THE ANTI-CHLAMYDIAL ACTIVITY OF DEFENSIN-LIKE PROTEINS

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Ph.D. Thesis

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Szeged
2014

1. Introduction

1.1. Chlamydiaceae

The family Chlamydiaceae consists of two clinically important genera, *Chlamydia* and *Chlamydophila*, with three species responsible for human diseases: *Chlamydia trachomatis (C. trachomatis)*, *Chlamydophila psittaci (C. psittaci)*, and *Chlamydophila pneumoniae*. Other species have been placed into the two genera, but they are uncommon human pathogens.
The Chlamydiaceae are obligate intracellular parasites that were once considered viruses because they are small enough to pass through 0.45 μm filters. Unlike other bacteria, Chlamydiaceae have a unique developmental cycle, forming metabolically inactive infectious forms (elementary bodies [EBs]) and metabolically active, non-infectious forms (reticulate bodies [RBs]). Much like spores, EBs are resistant to many harsh environmental factors. Although these bacteria lack the rigid peptidoglycan layer found in most other bacteria, their central dense core is surrounded by a cytoplasmic membrane and a double-layer outer membrane. The cell wall contains a lipopolysaccharide (LPS) that is common to all members of the family. The LPS has only weak endotoxin activity. The major outer membrane protein (MOMP) in the cell wall is an important structural component of the outer membrane and is unique for each species. Variable regions in the gene encoding this protein are found in *C. trachomatis* and are responsible for 18 serologic variants (called serovars). Similar variable 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. The EBs cannot replicate but are infectious; that is, they can bind to receptors on host cells and stimulate uptake by the infected cell. In this intracellular location, EBs convert into RBs, metabolically active, replicating form. Because the extensive cross-linked proteins are absent in RBs, this form is osmotically fragile; however, they are protected by their intracellular location.

Chlamydophila 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. *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. Although most of the acute infections with *C. pneumoniae* are probably asymptomatic, they may be frequently associated clinically with pneumonia, pharyngitis, sinusitis, and bronchitis. 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. 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.

Chlamydia 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, 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). 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. A prominent investigation has been carried out by Washington et al. studying the direct and indirect healthcare costs of chlamydial infections in men, and the infections and their sequelae in women. 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* infections can generally be treated effectively with antibiotics, though antibiotic resistance appears to be increasing. 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. 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. The development of new antimicrobial agents is required to overcome this problem.

1.2. 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)-γ or certain antibiotics. This dormant or "persistent form" allows *C. trachomatis* to remain dormant in the host cell, resulting in a long-term relationship with the infected host. It is known that serological diagnosis of chronic persistent, latent infections can somewhat be 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 anti-chlamydial therapy. Whittington et al. 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 two patient 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. 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. 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.

1.3. Chemokines

Chemokines have molecular masses of 8-10 kDa and show approximately 20-50 % sequence homology among each other 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: alpha- (CXC), beta- (CC), gamma- (XC) and delta- (CX3C) chemokines, depending on the presence of conserved NH_2-terminal cysteine residues. The CXC chemokine family is further divided into 2 classes, depending on the presence or absence of an NH_2-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-γ (MIG/CXCL9), the IFN-γ-induced protein 10 (IP-10/CXCL10) and the IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11) belong to the group of non-ELR CXC chemokines and has the ability to signal through the CXC chemokine receptor 3 (CXCR3), which is present on T cells and NK cells. The producers of CXCR3 ligands appear to be alveolar macrophages, epithelial cells and giant cells. 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 neuroimmunty. 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. MIG/CXCL9 and other CXC chemokines (IP-10/CXCL10 and I-TAC/CXCL11) additionally possess antibacterial activity *in vitro*. Several papers have been published regarding the antimicrobial effects of MIG/CXCL9 against *Escherichia coli, Listeria monocytogenes, Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrheae, Finegoldia magna, Bacillus anthracis, Porphyromonas gingivalis, Mycobacterium tuberculosis,* and *Chlamydia trachomatis.* However, the function of MIG/CXCL9 in *C. pneumoniae* infection has not been investigated thoroughly prior to our work. Skwor et al. found that chemokine protein levels for CCL11, 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. 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.

