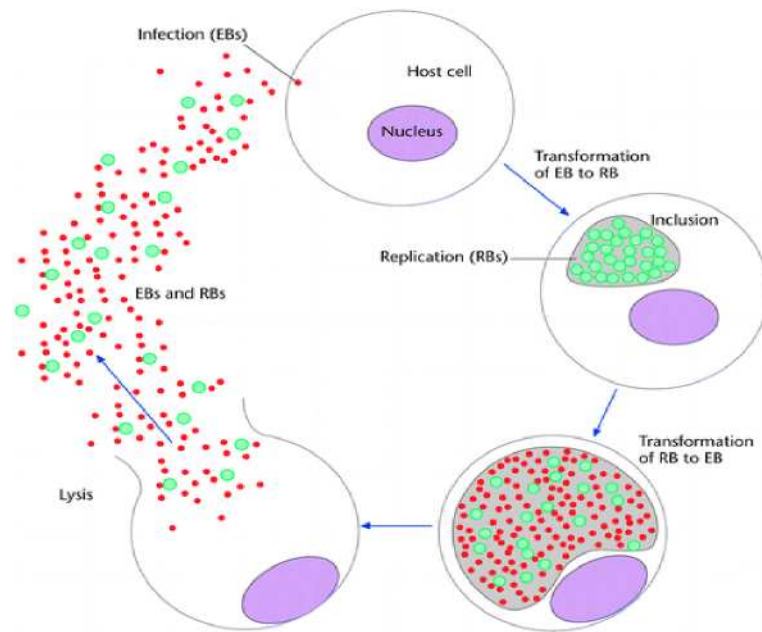
Figure 1. Life cycle of Chlamydiae [www.chlamydiae.com]

3.1.2. *Chlamydophila pneumoniae*

C. pneumoniae is a species of Chlamydophila bacteria that causes 10% of community-acquired pneumonia cases among adults. Until recently it was known as *Chlamydia pneumoniae,* and that name is used as an alternate. Previously the pathogen was known as the TWAR agent (Taiwan Acute Respiratory Agent) from the names of the two original isolates - Taiwan (TW-183) and an acute respiratory isolate designated AR-39 (Blasi et al., 2009).

C. pneumoniae causes by far the most common human chlamydial infection, with seropositivity in at least 50% of the general population over the age of 20 in the United States and elsewhere (Kanamoto et al., 1991; Grayston et al., 1992). Although most of the acute infections with *C. pneumoniae* are probably asymptomatic, they may be frequently associated clinically with pneumonia, pharyngitis, sinusitis, and bronchitis (Blasi et al., 2009). Moreover, *C. pneumoniae* has been suggested to be a risk factor for immune-reactive disorders such as adult onset asthma, reactive arthritis, and the acute chest syndrome of sickle cell anaemia (Hahn et al., 1991; Miller et al., 1991). Chronic infections caused by *C. pneumoniae* have also been linked to the development of heart diseases and atherosclerosis, the diseases which are responsible for the greatest morbidity and mortality in Western civilization (Burian et al.,

2001a; Burian et al 2001b; Rugonfalvi et al., 2002; Heltai et al 2004; Belland et al., 2004). The diagnosis of *C. pneumoniae* infections is difficult. The organisms do not grow in cell lines used for the isolation of *C. trachomatis,* and although *C. pneumoniae* will grow in HEp-2 cell line, this cell line is not used in most clinical laboratories. Detection of *C. pneumoniae* by nucleic acid amplification has long been successful; however, significant interlaboratory variation has been reported among laboratories with diverse experience in the use of these assays (Mahony et al., 1994). Microimmunofluorescence (MIF) test can be considered the only acceptable test for serodiagnosis. The criteria for the diagnosis of acute *C. pneumoniae* infection is a single IgM titre of >1:16 or a fourfold increase in IgG titer. A single increased IgG titre cannot be appropriately evaluated. Because IgG antibodies do not appear for 6 to 8 weeks after infection, serologic testing has limited value for the diagnosis of an acute infection.

The administration of macrolides (erythromycin, azithromycin, and clarithromycin), doxycycline, or levofloxacin is recommended for the treatment of *C. pneumoniae* infections, although evidence supporting their use is limited. Control of the exposure to *C. pneumoniae* is rather difficult since the bacterium is ubiquitous (Murray et al., 2009).

3.1.3. Chlamydia trachomatis

C. trachomatis has three human serotypes. Serovars Ab, B, Ba, or C cause the trachoma, an infection of the eyes, which is the leading infectious cause of blindness worldwide. Serovariants D-K and L1, L2, L3 cause sexually transmitted diseases, such as urethritis, pelvic inflammatory disease, and ectopic pregnancy (D-K), and lymphogranuloma venereum (LGV; L1-3).

C. trachomatis, especially serovars D-K, is of great public health significance because of the economic burden of chlamydial infections and the impacts of the untreated (or unsuccessfully treated) cases on human reproduction. Cervicitis and urethritis commonly occur in women, and about 40% of the untreated cases progress to pelvic inflammatory disease (PID). 20% of the PID cases results in infertility, while 18% of the women with this disease experience chronic pelvic pain, and 9% may suffer an ectopic pregnancy (Shaw et al., 2011).

A prominent survey has been carried out by Washington et al. investigating the direct and indirect healthcare costs of chlamydial infections in men, and the infections and their sequelae

in women (Washington et al., 1985; Randolph and Washington, 1990). Even though this study demonstrates the chlamydial infections to represent a substantial economic burden, it is still likely to underestimate the full costs of managing chlamydial infections and their consequences. At the individual level, *C. trachomatis* infection can generally be treated effectively with antibiotics, though antibiotic resistance appears to be increasing (Shaw et al., 2011). At the population level, public health control of the infection is rather problematic. With regard to the severe potential consequences of urogenital *C. trachomatis* infection in women, many countries offer screening. A variety of diagnostic methods are available for the detection of asymptomatic infections. Economic investigations suggest that the screening of asymptomatic individuals is a cost-effective strategy. (Malek, 2001)

Vaccination, which is currently unavailable, would be the best way to reduce the prevalence of *C. trachomatis* infections, as it would be much cheaper and would have a greater impact on controlling *C. trachomatis* infections worldwide (Hafner et al., 2008, Schautteet et al., 2011, Howie et al., 2011).

Regarding serovars A-C, trachoma is endemic in over 50 countries (Thylefors et al., 1995). Worldwide, over 80 million people have active trachoma, over 7 million have trichiasis (ingrown eyelashes), and over 1 million have blindness due to corneal scarring (Resnikoff et al., 2004, Wright et al., 2008). The prevalence of trachoma varies considerably. For example, in most countries of the Middle-East, Indian sub-continent, South-East Asia, Latin America and Australia, the prevalence of active trachoma has decreased markedly due to the improvements in public health. In contrast, in rural areas of North, East and South Africa trachoma is still hyperendemic. Sadly, active infection with *C. trachomatis* is mostly seen in children around four to six years; the prevalence in the hyperendemic areas can be 70% (West et al., 2001).

3.2. Chlamydial persistence and the difficulties in the therapy of chlamydial infections

Chlamydiae have the ability to cause prolonged and often subclinical infection. *In vitro,* it can be demonstrated that Chlamydiae can enter a latent state under stressful conditions such as exposure to interferon (IFN)- or certain antibiotics. This persistent form allows *C. trachomatis* to remain dormant in the host cell, resulting in a long-term relationship

with the host. It is known that serological diagnosis of chronic persistent, latent infections can be somewhat imprecise because of low detectable levels of specific antibodies. It has previously been reported that even very low levels of specific antibodies may be directly associated with a persistent infection. How often persistence occurs *in vivo* is unknown, but it may serve as an adaptive survival mechanism for the organism.

C. trachomatis infections can be effectively treated with antibiotics that are able to pass through the cell membranes and thus reach the RBs; cyclines, quinolones and macrolides are widely used for this purpose. In the case of pregnant women, amoxicillin is used in the antichlamydial therapy (Guaschino and Ricci, 2002).

Whittington et al. (2001) investigated the chance for therapeutic failures. They noted that 50 of 792 patients with *C. trachomatis* genital tract infections remained infected 42 days after receiving proper doxycycline or azythromycin treatment. The failure of the anti-chlamydial therapy cannot be explained only by re-infection. Several factors were indicated to be associated with infection continuation, such as the age of the patient, the renewal of sexual intercourse, low compliance, and drug pharmacokinetics and availability at the tissue level. However, studies have shown that failure could occur in spite of drug availability.

According to some studies, chlamydial resistance can have a role in therapy failure. Somani et al. published a case report of a pregnant woman and a couple who were respectively infected by *C. trachomatis* serovars E and F. In the therapy, the minimal concentrations of doxycycline, ofloxacin and azythromycin required to inhibit these patients' isolates were prominently higher than for susceptible control *C. trachomatis* strains. Based on this work, the authors suggested that certain *C. trachomatis* strains can bear heterotypic resistance (Somani et al., 2000). This indicates that the chlamydial population contains both susceptible and resistant organisms. Studies on this subject have revealed that only around 1% of the population is multiresistant (Jones et al., 1990; Lefevre et al., 1997). The mechanism which can be responsible for heterotypic resistance is yet unknown, but persistence can be suggested. *C. trachomatis* has been reported to adopt an intracellular form that is metabolically less active and thus less sensitive to antibiotics. This results in a long-lasting association between bacteria and their host.

3.3. Chemokines

Chemokines have molecular masses of 8-10 kDa and show approximately 20-50 % sequence homology at the protein level. The proteins also share common gene structures and tertiary structures. All chemokines possess a number of conserved cysteine residues involved in intramolecular disulfide bond formation, which allows chemokines to be grouped into 4 families (Figure 2.): alpha- (CXC), beta- (CC), gamma- (XC) and delta- (CX3C) chemokines, depending on the presence of conserved NH2-terminal cysteine residues (Baggiolini, 2001).
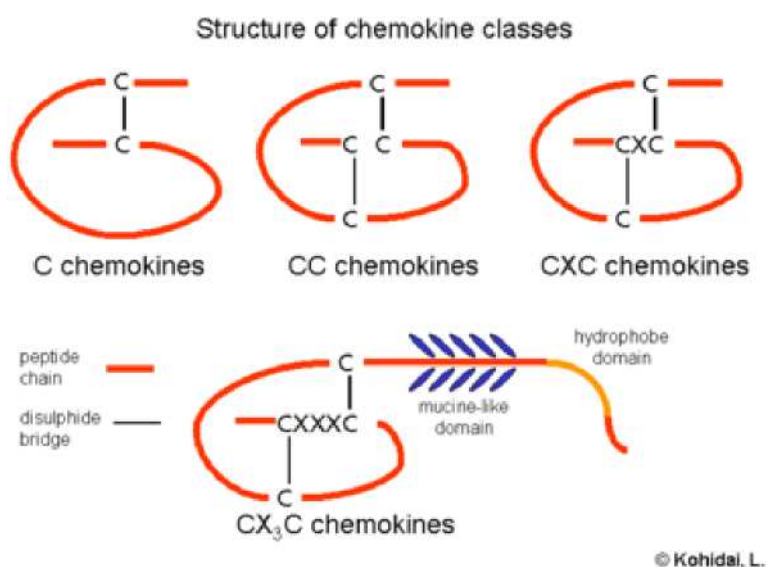
Figure 2. Structure of chemokine classes
[http://www.websters-online-dictionary.org/images/]

The CXC chemokine family is further divided into 2 classes, depending on the presence o absence of an NH2-terminal ELR sequence (Glu-Leu-Arg). The ELR-containing CXC chemokines (e.g. interleukin-8) chemoattract neutrophils, while the non-ELR CXC chemokines chemoattract lymphocytes. The monokine induced by IFN-ɣ (MIG/CXCL9), the

epithelial cells and giant cells (Park et al., 2002).

The binding of ligands to the receptors results in the activation and recruitment of the receptor bearing cells to the sites of inflammation. MIG/CXCL9 seems to have a multifaceted role in various immunological processes, such as autoimmune diseases, transplantational immunity, and neuroimmunity. It has also been revealed, that MIG/CXCL9 plays a role in the inhibition of fibrosis and angiogenesis, mainly in pancreatitis, and chronic hepatitis of various origins (Shen et al., 2013; Liang et al., 2012; Sahin et al., 2012). MIG/CXCL9 and other CXC chemokines (IP-10/CXCL10 and I-TAC/CXCL1 1) additionally possess antibacterial activity *in vitro* (Cole et al., 2001).

Several papers have been published regarding the antimicrobial effects of MIG/CXCL9 against *Escherichia coli, Listeria monocyto genes, Streptococcus pyo genes, Streptococcus pneumoniae, Neisseria gonorrheae, Fine goldia magna, Bacillus anthracis, Porphyromonas gingivalis,* and *Mycobacterium tuberculosis* (Cole et al., 2001; Egesten et al., 2007 and 2009; Eliasson et al., 2007 and 2010; Linge et al., 2008; Higaki and Morohashi, 2003; Murdoch, 2008; Karlsson et al., 2007 and 2009; Crawford et al., 2009 and 2010; Jauregui et al., 2013; Bai et al., 2010; Balogh et al., 2011; Handel et al., 2005). However, the function of MIG/CXCL9 in *C. pneumoniae* infection has not been investigated thoroughly prior to our work. Skwor et al. (2008) found that chemokine protein levels for CCL1 1, CCL8, MIG/CXCL9, and CCL2 were elevated in chronic scarring trachoma when compared with age and sex-matched controls. Mice deficient in the receptor for type I IFNs (IFNAR-/-) exhibited enhanced CD4 T cell recruitment to the cervical tissues, which was associated with an increased expression of MIG/CXCL9 in the genital secretion of IFNAR-/-mice, but not with the expression of CXCL10, which was reduced in the genital secretion of these mice (Nagarajan et al., 2008). In a mouse model after *C. trachomatis* infection, supernatants from upper genital tract homogenates contained significantly higher levels of MIG/CXCL9, CXCL10, and CCL5 than those in the lower genital tract (Maxion and Kelly, 2001).

3.4. Antimicrobial peptides

Antimicrobial peptides (AMPs), natural antibiotics produced by nearly all organisms from bacteria to plants and animals, are crucial effectors of innate immune systems, with different spectra of antimicrobial activity and with the ability to perform rapid killing. To date, more than 800 AMPs have been discovered in various organisms, including 270 in

plants. It has become clear in recent years that these peptides are able not only to kill a variety of pathogens, but also to modulate immune responses in mammals. However, their modes of action are poorly understood. In some species these peptides serve as the primary antimicrobial defence mechanism, whereas in others they serve as an adjunct to existing innate and adaptive immune systems (Brogden and Brogden, 2011). Cationic AMPs interact with negatively charged microbial membranes and permeabilize the membrane phospholipid bilayer, resulting in lysis and the death of microbes (Powers and Hancock, 2003; Hancock and Rozek, 2002). In view of their rapid and broad-spectrum antimicrobial properties, interest has emerged in AMPs as potential antibiotic pharmaceuticals to combat infections and microbial drug resistance (Hadley and Hancock, 2010; Capparelli et al., 2012).

Most plant AMPs are cysteine cluster proteins. This group includes major plant immunity effectors such as defensins, and also symbiotic peptides, including the nodule-specific cysteine rich (NCR) peptides, which are produced in Medicago-*Sinorhizobium meliloti* symbiosis and provoke irreversible differentiation of the endosymbiont. The NCR family is composed of about 500 divergent peptides in *Medicago truncatula* (Mergaert et al., 2003; Alunni et al., 2007; Nallu et al., 2013). Some cationic NCRs have been shown to possess genuine antimicrobial activities *in vitro,* killing various Gram-negative and Gram-positive bacteria highly efficiently (Tiricz et al., 2013).

The present study was designated to improve our current understanding of the pathological immune response to infection with chlamydiae and to find new natural compounds which have anti-chlamydial activities and might serve as alternative anti-microbial agents.

4. Aims

Our aims were the following

1. to detect whether *C. pneumonia*e can induce the production of CXCL chemokines *in vitro* and in *in vivo* mouse model;

2. to investigate the effect of co-treatment with *C. pneumoniae* and IFN-on the expression of MIG/CXCL9 in different cell types;

3. to study the anti-chlamydial activity of CXCL chemokines*in vitro* and to reveal the binding partner of MIG/CXCL9 on Chlamydia EBs;

4. to investigate the anti-chlamydial activity of plant peptides;

5. to find the chlamydial ligand responsible for the binding of NCR proteins.

5. Materials and methods

5.1. *C. pneumoniae* experiments

5.1.1. Inoculum preparation and culturing of *C. pneumoniae* from the lungs

C. pneumoniae strain TWAR CDC/CWL-029 [American Type Culture Collection (ATCC)] and a cardiovascular strain CV6, isolated from an atheroma of a patient with coronary artery disease (Maass et al., 1998), were propagated on HEp-2 cells (Dr Jacobs, National Institute for Biological Standards and Control, London, UK) in Modified Eagle Medium (Sigma, Saint Louis, Missouri, USA), supplemented with 10% fetal calf serum, 0.5% glucose, 0.3 mg L-glutamin e, 4 mM HEPES an d 25 ⎕g of gentamycin/ml. Two hours after

5.1.2. Mice and infection conditions

Eight-week-old female Balb/c mice were obtained from the Charles River Laboratory. The mice were fed a regular mouse chow diet *ad libitum* and housed under biosafety level 2 conditions. Before infection, mice were mildly sedated with an intraperitoneal injection o 200 l of sodium pentobarbital (7.5 mg/ml). They were then infected intranasally with 5×10^5 IFU of *C. pneumoniae* (CWL029) in 25 l of SPG. Uninfected mice were used as controls. After inoculation, 7 mice were anaesthetized and sacrificed on each of days 1, 2, 4, 7, 14 and 28. Sera were taken by cardiac puncture. The lungs were removed, cut up with scissors and then homogenized with seesand (acid purified; Fluka Chemie AG, Buchs, Switzerland) in a mortar with a pestle for 2 min (each sample). One half of the homogenized lungs was processed for real-time PCR, while the other half was re-suspended in 1 ml of SPG for the detection of viable *C. pneumoniae,* and for cytokine and chemokine measurements.

All experiments fully complied with the University of Szeged Guidelines for the Use of Laboratory Animals.

5.1.3. In vitro induction of MIG/CXCL9

J774A mouse macrophage cells (ATCC) and in-house-prepared mouse fibroblast cultures were grown in 24-well tissue culture plates in Modified Eagle Medium (Sigma) supplemented with 10% foetal calf serum, 0.5% glucose, 0.3 mg of L-glutamine/ ml, 4 mM HEPES and 25 g of gentamycin/ml. The semiconfluent tissues were infected with *C. pneumoniae* strain CWL029 or cardiovascular strain CV6 at a multiplicity of infection (m.o.i.) of 0.5 IFU or treated with the same volume of heat-inactivated EBs of the *C. pneumoniae* strains alone, or in the presence of different concentrations (10, 100 or 1000 IU/ml) of recombinant IFN-(Millipore, Bedford, Maryland, USA). Untreated cells served as controls. Supernatants of the treated cells were harvested after 24, 48 or (in some experiments) 72 h. Samples were centrifuged and stored at -80 °C un til use. MIG/CXCL9 concentrations of the samples were determined with the Quantikine mouse CXCL9/MIG kit (R&D Systems, Minneapolis, USA).

5.1.4. Measurement of the in vitro antibacterial activity of MIG/CXCL9, IP-10/CXCL10 and I-TA C/CXCL11

EBs of *C. pneumoniae* strain CWL-029 (4×10^4 IFU/ml) were incubated with recombinant MIG/CXCL9 (PeproTech London, UK) at various concentrations (10, 5, 2.5 or 1.25 g/ml) or IP-10/CXCL10 or I-TAC/CXCL1 1 (PeproTech) (10 g/ml) in SPG for 2 h at 37 °C. As controls, *C. pneumoniae* CWL-029 was also incubated either in buffer alone or mixed with heat-treated (90 C, 30 min) MIG/CXCL9 (10 g/ml). The time course of the anti-chlamydial effect of MIG/CXCL9 was tested after incubation periods of 0.25, 0.5, 1 and 2 h. To quantitate the anti-chlamydial effects of MIG/CXCL9, IP-10/CXCL10 and I - TAC/CXCL1 1, HEp2 cells were seeded in 24-well tissue culture plates with 1 3-mm cover glasses. After 24 h, the confluent cells were infected with MIG/CXCL9-treated *C. pneumoniae* CWL029 or the controls. After 48 h, the cells were fixed with acetone at -20 °C for 10 min. Fixed cells on cover glasses were stained as mentioned above.

5.1.5. Cytokine and chemokine measurements on the lungs

The supernatants of the lung homogenates and the sera were clarified by centrifugation (5 min, 12 000î*g*) and were assayed for IFN- content with the OptEIA ELISA set (BD Biosciences Pharmingen San Diego, CA, USA). The concentration of MIG/CXCL9 was determined using Quantikine mouse CXCL9/MIG kit (R&D Systems). The sensitivities of the IFN- and MIG/CXCL9 measurements lay in the ranges 125-1000 pg/ml and 31.2-2000 pg/ml, respectively. The clarified supernatants and sera were tested in duplicate in accordance with the manufacturer's instructions.

5.1.6. mRNA extraction and real-time PCR

Total RNA was extracted from the lung suspensions with the TRI Reagent (Sigma). First-strand cDNA was synthesized from 2 g of RNA by using reverse transcriptase, Superscript III (Invitrogen Carlsbad, Ca, USA) and 20 pM random primer in 20 l of reaction buffer. The synthesized first-strand cDNAs were amplified by quantitative real-time PCR, using 20 pM primer pair, with SYBR₋ Green JumpStart™ Taq ReadyMix™ (Sigma) in a

total volume of 20 l. Thermal cycling was initiated with a first denaturation step of 5 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The mouse MIG/CXCL9-specific primers were as follows: MIG/CXCL9 sense 5'-ACG TAG GTT TCG AGA CCA GGG ATT-3', MIG/CXCL9 antisense 5'-CAA CAC CAA GTG TTC TGC CAC CAA-3'; IP10/CXCL10 sense 5'-TGG CTA GTC CTA ATT GCC CTT GGT-3', IP10/CXCL10 antisense 5'-TCA GGA CCA TGG CTT GAC CAT CAT-3'; ITAC/CXCL11 sense 5'-TAC CCG AGT AAC AGC TGC GAC AAA-3', ITAC/CXCL1 1 antisense 5'-TAT GAG GCG AGC TTG CTT GGA TCT-3'. The real-time PCR amplification data were analysed by means of the Roche Molecular Biochemicals LightCycler Software version 3.5. The lowest cycle number at which the various transcripts were detectable, referred to as the threshold cycle (Ct), was compared with that of ß-actin, the difference being referred to as Ct. The relative expression level was given as 2-(ct), where Ct=Ct for the experimental sample minus t for the control sample.

5.1.7. Detection of MIG/CXCL9 -binding to Chlamydia EBs by FACS

Chlamydia EBs ($1x10^6$ IFU) were treated with 10 g of MIG/CXCL9 containing PBS for 2 h at 37 °C. After washing, the cells were stained with phycoerythrin(PE)-labelled anti-MIG/CXCL9 polyclonal antibody (R&D Systems) for 30 min at 4 °C. As controls, untreated and unstained, and untreated PE-labelled anti-MIG/CXCL9-stained cells were used. Cells were analysed with FACS StarPlus (Becton Dickinson) device.

5.1.8. Far-Western blot assay for identification of MIG/CXCL9-binding C. pneumoniae proteins

Concentrated *C. pneumoniae* CWL-029 EBs (1,75x 10_5 IFU) (prepared as described earlier) and a mock preparation were heated at 95 °C for 5 min in sample buffer, and polyacrylamide gel electrophoresis was performed (Laemmli, 1970). The proteins were separated by 10% SDS polyacrylamide gel in duplicate, and half of the gel carrying the separated proteins of the *C. pneumoniae* or the mock samples was blotted onto a polyvinylidene difluoride membrane (SERVA, Heidelberg, Germany). The membrane was blocked overnight at 4 °C with PBS containing 5% skimmed milk and 0.05% Tween 20. The

membrane was probed with a buffer [1% bovine serum albumin in PBS with 0.05% Tween 20 (PBST)] containing 10 g/ml MIG/CXCL9 (PeproTech) for 2 h. After washing 3 times with PBST, the filter was incubated with HRP-conjugated anti-MIG/CXCL9 IgG (R&D Systems). A control lane with separated *C. pneumoniae* EBs was also incubated with HRP-conjugated anti-MIG/CXCL9 without prior treatment with MIG/CXCL9. Following 3 further washings the colour was developed by using diaminobenzidine tetrahydrochloride (Sigma) with hydrogen peroxide in 10 mM Tris at pH 7.5. The second half of the gel with the separated proteins of the *C. pneumoniae* or the mock preparation was stained with Coomassie blue (Rosenfeld et al., 1992).

5.1.9. Identification of proteins by mass spectrometry

The gel slices containing the polypeptides of the concentrated *C. pneumoniae* EBs corresponding to proteins showing MIG/CXCL9 positivity in the blotting assay were cut out from the gel and analyzed 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 (DTT), and free sulphydryls were alkylated with iodoacetamide. Proteins were digested with modified porcine trypsin (Promega Madison, WI, USA) for 4 h at 37 °C. Samples were analyzed on LC-MSMS (liquid chromatography-tandem mass spectrometry) instruments. The LC-MSMS raw 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, methionine 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.

5.1.10. Statistical analysis

Statistical analysis of the data was carried out with GraphPad Prism 5 software, using the Wilcoxon-Mann-Whitney two-sample test. Differences were considered significant at *p*<0.05.

5.2. *C. trachomatis* experiments

5.2.1. Inoculum preparation

C. trachomatis Serovar D (ATCC-VR-885™) was propagated on HeLa cells as described earlier (Caldwell et al., 1981). The partially purified and concentrated EBs were aliquoted and stored at -80 °C until use. A mock preparation was prepared from an uninfected HeLa cellD monolayer processed in the same way as the infected cells. The titre of the infectious EBs was determined by indirect immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto tissue culture monolayers and, after a 48-h culture, cells were fixed with acetone and stained with murine monoclonal anti-Chlamydia LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled secondary anti-mouse IgG (Sigma). The number of inclusions was counted under a UV microscope, and the titre was expressed in inclusion forming units/ml (IFU/ml).

5.2.2. Measurement of in vitro antibacterial activity of NCR peptides

First, the toxicity of the NCR peptides was tested on non-infected HeLa cells in the highest concentration (10 g/ml) used during our experiments. The toxic peptides were excluded from the further experiments.

EBs of *C. trachomatis* D (4 î 10_4 IFU/ml) were incubated with chemically synthesized mature NCR030 (AFLPTSRNCITNKDCRQVRNYIARCRKGQCLQSPVR pI=10,37); NCR044 (AFIQLSKPCISDKECSIVKNYRARCRKGYCVRRRIR pI=10,32); NCR055 (VNDCIRIHCKDDFDCIENRLQVGCRLQREKPRCVNLVCRCLRR pI=9,21); NCR095 (ELVCDTDDDCLKFFPDNPYPMECINSICLSLTD pI=3,62); NCR1 37 (MTLRPCLTDKDCPRMPPHNIKCRKGHCVPIGKPFK pI=9,7); NCR1 68 (YPFQECKVDADCPTVCTLPGCPDICSFPDVPTCIDNNCFCT pI=3,61); NCR1 69

(EDIGHIKYCGIVDDCYKSKKPLFKIWKCVENVCVLWYK   pI=8,45);   NCR 183

(ITISNSSFGRIVYWNCKTDKDCKQHRGFNFRCRSGNCIPIRR   pI=10,1);   NCR192

(MKNGCKHTGHCPRKMCGAKTTKCRNNKCQCVQL   pI=9,54);   NCR247

(RNGCIVDPRCPYQQCRRPLYCRRR   pI=10,15);   or   NCR280

(MRVLCGRDGRCPKFMCRTFL pI=9,8) (Proteogenix Oberhausbergen, France) at various concentrations (10, 5, 2.5, or 1.25 ⏆g/ml) in sucrose-phosphate-glutamic acid buffer (SPG) for

7.5. The second half of the gel with the separated proteins of *C. trachomatis* or the mock preparation was stained with PageBlue Protein Staining Solution (Fermentas).

5.2.4. Identification of proteins by mass spectrometry

The gel slices containing the polypeptides of the concentrated *C. trachomatis* EBs corresponding to proteins exhibiting NCR247 positivity in the blotting assay were cut ou from the gel and analyzed by mass spectrometry. The proteins were identified as mentioned above in section *5.1.9.*

5.2.5. Detection of NCR peptide binding to Chlamydia EBs by FACS Chlamydia EBs (1 î 10₆ IFU) were treated with 1 g of FIT

6. Results

6.1. *C. pneumoniae* experiments

6.1.1. **C. pneumoniae** *infection induces CXC chemokine expression at mRNA and protein levels* **in vivo** *in Balb/c mice*

To investigate the production of ELR-negative CXC chemokines in *C. pneumoniae* infection, Balb/c mice were inoculated intranasally with*C. pneumoniae* CWL-029. On day 1, 2, 4, 7, 14 or 28 after infection, the mice were sacrificed and the lungs were collected for the determination of *C. pneumoniae* titres, MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL1 1 mRNA and MIG/CXCL9 and IFN- protein in the individual lungs.