1.4. 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 from 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 defense mechanism, whereas in others they serve as an adjunct to existing innate and adaptive immune systems. Cationic AMPs interact with negatively charged microbial membranes and permeabilize the membrane phospholipid bilayer, resulting in lysis and the death of microbes. In view of their rapid and broad-spectrum antimicrobial properties, interest has emerged in AMPs as potential antibiotic pharmaceuticals with which to combat infections and microbial drug resistance. 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*. Some cationic NCRs have been shown to possess genuine antimicrobial activities *in vitro*, killing various Gram-negative and Gram-positive bacteria highly efficiently.
In the present study, 7 of the 11 NCR peptides examined displayed dose- and time-dependent anti-chlamydial activity *in vitro*. NCR247 was also demonstrated to bind to the 60-kDa putative GroEL protein of *C. trachomatis* D.

Aims

Our aims were to **a,** detect whether *C. pneumonia*e can induce the production of CXCL chemokines *in vitro* in *in vivo* mouse model; **b,** investigate the effect of co-treatment with *C. pneumoniae* and IFN-γ on the expression of MIG/CXCL9 in different cell types; **c,** detect the anti-chlamydial activity of CXCL chemokines *in vitro* and to disclose the binding partner of MIG/CXCL9 on Chlamydia EBs; **d,** investigate the anti-chlamydial activity of plant peptides; **e,** find the chlamydial ligand responsible for the binding of NCR proteins

2. Materials and methods

2.1. *C. pneumoniae* experiments

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

C. pneumoniae strain TWAR CDC/CWL-029 and a cardiovascular strain CV6, isolated from an atheroma of a patient with coronary artery disease, were propagated on HEp-2 cells) in Modified Eagle Medium, supplemented with 10% fetal calf serum, 0.5% glucose, 0.3 mg L-glutamine, 4 mM HEPES and 25 μg of gentamycin/ml. Two hours after infection, the medium was supplemented with 1 μg of cycloheximide/ml. Infected cells were harvested on day 3 or 4 and were disrupted by two cycles of freeze-thawing and ultrasonication. After centrifugation at 500×*g* for 10 min to remove cell debris, the bacteria were concentrated by high-speed centrifugation at 30 000×*g* for 30 min. Pellets were re-suspended in sucrose-phosphate-glutamic acid (SPG) buffer. The concentrated *C. pneumoniae* was stored at −70 °C until use. The titres of the *C. pneumoniae* preparations were determined by an immunofluorescence assay. Serial dilutions of bacterial stocks were placed on Hep-2 cells in 24-well plates, centrifuged for 1 h at 800×*g* to increase the uptake of the bacterium by the cells, incubated for 2 days, fixed with acetone and stained with mouse monoclonal antibodies specific to the major outer membrane protein (MOMP) of *C. pneumoniae*, and then with fluorescein isothiocyanate-labelled secondary antibody. The number of *C. pneumoniae*

inclusions was counted under a UV microscope, and the titre was expressed in IFU/ml. Lung homogenates from individual mice were centrifuged (10 min, 400g), serial dilutions of the supernatants were inoculated onto HEp-2 cell monolayers, and the titre of *C. pneumoniae* was determined as previously described for the titration of *C. pneumoniae*.

2.1.2. 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, the mice were mildly sedated with an intraperitoneal injection of 200 μl of sodium pentobarbital (7.5 mg/ml). They were then infected intranasally with $5x10^5$ IFU of *C. pneumoniae* (CWL029) in 25 μl 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 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.

2.1.3. 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 supplemented with 10% foetal calf serum, 0.5% glucose, 0.3 mg of L-glutamine/ ml, 4 mM HEPES and 25 μg 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-γ. 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 until use. The MIG/CXCL9 concentrations of the samples were determined with the Quantikine mouse CXCL9/MIG kit.