In previous studies, culturable *C. pneumoniae* was demonstrated 14 days after primary infection in Balb/c mouse lungs (Kis et al., 2008). In our experiment, the infectious bacterial titres were below the level of detectability at 24 h after infection, but had increased to 5.21×10^4 IFU/lung by day 2. The peak titre of *C. pneumoniae* was measured on day 7, at 1.8×10^6 IFU/lung, and on day 14 after infection, the titre had decreased to $6.15 \times 10_3$ IFU/lung (Figure 3A).

The expression of MIG/CXCL9 mRNA was increased as early as on day 1, but the highest level of expression (156-fold) was detected on day 7 after infection (Figure 3B). The expression of IP-10/CXCL10 and I-TAC/CXCL1 1 mRNA was observed from the first day, but, in contrast with MIG/CXCL9 expression, peaked on day 4 at 85-fold and 579-fold, respectively (Figure 3C,D). The kinetics of production of MIG/CXCL9 protein correlated with the mRNA expression: it was increased from day 1, with the highest concentration on day 7 (Figure 3E). MIG/CXCL9 was also detected in the sera on days 7 and 14 (360 ± 124 pg/ml and 798 ± 252 pg/ml, respectively). The concentration of IFN- , the main in ducer of MIG/CXCL9 expression, was increased in the lungs during the infection, with a peak level of 12.5 ng/ml on day 7 (Figure 3F).
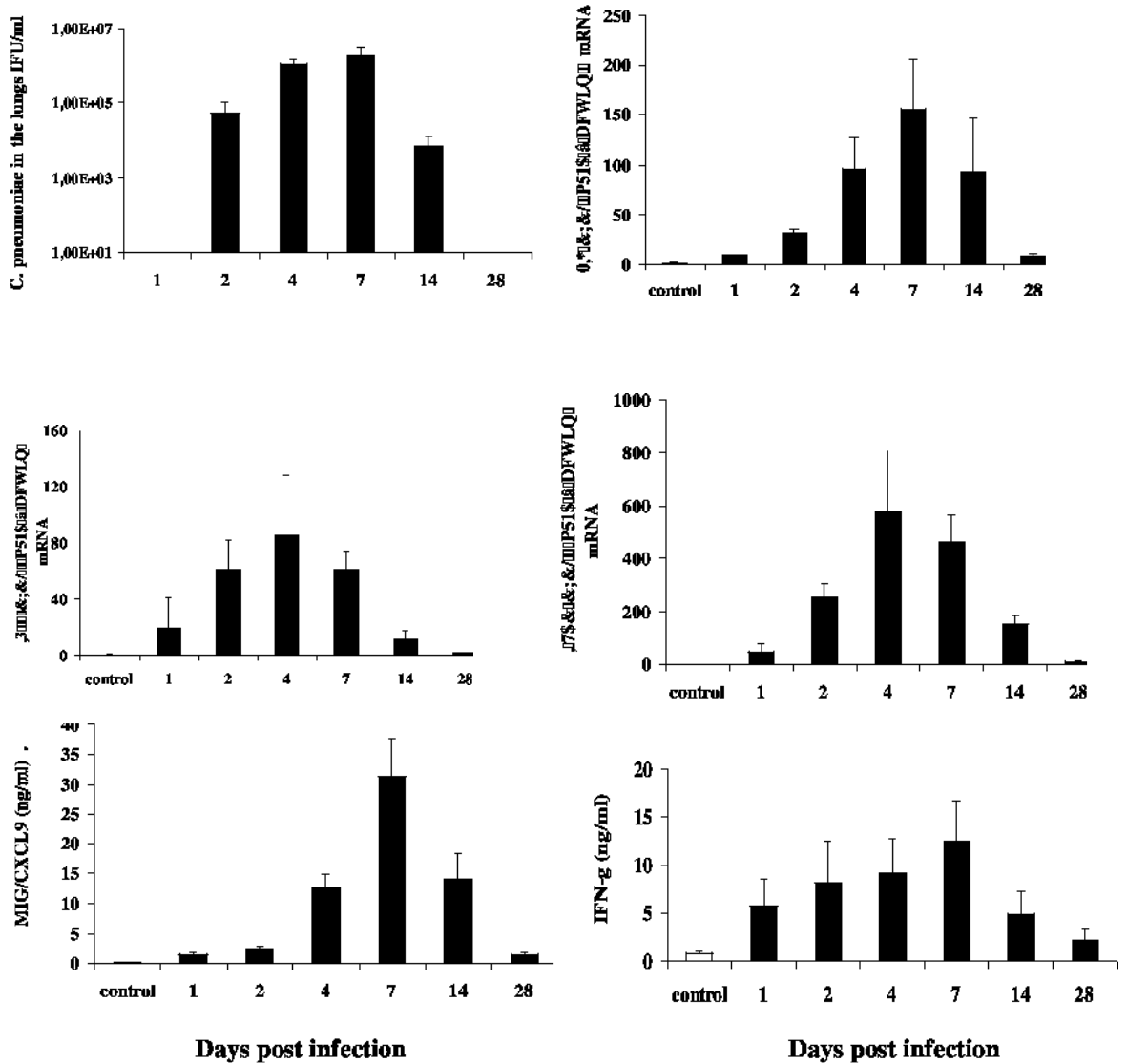
Figure 3. (A) Recovery of culturable *C. pneumoniae* from the lungs of mice after *C. pneumoniae* CWL-029 infection. Lung homogenates were inoculated onto HEp-2 cell monolayers and chlamydial inclusions were detected by indirect immunofluorescence, using LPS-specific monoclonal and FITC-labelled secondary antibodies. Expression of MIG/CXCL9 (B), IP-10/CXCL10 (C), and I-TAC/CXCL1 1 (D) in the lungs of infected mice. Total RNA extracted from the lungs was analysed by quantitative real-time PCR with the use of specific primers. Data are normalized for ß-actin RNA content and plotted as fold change over the results for uninfected mice. IFN- (E) and MIG/CXCL9 (F) production in the lungs of mice infected with *C. pneumoniae*. Lung homogenates were tested by using IFN- and MIG/CXCL9 ELISA kits according to the manufacturer's instructions. Bars denote

means and SDs of the results on 7 mouse lungs.

6.1.2. C. pneumoniae *induces MIG/CXCL9 in* in vitro *cell cultures*

To clarify whether *C. pneumoniae* is capable of inducing MIG/CXCL9 production *in vitro,* J774A mouse macrophage cells and mouse fibroblast tissue cultures were infected with *C. pneumoniae* CWL-029 strain, and the supernatants of the cells were tested by MIG/CXCL9-ELISA. After 24 h, no MIG/CXCL9 production was observed in either case (Figure 4A). However, *C. pneumoniae* induced MIG/CXCL9 secretion by the mouse fibroblasts from day 2 of in fection (122.7±22.3 pg/ml) (Figure 4B), and J774A cells produced MIG/CXCL9 from day 3 after infection (156.9±56.7 pg/ml) (data not shown). The heat-inactivated Chlamydia preparation also induced the production of MIG/CXCL9, at a level comparable to that for viable Chlamydia in both cell lines. During *in vivo* infection with *C. pneumoniae,* a large quantity of IFN-the main inducer of MIG/CXCL9) was detected in the mouse lungs. When the influence of IFN-on *C. pneumoniae*-induced MIG/CXCL9 production was investigated in *in vitro* cultures, co-treatment with different quantities of recombinant mouse IFN- (10, 100 or 1000 U/ml) and *C. pneumoniae* at a m.o.i. of 0.5 caused a dose-dependent increase in MIG/CXCL9 secretion in both cultures from day 1. Co-treatment of the mouse fibroblasts with *C. pneumoniae* and 10, 100 or 1000 U/ml of IFN-caused 51.8-, 27- and 25.8-fold increases in MIG/CXCL9 production, respectively, after 24 h relative to that evoked by treatment with the same quantities of IFN- alone. The enhancement of the ■ secretion of MIG/CXCL9 was similar when the co-treatment was carried out with heat-inactivated *C. pneumoniae* and different quantities of IFN-10, 100 or 1000 U/ml): 75-, 31.5- and 25.8-fold increases were observed after 24 h as compared with the increase induced by the different quantities of IFN- alone. Co-treatment of the J774A cells with *C. pneumoniae* and 10, 100 or 1000 U/ml of IFN- led to 21.6-, 7.4- and 10.8-fold increases in MIG/CXCL9 production, respectively, after 24 h in comparison with that evoked by the treatment with the same quantities of IFN- alone. The elevation of the secretion of MIG/CXCL9 was also more pronounced, as in the case of the elevation of mouse fibroblasts when the co-treatment was carried out with heat-inactivated *C. pneumoniae* and different quantities of IFN- 10, 100 or 1000 U/ml): 25.3-, 8.7- and 13.3-fold increases, respectively, after 24 h relative to the data for IFN- alone. Under all of the conditions mentioned above, the mouse fibroblasts produced significantly more MIG/CXCL9 than did the J774A ($p<0.05$;

Figure 4A,B). Similar results were seen under the conditions described above in both cell cultures after 48 h (Figure 4B).
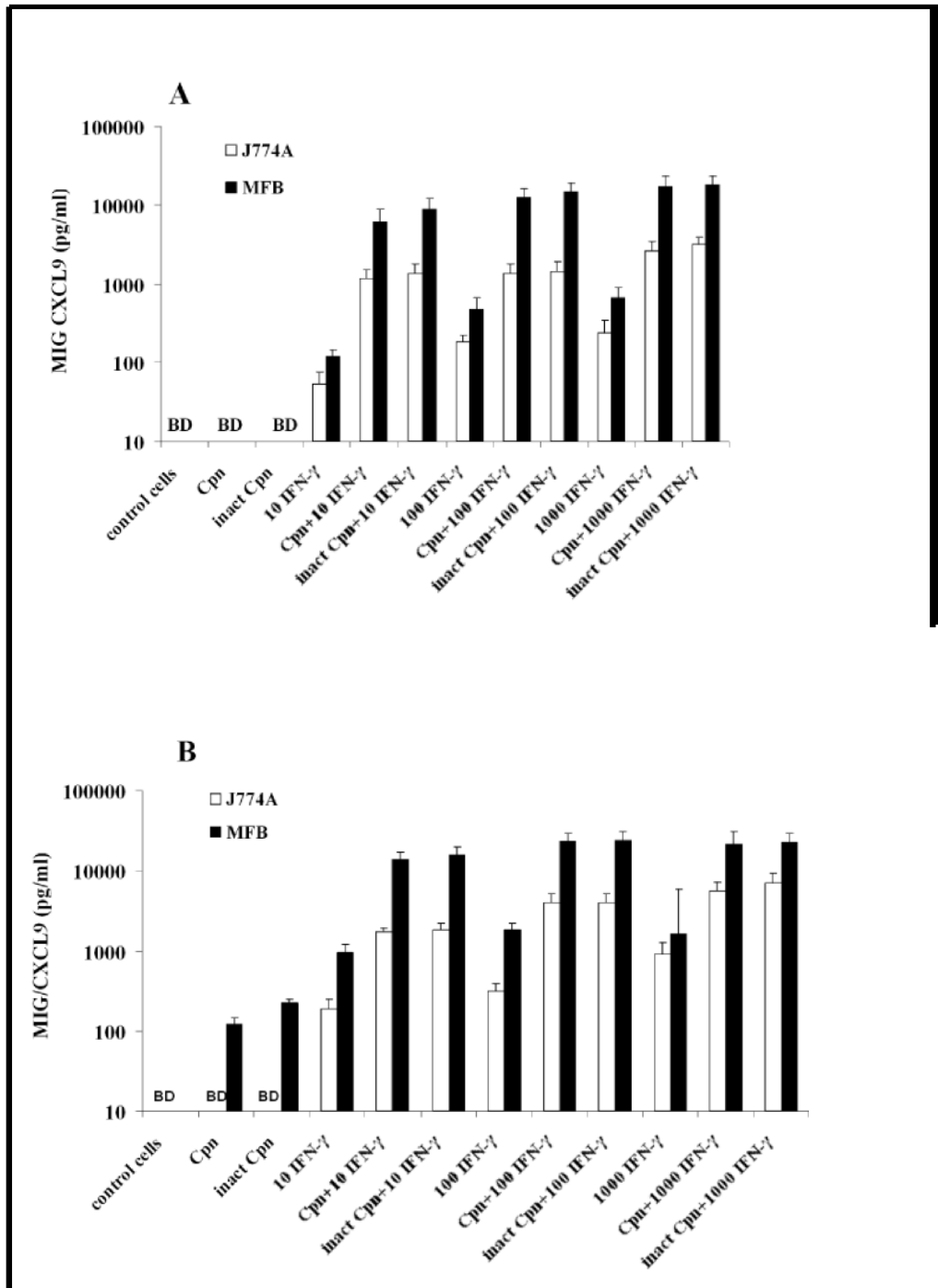
Figure 4. *C. pneumoniae*-induced MIG/CXCL9 production in J774A and mouse fibroblast (MFB) cells in the presence of IFN-. The cells were grown in 24-well tissue culture plates. The semi-confluent tissues were

infected with *C. pneumoniae* strain CW-L029 at a m.o.i. of 0.5 IFU, or were treated with the same amount of heat-inactivated *C. pneumoniae* alone, or in the presence of different quantities of IFN- (10, 100 or 1000 U/ml). Untreated J774A and MFB cells served as controls. Supernatants of the 3 biological replicates were taken afte 24 (A) and 48 (B) h. Samples were clarified by centrifugation, and the MIG/CXCL9 concentrations in th samples were detected with the ELISA kit accordingto the manufacturer's instruction. BD: below detectability All the data are representative of three separate experiments; SDs are shown. MFB cells produced a significantl higher level of MIG/CXCL9 than J774A cells in all combinations and at both time points ($p<0.05$).

A separate experiment revealed that the viable cardiovascular*C. pneumoniae* strain CV6 and its inactivated form also induced MIG/CXCL9 production in the mouse fibroblasts (156±34 and 187±67 pg/ml, respectively) after 48 h and also in the J774A cells (124±35 and 1 41±47 pg/ml, respectively) after 72 h (data not shown).

6.1.3. Effect of MIG/CXCL9 on C. pneumoniae *infectivity*

To determine whether MIG/CXCL9 has anti-chlamydial activity, *in vitro* bactericidal tests were carried out. Co-incubation of *C. pneumoniae* with recombinant mouse MIG/CXCL9 demonstrated a dose-dependent antibacterial activity of MIG/CXCL9 against*C. pneumoniae.* At a MIG/CXCL9 concentration of 10 g/ml, the number of viable *C. pneumoniae* inclusions was reduced by 98.4%, and at the lowest MIG/CXCL9 concentration tested (1.25 g/ml) a reduction of 79% was detected. The heat-treated recombinant MIG/CXCL9 did not exert an anti-chlamydial effect (Figure 5A). The experiments relating to the time course of the antibacterial effect of MIG/CXCL9 demonstrated a significant reduction (50%) in the number of viable Chlamydia inclusions after a 15-min co-incubation of MIG/CXCL9 and the Chlamydia EBs (Figure 5B). No anti-chlamydial effect was observed when other ELR-negative chemokines, IP-10/CXCL10 and I-TAC/CXCL1 1, were co-incubated with *C. pneumoniae* at a concentration of 10 g/ml (data not shown).
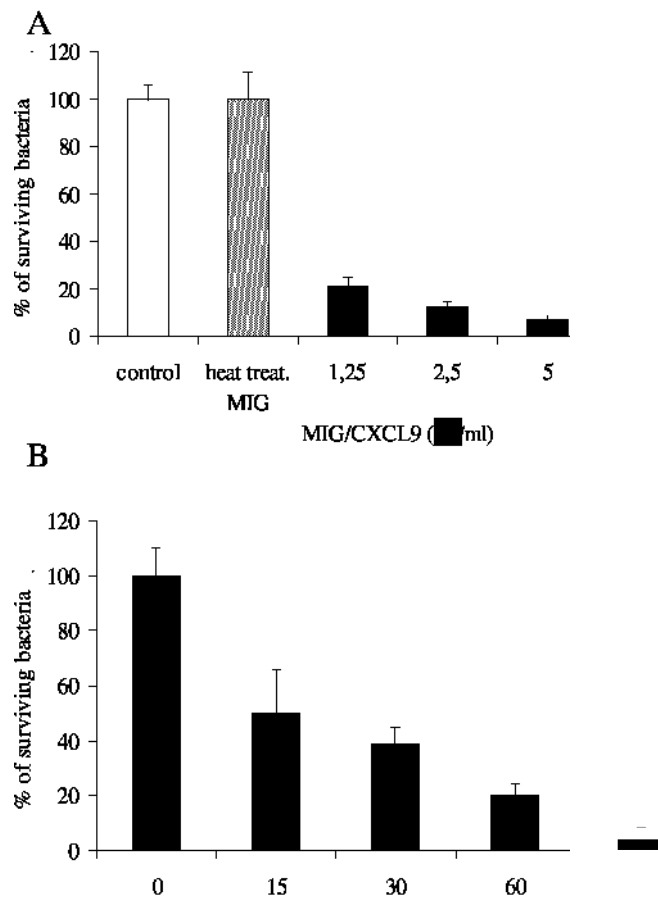
Figure 5. Concentration and time dependences of the anti-chlamydial effect of MIG/CXCL9. *C. pneumoniae* CWL-029 at $4x10^4$ IFU/ml was incubated with different quantities of recombinant MIG/CXCL9 for 2 h at 37 °C (A). *C. pneumoniae* was co-incubated with MIG/CXCL9 (5 g/ml) for 0, 15, 30, 60 or 120 min (B). The infectivity of the MIG/CXCL9-treated *C. pneumoniae* was determined by inoculating the mixture onto confluent HEp-2 cells on cover glasses. After a 24-h incubation, the fixed cells were stained with anti-chlamydia LPS antibody and the number of inclusions was counted under a UV microscope. All the data are representative of three separate experiments.

6.1.4. Identification of the chlamydial ligand responsible for MIG/CXCL9 binding

The concentrated *C. pneumoniae* EB preparation and the mock control preparation were separated by SDS-PAGE. After blotting, the membranes were probed with recombinant mouse MIG/CXCL9 and incubated with HRP-conjugated anti-MIG IgG. The control lane with Chlamydia EBs was stained with HRP-conjugated anti-MIG/CXCL9 IgG without incubation with MIG/CXCL9. The recombinant mouse MIG/CXCL9 was bound to 60 and 30

kDa protein bands in the Chlamydia lysate (Figure 6A). The recombinant mouse MIG/CXCL9 did not react with the mock lysate, and the Chlamydia EB lysate did not react with the HRP-conjugated anti-MIG/CXCL9. The gel slices containing the corresponding polypeptides of the concentrated *C. pneumoniae* EBs associated with the recombinant mouse MIG/CXCL9 were cut out from the gel and analysed by liquid chromatography-tandem mass spectrometry (LC-MSMS) in the Biological Research Centre, Hungarian Academy of Sciences, Szeged. Three kinds of chlamydial proteins were indicated by LC-MSMS and confirmed by post source decay analysis. The identified proteins included two forms of the 60 kDa cysteine-rich protein (also named OmcB/Omp2), which has been shown to be a chlamydial EB surface component to which heparin molecules can anchor (Stephens et al., 2001). Besides the 60 kDa whole form, a 30 kDa truncated form of the 60 kDa cysteine-rich protein (OmcB/Omp2) was identified. A third 60 kDa protein was identified as the putative GroEL protein of *C. pneumoniae* (Table 1) (Figure 6B).

Band	Name of the protein	Protein ID	MW(Da)	Sequence Coverage (%)	Number of unique peptides	Protein Score
1	60 kDa chaperonin (Putative GroEL protein *C. pneumoniae*)	P3 1681 AAA23 126	58204	21	14	915
	60 kDa cysteine-rich OMP *C. pneumoniae* (OmcB/ OMP2)	P23700 NP_224753	59719	6	3	242
2	Part of outer membrane protein 2 (OmcB/OMP2) *C. pneumoniae* (~ 30 kDa)	Q9S6B3 AAD20337	42273	6	3	172

Table 1. LC-MS/MS analysis of proteins combined with MIG/CXCL9 identified by SDS–PAGE separation. Band numbers refer to the gel bands shown in Figure 6. MW is calculated for the full sequence listed in the database and may not reflect the actual size of the protein.

A.

B.

putative GroEL protein [C. pneumoniae]

```
  1 MAAKNIKYNE EARKKIHKGV KTLAEAVKVT LGPKGRHVVI DKSFGSPQVT
 51 KDGVTVAKEI ELEDKHENMG AQMVKEVASK TADKAGDGTT TATVLAEAIY
101 SEGLRNVTAG ANPMDLKRGI DKAVKVVVDE LKKISKPVQH HKEIAQVATI
151 SANNDSEIGN LIAEAMEKVG KNGSITVEEA KGFETVLDVV EGMNFNRGYL
201 SSYFSTNPET QECVLEDALI LIYDKKISGI KDFLPVLQQV AESGRPLLII
251 AEEIEGEALA TLVVNRLRAG FRVCAVKAPG FGDRRKAMLE DIAILTGGQL
301 VSEELGMKLE NTTLAMLGKA KKVIVTKEDT TIVEGLGNKP DIQARCDNIK
351 KQIEDSTSDY DKEKLQERLA KLSGGVAVIR VGAATEIEMK EKKDRVDDAQ
401 HATIAAVEEG ILPGGGTALV RCIPTLEAFL PMLANEDEAI GTRIILKALT
451 APLKQIASNA GKEGAIICQQ VLARSANEGY DALRDAYTDM IDAGILDPTK
501 VTRSALESAR SIAGLLLTTE ALIADIPEEK SSSAPAMPSA GMDY
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60 kDa Cysteine-Rich OMP/ OmcB/ OMP2 [C. pneumoniae]

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  1 MSKLIRRVVT VLALTSMASC FASGGIEAAV AESLITKIVA SAETKPAPVP
 51 MTAKKVRLVR RNKQPVEQKS RGAFCDKEFY PCEEGRCQPV EAQQESCYGR
101 LYSVKVNDDC NVEICQSVPE YATVGSPYPI EILAIGKKDC VDVVITQQLP
151 CEAEFVSSDP ETTPTSDGKL VWKIDRLGAG DKCKITVWVK PLKEGCCFTA
201 ATVCACPELR SYTKCGQPAI CIKQEGPDCA CLRCPVCYKI EVVNTGSAIA
251 RNVTVDNPVP DGYSHASGQR VLSFNLGDMR PGDKKVFTVE FCPQRRGQIT
301 NVATVTYCGG HKCSANVTTV VNEPCVQVNI SGADWSYVCK PVEYSISVSN
351 PGDLVLHDVV IQDTLPSGVT VLEAPGGEIC CNKVVWRIKE MCPGETLQFK
401 LVVKAQVPGR FTNQVAVTSE SNCGTCTSCA ETTTHWKGLA ATHMCVLDTN
451 DPICVGENTV YRICVTNRGS AEDTNVSLIL KFSKELQPIA SSGPTKGTIS
501 GNTVVFDALP KLGSKESVEF SVTLKGIAPG DARGEAILSS DTLTSPVSDT
551 ENTHVY
```