2.1.4. Measurement of in vitro antibacterial activity of MIG/CXCL9

EBs of *C. pneumoniae* strain CWL-029 ($4x10^4$ IFU/ml) were incubated with recombinant MIG/CXCL9 at various concentrations (10, 5, 2.5 or 1.25 μg/ml) or IP-10/CXCL10 or I-TAC/CXCL11 (PeproTech) (10 μg/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 μg/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/CXCL11, HEp2 cells were seeded in 24-well tissue culture plates with 13-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.

2.1.5. Cytokine and chemokine measurements on the lungs

The supernatants of the lung homogenates and the sera were clarified by centrifugation (5 min, 12 000g) and were assayed for IFN-γ content with the OptEIA ELISA set (BD Biosciences Pharmingen San Diego, CA, USA). The concentration of MIG/CXCL9 was determined with the Quantikine mouse CXCL9/MIG kit. The sensitivities of the IFN-γ 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.

2.1.6. mRNA extraction and real-time PCR

Total RNA was extracted from the lung suspensions with the TRI Reagent. First-strand cDNA was synthesized from 2 μg of RNA by using reverse transcriptase, Superscript III and 20 pM random primer in 20 μl 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™ in a total volume of 20 μl. 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/CXCL11 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 ΔCt. The relative expression level was given as $2^{-(\Delta\Delta Ct)}$, where ΔΔCt=ΔCt for the experimental sample minus ΔCt for the control sample.

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

Chlamydia EBs ($1x10^6$ IFU) were treated with 10 μg 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 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 the FACS StarPlus device.

2.1.8. 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. 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 .The membrane was blocked overnight at 4 °C with 5% skimmed milk and 0.05% Tween 20 containing PBS. The membrane was probed with a buffer [1% bovine serum albumin in PBS with 0.05% Tween 20 (PBST)] containing 10 μg/ml MIG/CXCL9 for 2 h. After washing 3 times with PBST, the filter was incubated with HRP-conjugated anti-MIG/CXCL9 IgG). 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 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.

2.1.9. 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 $p<0.05$.

2.2. *C. trachomatis* experiments

2.2.1. Inoculum preparation

C. trachomatis D 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 cell monolayer processed in the same way as the infected cells. The titer 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 and FITC-labeled secondary anti-mouse IgG The number of inclusions was counted under a UV microscope, and the titer was expressed in inclusion forming units/ml (IFU/ml).

2.2.2. 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 μg/ml) used during our experiments. The toxic peptides were excluded from the further experiments. EBs of *C. trachomatis* D (4×10^4 IFU/ml) were incubated with chemically synthesized mature peptides at various concentrations (10, 5, 2.5, or 1.25 μg/ml) in sucrose-phosphate-glutamic acid buffer (SPG) for 2 h at 37 °C. As control, *C. trachomatis* D was incubated in buffer alone. The time courses of the anti-chlamydial effects of the NCR peptides were tested after incubation periods of 15, 30, 60 and 120 min. To quantify the anti-chlamydial effects of the NCR peptides, HeLa cells were seeded in 24-well tissue culture plates with 13-mm cover glasses. After 24 h, the confluent cells were infected with NCR-treated *C. trachomatis* D or the control. After 48 h, the cells were fixed with acetone at −20 °C for 10 min. Fixed cells on cover glasses were stained by the indirect immunofluorescence method described in "Inoculum preparation" section. The number of recoverable inclusions was counted under a UV microscope, and the titre was expressed in IFU/ml.

2.2.3. Far-Western blot assay for identification of NCR-binding Chlamydia proteins

Concentrated *C. trachomatis* (2×10^5 IFU) (prepared as described earlier) and a mock preparation were heated at 95 °C for 5 min in sample buffer, and polyacrylamide gel electrophoresis (PAGE) was performed. The proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel in duplicate, and half of the gel carrying the separated proteins of the *C. trachomatis* or the mock samples was blotted onto a polyvinylidene difluoride membrane. The membrane was blocked overnight at 4 °C with 5% skimmed milk and 0.05% Tween 20 containing PBS. The membrane was probed for 4 h with a buffer [1% bovine serum

albumin in PBS with 0.05% Tween 20 (PBST)] containing 10 μg/ml NCR247. After washing 3 times with PBST, the filter was incubated with anti-NCR247 rabbit IgG for 4 h and further incubated after washing 3 times with HRP-conjugated anti-rabbit antibody (Sigma). A control lane with separated *C. trachomatis* EBs was also incubated with anti-NCR247 and HRP-conjugated anti-rabbit antibody without prior treatment with NCR247 peptide. Following 3 further washings, the colour was developed by using diaminobenzidine tetrahydrochloride with hydrogen peroxide in 10 mM Tris at pH 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.

2.2.4. 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 out from the gel and analyzed by mass spectrometry.

2.2.5. Detection of NCR peptide binding to Chlamydia EBs by FACS

Chlamydia EBs (1×10^6 IFU) were treated with 1 μg of FITC-labelled NCR247 or FITC-labelled NCR035 peptide containing PBS for 2 h at 37 °C. As controls, untreated Chlamydia EBs were used. After 3 times washing with PBS, cells were analyzed with the FACS StarPlus device.

3. Results

3.1. *C. pneumoniae* experiments

3.1.1. C. pneumoniae *infection induces CXC chemokine expression at the 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/CXCL11 mRNA and MIG/CXCL9 and IFN-γ protein in the individual lungs. 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 on day 7, at 1.8×10^6 IFU/lung, and on day 14 after infection, the titre had decreased to 6.15×10^3 IFU/lung. 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. The expression of IP-10/CXCL10 and I-TAC/CXCL11 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. The kinetics of production of MIG/CXCL9 protein correlated with the mRNA expression: it increased from day 1, with the highest concentration on day 7. 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-γ, the main inducer of MIG/CXCL9 expression, was increased in the lungs during the infection, with a peak level of 12.5 ng/ml on day 7.

3.1.2. 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

the *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. However, *C. pneumoniae* induced MIG/CXCL9 secretion by the mouse fibroblasts from day 2 of infection (122.7±22.3 pg/ml), and J774A cells produced MIG/CXCL9 from day 3 after infection (156.9±56.7 pg/ml). 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-γ (the main inducer of MIG/CXCL9) was detected in the mouse lungs. When the influence of IFN-γ on *C. pneumoniae*-induced MIG/CXCL9 production was investigated in *in vitro* cultures, co-treatment with different quantities of recombinant mouse IFN-γ (10, 100 or 1000 U/ml) and *C. pneumoniae* at an 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-γ 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-γ 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-γ (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-γ alone. Co-treatment of the J774A cells with *C. pneumoniae* and 10, 100 or 1000 U/ml of IFN-γ 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-γ 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-γ (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-γ alone. Under all of the conditions mentioned above, the mouse fibroblasts produced significantly more MIG/CXCL9 than did the J774A ($p<0.05$). Similar results were seen under the conditions described above in both cell cultures after 48 h.

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 141±47 pg/ml, respectively) after 72 h.

3.1.3. 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 MIG/CXCL9 concentration of 10 μg/ml, the number of viable *C. pneumoniae* inclusions was reduced by 98.4%, and at the lowest MIG/CXCL9 concentration tested (1.25 μg/ml) a reduction of 79% was detected. The heat-treated recombinant MIG/CXCL9 did not exert an anti-chlamydial effect. 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. No anti-chlamydial effect was observed when other ELR-negative chemokines, IP-10/CXCL10 and I-TAC/CXCL11, were co-incubated with *C. pneumoniae* at a concentration of 10 μg/ml.

3.1.4. 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. 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*.

To show that the MIG/CXCL9 is able to bind to the native EBs, a FACS analysis was carried out. 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.