truncated form of outer membrane protein 2/ OmcB/ OMP2
[C. pneumoniae]

```
  1 IGKKDCVDVV ITQQLPCEAE FVSSDPETTP TSDGKLVWKI DRLGAGDKCK
 51 ITVWVKPLKE GCCFTAATVC ACPELRSYTK CGQPAICIKQ EGPDCACLRC
101 PVCYKIEVVN TGSAIARNVT VDNPVPDGYS HASGQRVLSF NLGDMRPGDK
151 KVFTVEFCPQ RRGQITNVAT VTYCGGHKCS ANVTTVVNEP CVQVNISGAD
201 WSYVCKPVEY SISVSNPGDL VLHDVVIQDT LPSGVTVLEA PGGEICCNKV
251 VWRIKEMCPG ETLQFKLVVK AQVPGRFTNQ VAVTSESNCG TCTSCAETTT
301 HWKGLAATHM CVLDTNDPIC VGENTVYRIC VTNRGSAEDT NVSLILKFSK
351 ELQPIASSGP TKGTISGNTV VFDALPKLGS KESVEFSVTL KGIAP
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Figure 6. Interaction of MIG/CXCL9 and *C. pneumoniae* EBs. Far-Western blot analysis for identification of the chlamydial ligands responsible for MIG/CXCL9 binding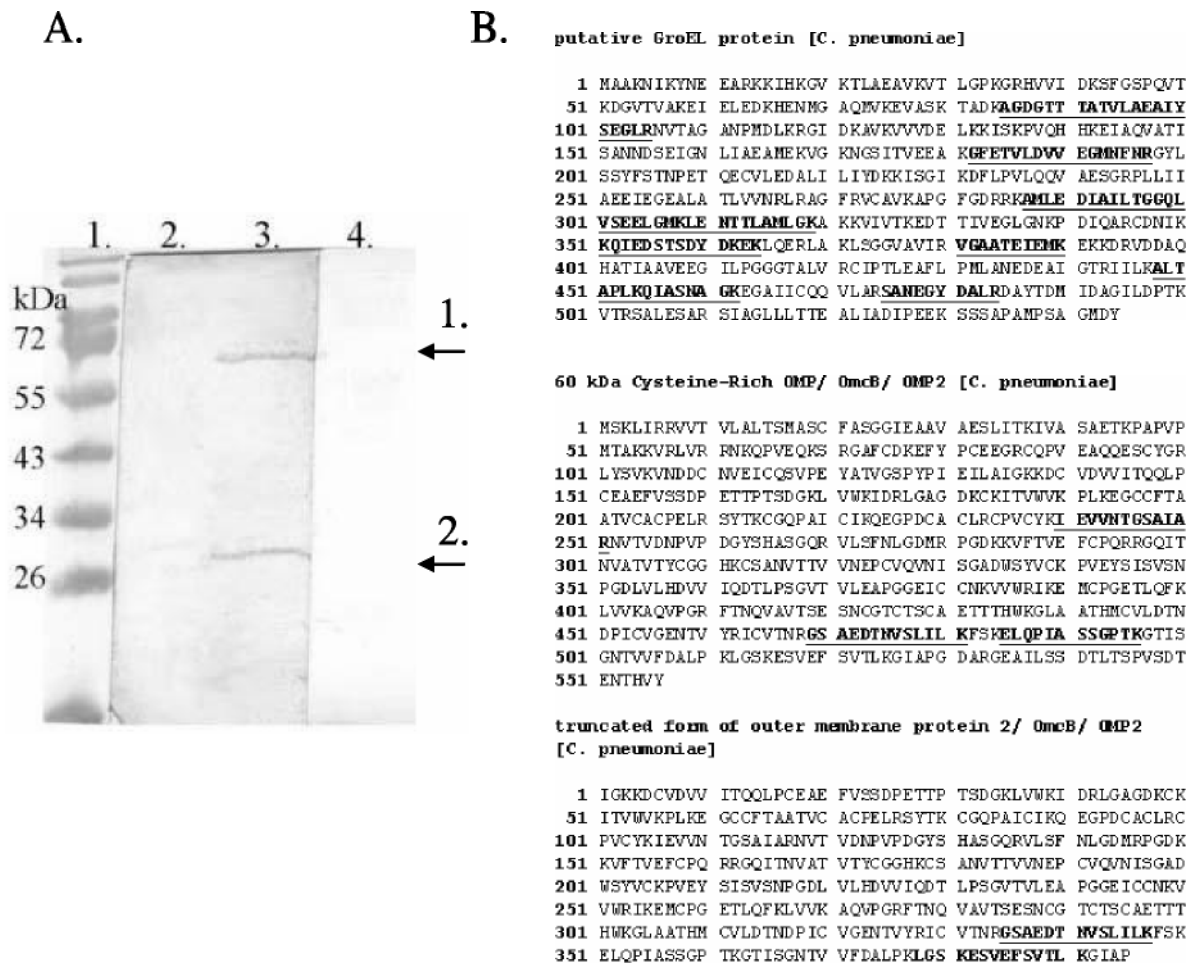. Concentrated *C. pneumoniae* and mock control preparations were separated by SDS-PAGE. After blotting, the membrane was probed with recombinant mouse MIG/CXCL9 and incubated with HRP-conjugated anti-MIG IgG (lane 1: molecular weight marker, lane 2: mock preparation, lane 3: Chlamydia EBs treated with MIG/CXCL9 and HRP-conjugated anti-MIG/CXCL9, and lane 4: Chlamydia EBs treated with HRP-conjugated anti-MIG/CXCL9 only (A). Identified MIG/CXCL9-binding proteins of *C. pneumoniae.* Peptide fragments that match the defined protein sequences are underlined (B).

To show that the MIG/CXCL9 is able to bind to the native EBs, a FACS analysis was carried out. Figure 7B reveals that the Chlamydia EBs interact with MIG/CXCL9 as EBs incubated with MIG/CXCL9 were readily detected after staining with PE-conjugated anti-MIG/CXCL9 IgG. Chlamydia EBs untreated with MIG/CXCL9 were not stained with PE-conjugated anti-MIG/CXCL9 IgG (Figure 7C).
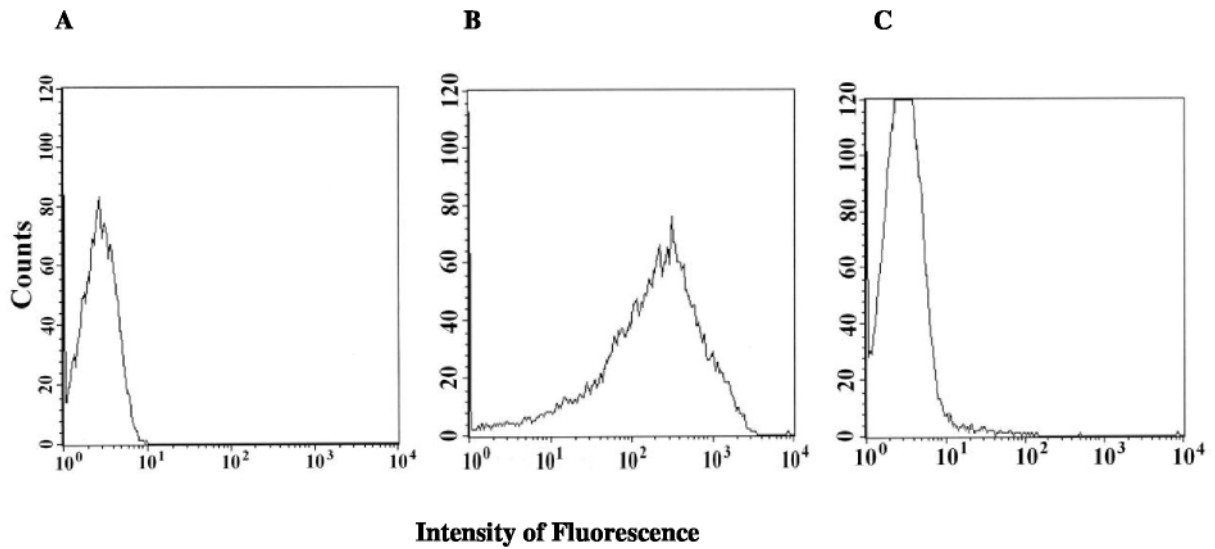

Figure 7. FACS analysis for the detection of MIG/CXCL9 binding to whole *C pneumoniae* EBs. Untreated and unstained Chlamydia EBs (A). MIG/CXCL9 protein-treated and PE-labelled anti-MIG/CXCL9-stained *C. pneumoniae* EBs (B). Chlamydia EBs stained with PE-labelled anti-MIG/CXCL9 antibody (C).

6.2. *C. trachomatis* experiments

6.2.1. Anti-chlamydial effect of plant peptides.

To determine whether they possess anti-chlamydial activity, 11 NCR peptides (NCR030, NC0R44, NCR055, NCR095, NCR137, NCR168, NCR169, NCR183, NCR192 NCR247 and NCR280) were co-incubated individually with *C. trachomatis* EBs at 10 g/ml for 2 h at 37 °C. Coun ting of the number of viable *C. trachomatis* inclusions demonstrated that 7 of the 11 peptides (NCR044, NCR055, NCR095, NCR183, NCR192, NCR247 and NCR280) were effective killers of *C. trachomatis in vitro,* while NCR030 and NCR168 displayed weaker activity, and NCR137 and NCR169 did not exert an anti-chlamydial effect (Figure 8A). *C. trachomatis* inclusions were then treated for 2 h with concentrations of the peptides ranging from 1.25 g/ml to 10 g/ml (Figure 8B). NCR044, NCR055 and NCR183
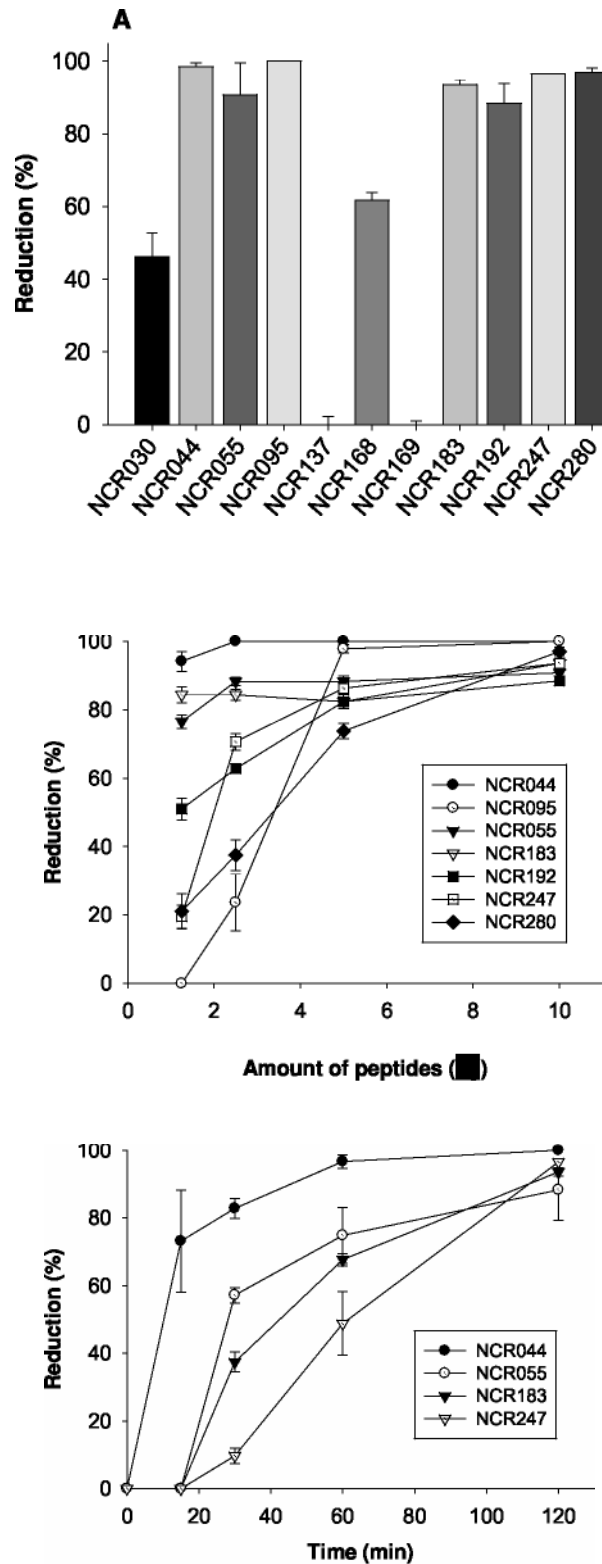
Figure 8. Concentration and time dependences of the anti-chlamydial effects of NCR peptides. *C. trachomatis* at 4×10^4 IFU/ml was incubated with 10 g/ml of synthetic NCR peptide for 2 h at 37 °C *(A). C. trachomatis* EBs

were incubated with different quantities of synthetic NCR peptides for 2 h at 37 °C (B) *C. trachomatis* was co-incubated individually with different NCR peptides (5 g/ml) for 0, 15, 30, 60 or 120 min (C). The infectivity of the NCR peptide-treated *C. trachomatis* was determined by inoculating the mixture onto confluent HeLa cells on cover glasses. After a 24-h incubation, the fixed cells were stained with anti-chlamydia LPS antibody and the number of inclusions was counted under a UV microscope. All the data are representative of three separat experiments.

6.2.2. Identification of the chlamydial ligand responsible for NCR247 binding

Further investigations were carried out with NCR247, which displayed antichlamydial activity in the previous tests.

To identify the chlamydial ligand responsible for NCR peptide binding, concentrated *C. trachomatis* EB preparations and mock control preparations were separated by SDS-PAGE. After blotting, the membranes were probed with synthetic NCR247 peptide and incubated with anti-NCR247 IgG, and then with HRP-labelled anti-rabbit antibody. The control lane with Chlamydia EBs was stained with anti-NCR247 IgG and HRP-labelled anti-rabbit antibody without incubation with synthetic NCR247 peptide. The synthetic NCR247 peptide was bound to a 60-kDa protein band in the Chlamydia lysate (Figure 9A, lane 4). The synthetic NCR247 did not react with the mock lysate (lane 2), and the Chlamydia EB lysate did not react with the HRP-conjugated anti-rabbit antibody (lane 3). The gel slice containing the corresponding polypeptide of the concentrated *C. trachomatis* EBs associated with the synthetic NCR247 peptide was cut out from the gel and analyzed by LC-MSMS. A 60 kDa putative GroEL protein of Chlamydia was indicated by LC-MSMS and confirmed by post source decay analysis (Figure 9B).
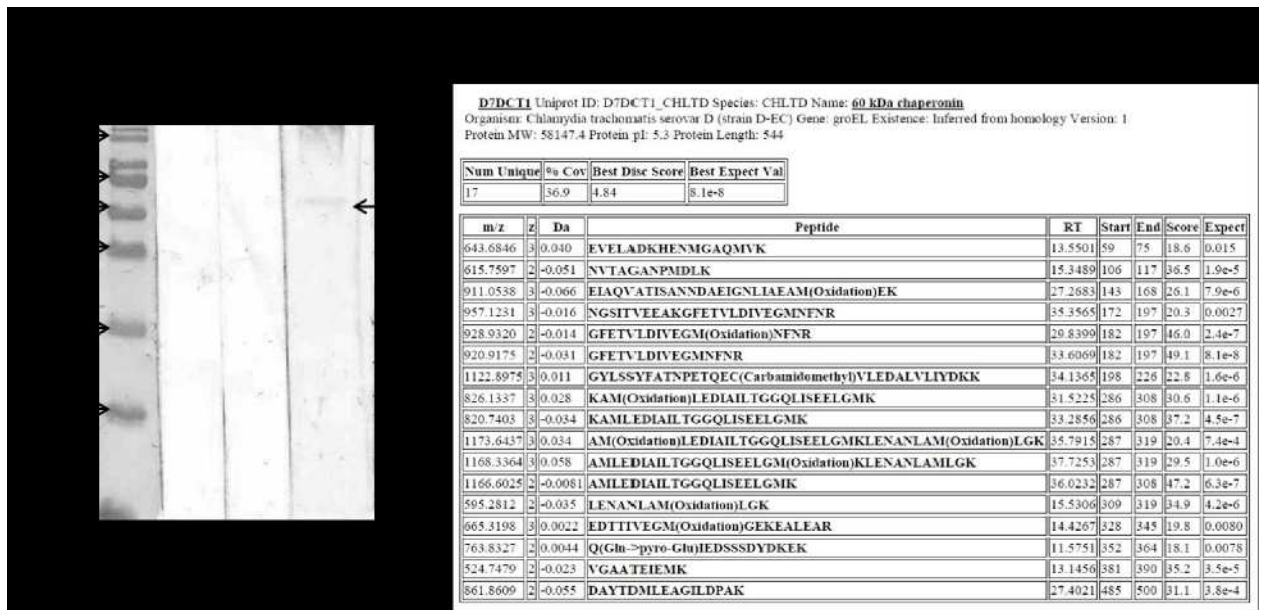
Figure 9. Interaction of NCR247 peptide and *C. trachomatis* EBs. Far-Western blot analysis of the chlamydial ligands responsible for NCR247 peptide binding. (A) Concentrated *C. trachomatis* and mock control preparations were separated by SDS-PAGE. After blotting, the membrane (lane 2,4) was probed with synthetic NCR247 peptide and incubated with anti-NCR247 IgG and HRP-labelled anti-rabbit antibody. (lane 1-molecular weight marker, lane 2 - mock preparation, lane 4 - Chlamydia EBs lysate). A control lane (lane 3) with separated *C. trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit antibody without prior treatment with the NCR247 peptide. Identification of the *C. trachomatis* proteins by LCMSMS (B). Peptide fragments that match the defined protein sequences are to be found in the Table.

6.2.3. FACS analysis for the detection of NCR247 binding to the whole *C. trachomatis* EBs

To show that NCR247 is able to bind not only to the degraded Chlamydia particles but to the native, viable Chlamydia EBs, a FACS analysis was carried out. Figure 10 reveals that Chlamydia EBs interacted with FITC-conjugated NCR247 peptide. Untreated or FITC-labelled NCR035 peptide-treated (this peptide showed no anti-chlamydial effect earlier) Chlamydia EBs did not demonstrate increased fluorescence.
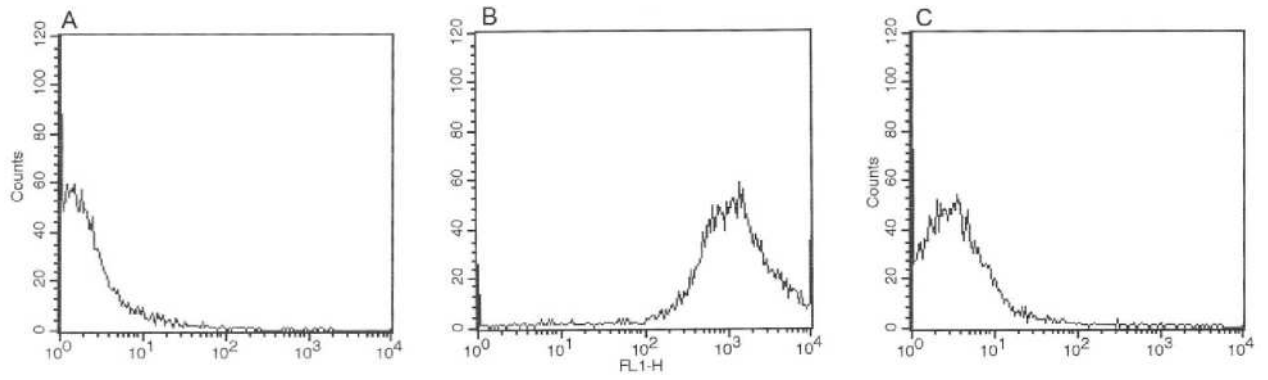
Figure 10. FACS analysis of NCR247 peptide binding to whole *C. trachomatis* EBs. Untreated and unstained Chlamydia EBs (A). FITC-labelled NCR247 peptide treated *C. trachomatis* EBs (B). Chlamydia EBs treated with FITC-labelled NCR035 peptide (C).

7. Discussion

Innate or natural immunity is the principal pathway for the effective elimination of most bacterial pathogens from the lung (Nelson et al., 1995). Antimicrobial peptides such as defensins are natural peptide antibiotics. When the host is challenged by microbes, these peptides act as the first line of innate defence before the activation of adaptive immunity. The defensins and certain chemokines, e.g. MIG/CXCL9, have similar characteristics, including size, disulphide bonding, IFN inducibility, a cationic charge at neutral pH, and the antimicrobial activity (Cole et al., 2001). Innate immune responses are enhanced in the presence of type 1 cytokines, including IL-12 and IFN- (Moore et al., 2002; Rubins and Pomeroy, 1997). Type 1 cytokine responses are indispensable for the effective killing of intracellular pathogens (Deng et al., 2001). Our aims were to reveal whether *C. pneumonia*e can induce the production of CXCL chemokines *in vitro* and in *in vivo* mouse model, and to detect the possible anti-chlamydial activity of CXCL chemokines *in vitro.* Our study revealed that *C. pneumoniae* can induce MIG/CXCL9 expression both *in vitro* and *in vivo,* and that MIG/CXCL9 has anti-chlamydial activity. Moreover, the potential chlamydial ligand of MIG/CXCL9 was suggested.

The time course of the effect of IFN-, which is the main inducer of MIG/CXCL9 production following *C. pneumoniae* infection, was described earlier (Burian et al., 2003; Geng et al., 2000). The *in vivo* kinetics of MIG/CXCL9 mRNA expression was similar to that of the recovery of viable Chlamydiae from the lungs after infection: the expression of the MIG/CXCL9 mRNA and the number of culturable Chlamydia inclusions were highest on day 7, while at 4 weeks after infection the MIG/CXCL9 mRNA expression remained higher in the infected mice than in the controls, whereas the number of culturable *C. pneumoniae* inclusions had decreased to an undetectable level. The expression of IP-10/CXCL10 and I - TAC/CXCL11 mRNA, in contrast with MIG/CXCL9 expression, peaked on day 4. The finding that IP-10/CXC10 expression was highest on day 4 coincided with the result published by Maxion et al., that the level of IP-10/CXCL10 was highest on day 4 in the upper genital tract of mice after *C. trachomatis* infection (Maxion et al., 2002). The highest concentration of MIG/CXCL9 at a protein level was likewise observed on day 7. The Th1 cytokine IFN- plays important roles in combating acute Chlamydia infection and in the establishment of persistence (Rottenberg, 2002). IFN- may also play a regulatory role in

chlamydial defence, via its ability to alter the Th1/Th2 balance. Accordingly, IFN- gene knockout mice exhibited Th2-cytokine-dependent immunopathology together with disseminated chlamydial infection (Wang et al., 1999). IFN- may modify the outcome of infection by enhancing the expression of chemokines and adhesion molecules. IFN-administered therapeutically to patients with chronic granulomatous disease improved the ability of phagocytes to clear bacterial infections (Billiau and Matthys, 2009). Although there is still some controversy, it is quite plausible that the increase in antimicrobial activity results from oxygen-independent bactericidal mechanisms, including the IFN--inducible ELR-negative CXC chemokines. If, as might be expected, even higher concentrations prevail in the microenvironment of chemokine-secreting cells, antimicrobial effects could occur through the individual or combined activities of one or more of these chemokines. It is important that MIG/CXCL9 is not compartmentalized in the lungs, since we detected it in the circulation between days 7 and 14 after *C. pneumoniae* infection, which suggests an important role of MIG/CXCL9 in the resolution of the infection. An elevated MIG/CXCL9 content has also been measured in the human plasma during *Burkholderia pseudomallei* infection (Lauw et al., 2000).

We found that *in vitro* infection with *C. pneumoniae* induced MIG/CXCL9 production from day 3 in J774A cells, and from day 2 in infected mouse fibroblasts. It is interesting that mouse fibroblasts produced significantly more MIG/CXCL9 than did the macrophage cell line J774A (Figure 4A,B). It has been reported that cellular processes of non-immune cells (epithelial and endothelial cells and fibroblasts) triggered by chlamydial infection provoke the influx of inflammatory neutrophils, T cells, B cells and macrophages stimulated by the milieu of pro-inflammatory cytokines and chemokines (Stephens, 2003). It was surprising that co-treatment of the mouse fibroblasts with a dose of *C. pneumoniae,* which was unable to induce MIG/CXCL9 production on day 1, and with 10 U of IFN- resulted in a 51.8-fold increase in MIG/CXCL9 release as compared with treatment with 10 U of IFN- alone. Our observation that heat-inactivated *C. pneumoniae* together with IFN- induced a 75-fold increase in MIG/CXCL9 secretion relative to IFN- treatment alone suggests the heat-stable LPS as the putative chlamydial component responsible for the stimulation of MIG/CXCL9 production. Besides the respiratory *C. pneumoniae* strain CWL029, the cardiovascular strain CV6 was shown to stimulate the production of MIG/CXCL9. Similarly to our results, Högdahlet al.

found that *C. pneumoniae* T45, a respiratory strain isolate, induces the production of chemokines such as IL-8, MCP-1, RANTES, IP-10 and MIG/CXCL9 in human coronary artery endothelial cells after 48 h (Högdahl et al., 2008).

Our experiments demonstrated that recombinant MIG/CXCL9 can inhibit*C. pneumoniae* in a dose-dependent manner. A previous work has revealed that MIG/CXCL9 and IP-10/CXCL10 bear antibacterial effect against *Escherichia coli* and *Listeria monocyto genes* (Cole et al., 2001). It was recently reported that CXCL9, CXCL10 and CXCL1 1 kill *Streptococcus pyo genes,* and that, in particular, CXCL9 was produced in a bactericidal concentration by inflamed pharyngeal cells both *in vivo* and *in vitro* (Egesten et al., 2009). The role of MIG/CXCL9 during bacterial infection has not been fully elucidated. In contrast with the examples mentioned above, in the case of *Fine goldia magna* MIG/CXCL9 displays no antibacterial activity. Moreover, the SufA of *F. magna* cleaves MIG/CXCL9 (Karls son et al., 2009, Karlsson et al., 2007). In our *in vivo* experiments, MIG/CXCL9 concentration in the whole lung suspension in the ELISA test was lower than the lowest bactericidal concentration in the *in vitro* test, but the local chemokine concentration should be higher in the affected area of the lung. Indeed, as MIG/CXCL9 has affinity for heparin, the local concentration might be even higher. Thus, interactions with glucosaminoglycans (GAGs) can retain MIG/CXCL9 at epithelial surfaces and further increase the local concentration. It has been suggested that the binding of chemokines to GAGs may furnish a chemotactic gradient to recruit leukocytes during inflammation (Egesten et al., 2007; Handel et al., 2005). Since MIG/CXCL9 was antichlamydial only when it was co-incubated (for at least 15 min) with the EBs, it is likely that the expressed MIG/CXCL9 does not exert an effect on already infected epithelial cells, but rather prevents the spread of infectious EBs extracellularly.

By means of mass spectrometry, we identified three kinds of chlamydial proteins. We found that MIG/CXCL9 binds to OmcB, which has been identified as a major component of the chlamydial outer membrane complex, and is conserved among all chlamydial species (Wagels et al., 1994). OmcB was initially thought to be an intimate part of the outer membrane fraction of the chlamydial outer membrane complex. However, according to others, OmcB was resistant to trypsin digestion, which supported its lack of surface exposure (Everett and Hatch, 1995). Nonetheless, Moelleken and Hegemann established a yeast adhesion system in which the binding of live OmcB-presenting yeast cells to human cells could be studied. They

showed that the OmcB protein from *C. pneumoniae* mediates adhesion to HEp-2 cells (Moelleken and Hegemann, 2008). Moreover, OmcB induces a strong antibody response suggesting that it is readily accessible to the humoral immune response, which is indicative o a surface localization (Cunningham and Wards, 2003; Portig et al., 2003). Since OmcB protein is present on the surface, MIG/CXCL9 can bind to it. This is supported by our finding that the truncated form of OmcB (the 30 kDa protein) was the other protein identified by LCMSMS. During the denaturation process for the binding assay, this intermediate form of OmcB could be released from the EBs. Our results are also in accordance with MIG/CXCL9 having affinity for heparin (Handel et al., 2005). It is interesting that heparin-like (GAG) structures on the mammalian cell surface play an important role in the interactions with many bacterial pathogens and are recognized by chlamydial OmcB (Moelleken and Hegemann, 2008). Moreover, the M1 protein, the major surface protein of *S. pyo genes,* which is necessary for the adhesion of bacteria to epithelial cells via GAGs, induces an enhanced production of MIG/CXCL9 (Eliasson et al., 2007 and 2010). The third protein identified by mass spectrometry is the putative GroEL protein of *C. pneumoniae.* Wuppermann et al. recently described the localization of GroEL1 on the surface of the EBs, and the ability of GroEL1 to bind to MIG/CXCL9 therefore cannot be excluded (Wuppermann et al., 2008). Since our results showed that MIG/CXCL9 binds not only to the full OmcB protein, but also to its truncated form, and the role of OmcB protein in the initiation of infection is well documented, we concluded that binding to this protein is of major significance.

Our FACS experiment confirmed that MIG/CXCL9 binds not only to the denatured proteins of the EBs, but also to the viable native form, supporting the antibacterial capability of this chemokine.

Overall, these results indicate that *C. pneumoniae* has the capability to induce the production of MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL1 1 *in vivo,* and the production of MIG/CXCL9 *in vitro.* MIG/CXCL9 production did not require the presence of viable Chlamydiae. Our work was the first report about the anti-chlamydial activity of recombinant MIG/CXCL9, and about the role of the 60 kDa proteins in the binding of MIG/CXCL9 to the chlamydial cells. Thus, MIG/CXCL9 released by sentinel and non-immune cells could directly target and inactivate microbial pathogens, while also serving as a signal for the recruitment of leukocytes to infected tissues.

Because of the severity of *C. trachomatis*-caused infections and their consequences we were concerned about searching for new anti-chlamydial compounds. As mentioned above, *C. trachomatis* is the leading cause of sexually transmitted bacterial diseases in both developed and developing countries, with more than 90 million new cases of genital infections occurring annually. The development of effective new antimicrobial compounds is indispensable to avoid the late severe sequelae of the infections, such as ectopic pregnancy and infertility (WHO, 1996). AMPs appear to be potentially promising candidates for this purpose. It was demonstrated that the synthetic tomato defensin peptide exhibits potent antibacterial activity against Gram-positive bacteria, such as *Staphylococcus. aureus* A170, *Streptococcus. epidermidis,* and *Listeria. monocyto genes,* and Gram-negative bacteria, including *Salmonella enterica serovar* Paratyphi, *E. coli,* and *Helicobacter pylori* (Rigano et al., 2012). In addition, the synthetic peptide shows minimal (<5%) hemolytic activity and absence of cytotoxic effects against THP-1 cells. Although their antimicrobial activity against bacteria, fungi and protozoa has been extensively studied (Rigano et al., 2012), their anti-chlamydial action has not yet been tested. In the present study, therefore, we investigated the *in vitro* activity of 11 NCR peptides against *C. trachomatis*. Seven of these peptides exerted significant antichlamydial activity at a 10 g/ml concentration. A number of synthetic NCR peptides from *Medicago truncatula* have been reported to be potent killers of various Gram-negative *(Escherichia coli, Salmonella* Typhimurium, *Agrobacterium tumefaciens, Pseudomonas aeruginosa* and *Xanthomonas campestris)* and Gram-positive *(Bacillus megaterium, Bacillus cereus, Clavibacter michiganensis, Staphylococcus aureus* and *Listeria monocyto genes)* bacteria, including human/animal and plant pathogens (Tiricz et al., 2013). Furthermore, AMPs were effective against *Staphylococcus epidermidis* in *in vivo* mouse model, and they also displayed anti-inflammatory activity (Capparelli et al., 2012).

Our LC-MSMS experiment identified the GroEL protein of *C. trachomatis* as the chlamydial ligand of the NCR247 peptide. The GroEL protein is one of the few proteins that have so far been confirmed as relevant in chlamydial pathogenesis; it is also referred to as heat shock protein 60 (Hsp60) (Ward, 1999). This protein belongs to group I chaperones produced by almost all prokaryotic and eukaryotic cells, which assist as intracellular proteins, in the correct folding of nascent or denatured proteins under both normal and stress conditions (Zügel and Kaufmann, 1999). Several reports have indicated that molecular chaperones

produced by pathogenic bacteria, can function as intracellular, cell surface, or extracellula signals in the course of infection processes (Henderson et al., 2006). The immune responses to chlamydial GroEL correlate significantly with disease sequelae in humans, and 80 to 90% of patients infected with *C. trachomatis* have antibodies directed against GroEL (Horner et al., 1997). The high degree of antigenicity of GroEL in patients implies that the protein i easily accessible to the immune system, perhaps becauseit is localized on the surface of the chlamydial particles. Early studies on isolated outer membrane complexes from *C. trachomatis* and *C. psittaci* EBs had indeed pointed to the possibility that GroEL might be associated with chlamydial membranes (Bavoil et al., 1990). Taken together, GroEL is accessible for the binding of NCR247 peptide.

Our study indicates that certain NCR peptides possess substantial *in vitro* activity against *C. trachomatis* D. Studies of chlamydial infection in animal models are clearly needed to establish whether they have parallel *in vivo* results and whether these peptides can be useful lead compounds for the development of anti-chlamydial drugs.

The following of our results are considered novel

1. We found that MIG/CXCL9 showed dose-dependent anti-chlamydial activity in *in vitro C. pneumoniae* infection.

2. During our experiments it was revealed that the 60 kDa chlamydial proteins were responsible for the binding of MIG/CXCL9.

3. Seven of the NCR peptides examined showed dose dependent anti-chlamydial effect in *in vitro C. trachomatis* infection.

4. We found the chlamydial GroEL protein to be responsible for the binding of NCR247 peptide.

8. Summary

Chlamydophila pneumoniae and *Chlamydia trachomatis* are obligate intracellular parasites that belong to the family of Chlamydiaceae. These bacteria have the ability to cause prolonged and often subclinical infections.

C. pneumoniae causes approximately 10% of community-acquired pneumonia cases among adults. Although most of the acute infections with *C. pneumoniae* are probably asymptomatic, they may be frequently associated clinically with pneumonia and bronchitis; moreover, *C. pneumoniae* infections have also been linked to the development of heart diseases and atherosclerosis.

C. trachomatis has three human serotypes. Serovars D-K cause sexually transmitted diseases, which are of great public health significance. Cervicitis and urethritis commonly occur in women and about 40% of the untreated cases progress to pelvic inflammatory disease (PID). Infertility is the result in 20% of the PID cases, while 18% of the women with this disease experience chronic pelvic pain, and 9% may suffer an ectopic pregnancy. At the individual level, the diseases caused by *C. trachomatis* can generally be treated effectively with antibiotics, but at the population level, public health control of the infections is rather problematic. Vaccination, which is currently unavailable, would be the best way to reduce the prevalence of *C. trachomatis* infections, as it would be much cheaper and more effective in controlling chlamydial diseases. *C. trachomatis* has been reported to adopt an intracellular form that is metabolically less active and thus less sensitive to antibiotics. The development of new antimicrobial agents is required for the effective treatment of persistent chlamydial infections.

CXC chemokines that lack the ELR (glutamic acid-leucine-arginine) motif, including the monokine induced by IFN- (MIG/CXCL9), the IFN-induced protein of 10 kDa (IP-10/CXCL10), and the IFN-inducible T-cell -chemoattractant (ITAC/CXCL1 1), have been shown to mediate the generation of type 1 immune responses and to possess defensin-like bactericidal effects. Our study revealed that the infection of mice with *C. pneumoniae* via the intranasal route resulted in the local expression of MIG/CXCL9, IP-10/CXCL10, and I -TAC/CXCL1 1. MIG/CXCL9 was also detected at a protein level from day 1, with the highest concentration in the supernatants of the infected lungs on day 7. The expression of IFN-█ displayed similar kinetics. *C. pneumoniae* and its inactivated form also induced the

production of MIG/CXCL9 in mouse fibroblasts and in the murine macrophage cell line J774A *in vitro.* Co-treatment of the tissue cultures with *C. pneumoniae* and different quantities of IFN- resulted in strong increases in MIG/CXCL9 production. Recombinan MIG/CXCL9 exerted dose-dependent antibacterial activity against *C. pneumoniae.* In our *in vivo* experiments, MIG/CXCL9 concentration in the whole lung suspension in the ELISA test was lower than the lowest bactericidal concentration in the *in vitro* test, but the local chemokine concentration should be higher in the affected area of the lung. Since MIG/CXCL9 seemed to bear anti-chlamydial effect only when it was co-incubated (for at least 15 min) with the EBs, it is likely that the expressed MIG/CXCL9 does not exert an effect on already infected epithelial cells, but rather prevents the spread of infectious EBs extracellularly. Chlamydial proteins at a molecular weight of 60 kDa were identified by Far-Western blot assay and liquid chromatography-tandem mass spectrometry as binding molecules of MIG/CXCL9. The results of these experiments suggest that MIG/CXCL9 might play an important role in the innate and acquired defence mechanisms against *C. pneumoniae.* Antimicrobial peptides (AMPs) are natural antibiotics produced by nearly all organisms, from bacteria to plants and animals. Most plant AMPs are cysteine cluster proteins. This group includes major plant immunity effectors such as defensins, and also symbiotic peptides, including the nodule-specific cysteine rich (NCR) peptides. Some cationic NCRs have been shown to possess genuine antimicrobial activities *in vitro,* killing various Gram-negative and Gram-positive bacteria highly efficiently. In our present study, 7 of the 11 NCR peptides examined displayed dose- and time-dependent anti-chlamydial activity *in vitro.* NCR247 was demonstrated to bind to the 60-kDa putative GroEL protein of *C. trachomatis.*

9. Összefoglalás

A *Chlamydophila pneumoniae* és *Chlamydia trachomatis* a Chlamydiaceae családba tartozó obligát intracellularis paraziták. Ezen baktériumok gyakran elhúzódó, tünetmentes fertőzéseket hoznak létre.

A *C. pneumoniae* felelős a felnőttek közösségben szerzett tüdőgyulladásának kb. 10%-áért. Habár az akut fertőzések nagy része valószínűleg tünetmentes, a betegség manifesztálódhat pneumonia vagy bronchitis képében; sőt, a *C. pneumoniae* által okozott infekcióknak szerepük lehet egyes szívbetegségek és az atherosclerosis kialakulásában is.

A *C. trachomatis*-nak három human szerotípusa ismert. A D-K szerovariánsok szexuális úton átvihető fertőzéseket okoznak, amelyeknek népegészségügyi jelentősége kiemelkedő. Nőknél a fertőzés gyakran cervicitist és urethritis t okoz, amelyek kezeletlen esetben 40%-ban progrediálnak kismedencei gyulladássá (pelvic inflammatory dis ease, PID). A PID esetek 9%-ában ectopiás terhesség, 18%-ában krónikus kismedencei fájdalom, 20%-ban terméketlenség a következmény. Habár egyéni szinten jól kezelhetőek a *C. trachomatis* infekciók, populációs szinten rendkívüli nehézséggel jár a fertőzések kordában tartása. A jelenleg még nem elérhető vakcináció lenne a leghatékonyabb módszer a *C. trachomatis* által okozott betegségek prevalenciájának csökkentésére. Mivel a *C. trachomatis* képes egy metabolikusan kevéssé aktív és így az antibiotikumokra fokozottan rezisztens formában túlélni a sejten belül, új antimikrobiális szerek kifejlesztése kiemelkedően fontos.

Az ELR (glutaminsav-leucin-arginin) motívumot nem tartalmazó CXC chemokinek, így a MIG/CXCL9 (monokine induced by IFN- az IP-10/CXCL10 (IFN-induced protein of 10 kDa), és az ITAC/CXCL1 1 (IFN-inducible T-cell -chemoattractant) az 1-es típusú immunválasz kiváltásában játszanak szerepet, valamint defenzin-szerű baktericid hatással bírnak. Kísérleteink során egereket fertőztünk intranasalisan *C. pneumoniae*-val, amely hatására a MIG/CXCL9, IP-10/CXCL10, és I-TAC/CXCL11 lokálisan expresszálódott az egerek tüdejében. A fertőzés utáni első naptól a MIG/CXCL9 protein szinten is detektálható volt, legmagasabb koncentrációban a fertőzést követő 7. napon. Az IFN- expressziója hasonló kinetikát mutatott.

C. pneumoniae élő és elölt formája hatására *in vitro* is megindult a MIG/CXCL9 termelődés az általunk használt egér fibroblast és J774A macrophag sejtvonalban. A sejtkultúrák egyidejű kezelése *C. pneumoniae*-val és IFN- különböző koncentrációival szignifikáns emelkedést

indukált a MIG/CXCL9 termelődésben. Az alkalmazott rekombináns MIG/CXCL9 dózis-dependens antibakteriális hatással rendelkezett a *C. pneumoniae*-val szemben. *In vivo* kísérleteinkben a MIG/CXCL9 koncentrációja alacsonyabb volt, mint az *in vitro* tesztekben hatásos koncentráció, de a lokális chemokin szint valószínűleg magasabb az érintett tüdőterületeken. Mivel a MIG/CXCL9 csak akkor mutatott anti-chlamydialis aktivitást, ha legalább 15 percig együtt inkubáltuk a chlamydia elementáris testekkel, valószínűsíthető, hogy az expresszált MIG/CXCL9 nem a már fertőzött sejteken fejti ki hatását, hanem extracellularisan akadályozza a fertőző elementáris testek terjedését.

Far-Western blot és LC-MSMS (folyadékkromatográfia-tömegspektrometria) vizsgálat során 60 kDa molekulatömegű chlamydialis fehérjéket azonosítottunk a MIG/CXCL9 kötő partnereként. Ezen eredmények azt sugallják, hogy a MIG/CXCL9 fontos szerepet játszhat mind a veleszületett, mind pedig a szerzett anti-chlamydialis védekező mechanizmusokban.

Az antimikrobialis peptidek olyan természetes anitibiotikumok, amelyek termelésére rengeteg élő szervezet képes, a baktériumoktól a növényeken át az állatokig. A legtöbb antimikrobialis peptid ciszteinben gazdag fehérje. Ebbe a csoportba tartoznak olyan kiemelkedően fontos növényi immunitást szabályzó fehérjék, mint a defenzinekvagy a gümő-specifikus ciszteingazdag (Nodule-specific Cysteine-Rich, NCR) peptidek. Néhány kationos NCR ismert antimikrobiális hatással rendelkezik *in vitro* Gram-negatív és Gram-pozitív baktériumokkal szemben. Kísérleteink során 7 növényi peptid fejtett ki dózis-, valamint időfüggő anti-chlamydialis hatást *in vitro*. Az NCR 247 peptid kötő partnereként a *C. trachomatis* 60 kDa molekulatömegű GroEL fehérjéjét azonosítottuk.

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11. References

1. Alunni B, Kevei Z, Redondo-Nieto M, Kondorosi A, Mergaert P, Kondorosi E. Genomic organization and evolutionary insights on GRP and NCR genes, two large nodule-specific gene families in Medicago truncatula. Mol Plant Microbe Interact 20:1138-1 148. (2007)

2. Baggiolini M. Chemokines in pathology and medicine. J Intern.Med. 250, 91-104. (2001)

3. Bai X, Chmura K, Ovrutsky AR, Bowler RP, Scheinman RI, Oberley-Deegan RE, Liu H, Shang S, Ordway D, Chan ED. Mycobacterium tuberculosis increases IP-10 and MIG protein despite inhibition of IP-10 and MIG transcription. Tuberculosis (Edinb). 91(1):26-35. (2011)

4. Balogh EP, Faludi I , Vir^{y}k DP, Endrész V, Burián K. Chlamydophila pneumoniae induces production of the defensin-like MIG/CXCL9, which has in vitro antichlamydial activity. Int J Med Microbiol. 301(3):252-9. (2011)

5. Balogh EP, Mosolyg^y T, Tiricz H, Szab^y ÁM, Karai A, Kerekes F, Vir^{y}k DP, Kondorosi É, Burián K. Anti-chlamydial effect of plant peptides. Acta Microbiologica et Immunologica Hungarica. (ahead of print) (2014)

6. Bavoil P, Stephens RS, Falkow S.: A soluble 60 kiloDalton antigen of Chlamydia. Mol Microbiol. 4(3):461-9. (1990)

7. Belland RJ, Ouellette SP, Gieffers J, Byrne GI. *Chlamydia pneumoniae* and atherosclerosis. Cell Microbiol. 6:117-127. (2004)

8. Billiau A, Matthys P. Interferon-gamma: a historical perspective. Cytokine Growth

Factor Rev. 20, 97-113. (2009)

9. Blasi F, Tarsia P, Aliberti S. *Chlamydophila pneumoniae.* Clin Microbiol Infect. 15:29-35. (2009)

10. Brogden NK, Brogden KA. Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? Int J Antimicrob Agents 38:217-225 (2011)

11. Burian K, Kis Z, Virok D, Endresz V, Prohaszka Z, Duba J, Berencsi K, Boda K, Horvath L, Romics L, Fust G, Gonczo, E. Independent and joint effects of antibodies to human heat-shock protein 60 and *Chlamydia pneumoniae* infection in the development of coronary atherosclerosis. Circulation 103:1503-1508. (2001)

12. Burián K, Hegyesi H, Buzás E, Endrész V, Kis Z, Falus A, Gönczöl E. *Chlamydophila (Chlamydia) pneumoniae* induces histidine decarboxylase production in the mouse lung. Immunol Lett. 89:229-236. (2003)

13. Burian K, Endresz V, Deak J, Kormanyos Z, Pal A, Nelson D, Virok D. Transcriptome analysis indicates an enhanced activation of adaptive and innate immunity by chlamydia-infected murine epithelial cells treated with interferon . J Infect Dis. 202(9):1405-14. (2010)

14. Burian K, Berencsi K, Endresz V, Gyulai Z, Valyi-Nagy T, Valyi-Nagy I, Bakay M, Geng Y, Virok D, Kari L, Hajnal-Papp R, Trinchieri G, Gonczol E. Chlamydia pneumoniae exacerbates aortic inflammatory foci caused by murine cytomegalovirus infection in normocholesterolemic mice. Clin Diagn Lab Immunol. 8(6): 1263-6. (2001)

15. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun 31:1161-

1176. (1981)

16.   Capparelli R, De Chiara F, Nocerino N, Montella RC, Iannaccone M, Fulgione A Romanelli A, Avitabile C, Blaiotta G, Capuano F. New perspectives for natural antimicrobial peptides: application as antinflammatory drugs in a murine model. BMC Immunol 13:61. (2012)

17. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR-CXC chemokines display defensin-like antimicrobial activity. J. Immunol. 167:623-627. (2001)

18.   Crawford MA, Burdick MD, Glomski IJ, Boyer AE, Barr JR, Mehrad B, Strieter RM, Hughes MA. Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. PLoS Pathog. 18;6(11):e1001199. (2010)

19.   Crawford MA, Zhu Y, Green CS, Burdick MD, Sanz P, Alem F, O'Brien AD, Mehrad B, Strieter RM, Hughes MA. Antimicrobial effects of interferon-inducible CXC chemokines against Bacillus anthracis spores and bacilli. Infect Immun. 77(4): 1664-78. (2009)

20. Cunningham AF, Ward ME. Characterization of human humoral responses to the major outer membrane protein and OMP2 of *Chlamydophila pneumoniae*. FEMS Microbiol Lett. 227: 73-79. (2003)

21. Deng JC, Tateda K, Zeng X, Standiford TJ. Transient transgenic expression of gamma interferon promotes *Legionella pneumophila* clearance in immunocompetent hosts. Infect Immun. 69:6382-6390. (2001)

22. Egesten A, Olin AI, Linge HM, Yadav M, Mörgelin M, Karlsson A, Collin M. SpeB of *Streptococcus pyo genes* differentially modulates antibacterial and receptor

activating properties of human chemokines. PLoS One 4:e4769. (2009)

23. Egesten A, Eliasson M, Johansson HM, Olin AI, Morgelin M, Mueller A, Pease JE, Frick IM, Björck L. The CXC chemokine MIG/CXCL9 is important in innate immunity against *Streptococcus pyogenes*. J Infect Dis. 195:684-693. (2007)

24. Eliasson M, Frick IM, Collin M, Sørensen OE, Björck L, Egesten A.M1 protein of *Streptococcus pyo genes* increases production of the antibacterial CXC chemokine MIG/CXCL9 in pharyngeal epithelial cells. Microb Pathog. 43:224-233. (2007)

25. Eliasson M, Mörgelin M, Farber JM, Egesten A, Albiger B. Streptococcus pneumoniae induces expression of the antibacterial CXC chemokine MIG/CXCL9 via MyD88-dependent signaling in a murine model of airway infection. Microbes Infect. 12(7):565-73. (2010)

26. Everett KD, Hatch TP. Architecture of the cell envelope of *Chlamydia psittaci* 6BC. J Bacteriol. 177:877-882. (1995)

27. Geng Y, Berencsi K, Gyulai Z, Valyi-Nagy T, Gonczol E, Trinchieri G. Roles of interleukin-12 and gamma interferon in murine *Chlamydia pneumoniae* infection. Infect Immun. 68:2245-2253. (2000)

28. Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH, Wang SP. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. J Infect Dis. 161:618-625. (1990)

29. Guaschino S, Ricci G. How, and how efficiently, can we treat Chlamydia trachomatis infections in women? Best Pract Res Clin Obstet Gynaecol. 16(6):875-88. (2002)

30. Hadley EB, Hancock RE. Strategies for the discovery and advancement of novel cationic antimicrobial peptides. Curr Top Med Chem. 10:1872-1881. (2010)

31. Hafner L, Beagley K, Timms P. Chlamydia trachomatis infection: host immune responses and potential vaccines.Mucosal Immunol. 1(2): 116-30. (2008)

32. Hahn DL, Dodge RW, Golubjatnikov R. Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. JAMA. 266: 225-230. (1991)

33. Hancock RE, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiol Lett. 206: 143-149. (2002)

34. Handel TM, Johnson Z, Crown SE, Lau EK, Proudfoot AE. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. Annu Rev Biochem. 74:385-410. (2005)

35. Heltai K, Kis Z, Burian K, Endresz V, Veres A, Ludwig E, Gönczöl E, Valyi-Nagy I. Elevated antibody levels against Chlamydia pneumoniae, human HSP60 and mycobacterial HSP65 are independent risk factors in myocardial infarction and ischaemic heart disease. Atherosclerosis. 173(2):339-46. (2004)

36. Henderson B, Allan E, Coates AR. Stress wars: the direct role of host and bacterial molecular chaperones in bacterial infection. Infect Immun. 74:3693-3706. (2006)

37. Higaki S, Morohashi M. Characteristics of anaerobes from skin specimens. Drugs Exp Clin Res. 29:153–155. (2003)

38. Horner PJ, Cain D, McClure M, Thomas BJ, Gilroy C, Ali M, Weber JN, Taylor-Robinson D. Association of antibodies to Chlamydia trachomatis heat-shock protein 60 kD with chronic nongonococcal urethritis. Clin Infect Dis. 24:653-660. (1997)

39. Howie SEM, Horner PJ, Horne AW, Entrican G. Immunity and vaccines agains sexually transmitted Chlamydia.trachomatis infection. Curr Opin Infect Dis. 24(1): 56–61. (2011)

40. Högdahl M, Söderlund G, Kihlström E. Expression of chemokines and adhesion molecules in human coronary artery endothelial cells infected with *Chlamydia (Chlamydophila) pneumoniae*. APMIS. 116:1082-1088. (2008)

41. Jauregui CE, Wang Q, Wright CJ, Takeuchi H, Uriarte SM, Lamont RJ. Suppression of T-cell chemokines by Porphyromonas gingivalis. Infect Immun. 81(7):2288-95. (2013)

42. Jones RB, Van der Pol B, Martin DH, Shepard MK. Partial characterization of Chlamydia trachomatis isolates resistant to multiple antibiotics. J Infect Dis. 162(6):1309-15. (1990)

43. Kanamoto Y, Ouchi K, Mizui M, Ushio M, Usui T. Prevalence of antibody to *Chlamydia pneumoniae* TWAR in Japan. J Clin Microbiol. 29:816-818. (1991)

44. Karlsson C, Eliasson M, Olin AI, Mörgelin M, Karlsson A, Malmsten M, Egesten A, Frick IM. SufA of the opportunistic pathogen *Fine goldia magna* modulates actions of the antibacterial chemokine MIG/CXCL9, promoting bacterial survival during epithelial inflammation. J Biol Chem. 284:29499-29508. (2009)

45. Karlsson C, Andersson ML, Collin M, Schmidtchen A, Björck L, Frick IM. SufA--a novel subtilisin-like serine proteinase of Finegoldia magna. Microbiology. 153(Pt 12):4208-18. (2007)

46. Kis Z, Burían K, Treso B, Acs K, Prohaszka Z, Fust G, Gonczol E, Endresz V. Inflammatory- and immune responses in relation to bacterial replication in mice following re-infections with *Chlamydophila pneumoniae*. Inflamm Res. 57:287-295.

(2008)

47. Laemmli UK. Cleavage of structural proteins during the assembly of the head ofbacteriophage, T4. Nature. 227:680-685. (1970)

48. Lauw FN, Simpson AJ, Prins JM, van Deventer SJ, Chaowagul W, White NJ, van der Poll T. The CXC chemokines gamma interferon (IFN-gamma)-inducible protein 10 and monokine induced by IFN-gamma are released during severe melioidosis. Infect Immun. 68:3888-3893. (2000)

49. Lefevre JC, Lepargneur JP, Guion D, Bei S. Tetracycline-resistant Chlamydia trachomatis in Toulouse, France. Pathol Biol (Paris). 45(5):376-8. (1997)

50. Liang YJ, Luo J, Lu Q, Zhou Y, Wu HW, Zheng D, Ren YY, Sun KY, Wang Y, Zhang ZS. Gene profile of chemokines on hepatic stellate cells of schistosomeinfected mice and antifibrotic roles of CXCL9/10 on liver non-parenchymal cells. PLoS One. 7(8):e42490. (2012)

51. Linge HM, Collin M, Giwercman A, Malm J, Bjartell A, Egesten A. The antibacterial chemokine MIG/CXCL9 is constitutively expressed in epithelial cells of the male urogenital tract and is present in seminal plasma. J Interferon Cytokine Res. 28(3):191-6. (2008)

52. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. J Am Coll Cardiol. 3 1:827-832. (1998)

53. Mahony JB, Luinstra KE, Waner J, McNab G, Hobranzska H, Gregson D, Sellors JW, Chernesky MA. Interlaboratory agreement study of a double set of PCR plasmid primers for detection of Chlamydia trachomatis in a variety of genitourinary specimens. J Clin Microbiol. 32(1):87-91. (1994)

54. Malek M. Economic implications of Chlamydia trachomatis. In: Moss TR. International handbook of Chlamydia. Exeter, UK: Polestar Wheatons Ltd. (2001)

55. Maxion HK, Kelly KA. Chemokine expression patterns differ within anatomically distinct regions of the genital tract during *Chlamydia trachomatis* infection. Infect Immun. 70:1538-1546. (2001)

56. Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E. A novel family in Medicago truncatula consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. Plant Physiol 132: 161-173. (2003)

57. Miller ST, Hammerschlag MR, Chirgwin K, Rao SP, Roblin P, Gelling M, Stilerman T, Schachter J, Cassell G. Role of *Chlamydia pneumoniae* in acute chest syndrome of sickle cell disease. J Pediatr. 118:30-33. (1991)

58. Moelleken K, Hegemann JH. The *Chlamydia* outer membrane protein OmcB is required for adhesion and exhibits biovar-specific differences in glycosaminoglycan binding. Mol Microbiol. 67:403-419. (2008)

59. Moore TA, Perry ML, Getsoian AG, Newstead MW, Standiford TJ. Divergent role of gamma interferon in a murine model of pulmonary versus systemic *Klebsiella pneumoniae* infection. Infect Immun. 70:6310-63 18. (2002)

60. Murdoch DA. Gram-positive anaerobic cocci. Clin Microbiol Rev. 11:81–120. (1998)

61. Murray PR, Rosenthal KS, Pfaller MA, Medical Microbiology. P. 441-449. (2009)

62. Nagarajan UM, Prantner D, Sikes JD, Andrews Jr. CW, Goodwin AM, Nagarajan S, Darville T. Type I interferon signaling exacerbates *Chlamydia muridarum* genital

infection in a murine model. Infect Immun. 76:4642-4648. (2008)

63. Nallu S, Silverstein KA, Samac DA, Bucciarelli B, Vance CP, VandenBosch KA. Regulatory patterns of a large family of defensin-like genes expressed in nodules of Medicago truncatula. PLoS One 8:e60355. (2013)

64. Nelson S, Mason CM, Kolls J, Summer WR. Pathophysiology of pneumonia. Clin Chest Med. 16:1-12. (1995)

65. Park MK, Amichay D, Love P, Wick E, Liao F, Grinberg A, Rabin RL, Zhang HH, Gebeyehu S, Wright TM, Iwasaki A, Weng Y, DeMartino JA, Elkins KL, Farber JM. The CXC chemokine murine monokine induced by IFN-gamma (CXC chemokine ligand 9) is made by APCs, targets lymphocytes including activated B cells, and supports antibody responses to a bacterial pathogen in vivo. J Immunol. 169(3): 1433-43. (2002)

66. Penttilä T, Vuola JM, Puurula V, Anttila M, Sarvas M, Rautonen N, Mäkelä PH, Puolakkainen M. Immunity to Chlamydia pneumoniae induced by vaccination with DNA vectors expressing a cytoplasmic protein (Hsp60) or outer membrane proteins (MOMP and Omp2). Vaccine. 19(9-10):1256-65. (2000)

67. Portig I, Goodall JC, Bailey RL, Gaston JS. Characterization of the humoral immune response to *Chlamydia* outer membrane protein 2 in chlamydial infection. Clin Diagn Lab Immunol. 10:103-107. (2003)

68. Powers JP, Hancock RE. The relationship between peptide structure and antibacterial activity. Peptides. 24:1681-1691. (2003)

69. Randolph AG, Washington AE. Screening for Chlamydia trachomatis in adolescent males: a cost-based decision analysis. Am J Public Health. 80(5):545-50. (1990)

70. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, Mariotti SP. Global data on visual impairment in the year 2002. Bull World Health Organ. 82(1 1):844-51. (2004)

71. Rigano MM, Romanelli A, Fulgione A, Nocerino N, D'Agostino N, Avitabile C Frusciante L, Barone A, Capuano F, Capparelli R. A novel synthetic peptide from a tomato defensin exhibits antibacterial activities against Helicobacter pylori. J Pept Sci. (2012)

72. Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem. 203:173-179. (1992)

73. Rottenberg ME, Gigliotti-Rothfuchs A, Wigzell H. The role of IFN-gamma in the outcome of chlamydial infection. Curr Opin Immunol. 14:444-451. (2002)

74. Rubins JB, Pomeroy C. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infect Immun. 65:2975-2977. (1997)

75. Rugonfalvi-Kiss S, Endrész V, Madsen HO, Burián K, Duba J , Prohászka Z, Karádi I, Romics L, Gönczöl E, Füst G, Garred P. Association of Chlamydia pneumoniae with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. Circulation. 106(9): 1071-6. (2002)

76. Sahin H, Borkham-Kamphorst E, Kuppe C, Zaldivar MM, Grouls C, Al-samman M, Nellen A, Schmitz P, Heinrichs D, Berres ML, Doleschel D, Scholten D, Weiskirchen R, Moeller MJ, Kies sling F, Trautwein C, Wasmuth HE. Chemokine Cxcl9 attenuate liver fibrosis-associated angiogenesis in mice. Hepatology. 55(5): 16 10-9. (2012)

77. Schautteet K, De Clercq E, Vanrompay D. Chlamydia trachomatis vaccine research through the years. Infect Dis Obstet Gynecol. 2011:963513. (2011).

78. Shaw K, Coleman D, O'Sullivan M, Stephens N. Public health policies and management strategies for genital Chlamydia trachomatis infection. Risk Manag Healthc Policy. 4:57-65. (2011)

79. Shen J, Gao J, Chen C, Lu H, Hu G, Shen J, Zhu S, Wu M, Wang X, Qian L, Yu Y Han W, Wan R, Wang X. Cytokine. Antifibrotic role of chemokine CXCL9 in experimental chronic pancreatitis induced by trinitrobenzene sulfonic acid in rats. 64(1):382-94. (2013)

80. Skwor TA, Atik B, Kandel RP, Adhikari HK, Sharma B, Dean D. Role of secreted conjunctival mucosal cytokine and chemokine proteins in different stages of trachomatous disease. PLoS Negl Trop Dis. 16;2:e264. (2008)

81. Söbirk SK, Mörgelin M, Egesten A, Bates P, Shannon O, Collin M. Human chemokines as antimicrobial peptides with direct parasiticidal effect on Leishmania mexicana in vitro. PLoS One. 8(3):e58 129. (2013)

82. Somani J, Bhullar VB, Workowski KA, Farshy CE, Black CM. Multiple drug-resistant Chlamydia trachomatis associated with clinical treatment failure. J Infect Dis. 181(4):1421-7. (2000)

83. Stephens RS, Koshiyama K, Lewis E, Kubo A. Heparin-binding outer membrane protein of chlamydiae. Mol Microbiol. 40:691-699. (2001)

84. Stephens RS. The cellular paradigm of chlamydial pathogenesis. Trends Microbiol. 11:44-51. (2003)

85. Thylefors B, Négrel AD, Pararajasegaram R, Dadzie KY. Global data on blindness. Bull World Health Organ. 73:115. (1995)

86. Tiricz H, Szucs A, Farkas A, Pap B, Lima RM, Mar^{y}ti G, Kondorosi É, Kereszt A. Antimicrobial nodule-specific cysteine-rich peptides induce membrane depolarization-associated changes in the transcriptome of Sinorhizobium meliloti. Appl Environ Microbiol. 79:6737-6746. (2013)

87. Wagels G, Rasmussen S, Timms P. Comparison of *Chlamydia pneumoniae* isolates by western blot (immunoblot) analysis and DNA sequencing of the omp 2 gene. J Clin Microbiol. 32:2820-2823. (1994)

88. Ward ME. Mechanisms of chlamydia-induced disease. p.171–210. In Stephens RS, ed. Chlamydia: intracellular biology, pathogenesis, and immunity. ASM Press Washington, DC. (1999)

89. Washington AE, Sweet RL, Shafer MA. Pelvic inflammatory disease and its sequelae in adolescents. J Adolesc Health Care. 6(4):298-3 10. Review. (1985)

90. West SK, Muñoz B, Mkocha H, Hsieh YH, Lynch MC. Progression of active trachoma to scarring in a cohort of Tanzanian children. Ophthalmic Epidemiol. 8(2-3):137-44. (2001)

91. WHO: Global Prevalence and Incidence of Selected SexuallyTransmitted Diseases: Overviews and Estimates. Geneva, Switzerland: World Health Organization. (1996)

92. Wang S, Fan Y, Brunham RC, Yang X. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. Eur JImmunol. 29:3782-3792. (1999)

93. Watson MW, PR Lambden, JS Everson, IN Clarke. Immunoreactivity of the 60 kDa cysteine-rich proteins of Chlamydia trachomati, Chlamydia psittaci and Chlamydia pneumoniae expressed in Escherichia coli. Microbiology. 140:2003–2011. (1994)

94.   Whittington WL, Kent C, Kissinger P, Oh MK, Fortenberry JD, Hillis SE, Litchfield B, Bolan GA, St Louis ME, Farley TA, Handsfield HH. Determinants of persisten and recurrent Chlamydia trachomatis infection in young women: results of a multicenter cohort study. Sex Transm Dis. 28(2): 117-23. (2001)

95.   Wright HR, Turner A, Taylor HR. Trachoma. Lancet.7;371(9628):1945-54. (2008)

96. Wuppermann FN, Mölleken K, Julien M, Jantos CA, Hegemann JH. *Chlamydia pneumoniae* GroEL1 protein is cell surface associated and required for infection of HEp-2 cells. J Bacteriol. 190:3757-3767. (2008)

97. Zügel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev. 12:19-39. (1999)

12. Annexes

I.

II.