3.2. *C. trachomatis* experiments

3.2.1. 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 μg/ml for 2 h at 37 °C. Counting 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. *C. trachomatis* inclusions were then treated for 2 h with concentrations of the peptides ranging from 1.25 μg/ml to 10 μg/ml. NCR044, NCR055 and NCR183 were found to exert the strongest anti-chlamydial activities by reducing the viability to 95%, 78% and 85%, respectively, at 1.25 μg/ml, whereas the other peptides revealed no effect at 1.25 μg/ml concentration. NCR192 and NCR247 had significant anti-chlamydial effects at 2.5 μg/ml concentration. The time course of killing was investigated in the cases of NCR044, NCR055, NCR183 and NCR247 at 5 μg/ml concentration. NCR044 elicited the fastest effect, achieving an 80% reduction in the number of viable Chlamydia inclusions after a 15-min co-incubation with *C. trachomatis* EBs. The other three peptides required longer times to attain the killing effect. Of the tested peptides therefore, NCR044 exhibited the strongest anti-chlamydial activity, acting at the lowest concentration and most rapidly.

3.2.2. Identification of the chlamydial ligand responsible for NCR247 binding

Further investigations were carried out with NCR247, which displayed anit-chlamydial 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-labeled anti-rabbit antibody. The control lane with Chlamydia EBs was stained with anti-NCR247 IgG and HRP-labeled 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. The synthetic NCR247 did not react with the mock lysate, and the Chlamydia EB lysate did not react with the HRP-conjugated anti-rabbit antibody. 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.

3.2.3. 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. Chlamydia EBs interacted with FITC-conjugated NCR247 peptide. Untreated or FITC-labeled NCR035 peptide-treated (this peptide showed no anti-chlamydial effect earlier) Chlamydia EBs did not demonstrate increased fluorescence.

4. Discussion

Innate or natural immunity is the principal pathway for the effective elimination of most bacterial pathogens from the lung. 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. Innate immune responses are enhanced in the presence of type 1 cytokines, including IL-12 and IFN. Type 1 cytokine responses are indispensable for the effective killing of intracellular pathogens. 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- γ, which is the main inducer of MIG/CXCL9 production following *C. pneumoniae* infection, was described. 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. The highest concentration of MIG/CXCL9 at a protein level was likewise observed on day 7. The Th1 cytokine IFN- γ plays important roles in combating acute Chlamydia infection and in the establishment of persistence. IFN- γ may also play a regulatory role in chlamydial defence, via its ability to alter the Th1/Th2 balance. Accordingly, IFN-γ gene knockout mice exhibited Th2-cytokine-dependent immunopathology together with disseminated chlamydial infection. IFN- γ may modify the outcome of infection by enhancing the expression of chemokines and adhesion molecules. IFN- γ administered therapeutically to patients with chronic granulomatous disease improved the ability of phagocytes to clear bacterial infections. 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-γ-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. 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 .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 . 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-γ resulted in a 51.8-fold increase in MIG/CXCL9 release as compared with treatment with 10 U of IFN-γ alone. Our observation that heat-inactivated *C. pneumoniae* together with IFN-γ induced a 75-fold increase in MIG/CXCL9 secretion relative to IFN-γ 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, Högdahl et 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 .

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 monocytogenes*. It was recently reported that CXCL9, CXCL10 and CXCL11 kill *Streptococcus pyogenes*, and that, in particular, CXCL9 was produced in a bactericidal concentration by inflamed pharyngeal cells both *in vivo* and *in vitro*. The role of MIG/CXCL9 during bacterial infection has not been fully elucidated. In contrast with the examples mentioned above, in the case of *Finegoldia magna* MIG/CXCL9 displays no antibacterial activity. Moreover, the SufA of *F. magna* cleaves MIG/. 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 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. Since MIG/CXCL9 was anti-chlamydial 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. 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. 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. Moreover, OmcB induces a strong antibody response, suggesting that it is readily accessible to the humoral immune response, which is indicative of a surface localization. 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 LC-MSMS. 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. It is interesting that heparin-like structures on the mammalian cell surface play an important role in the interactions with many bacterial pathogens and are recognized by chlamydial OmcB. Moreover, the M1 protein, the major surface protein of *S. pyogenes*, which is necessary for the adhesion of bacteria to epithelial cells via GAGs, induces an enhanced production of MIG/CXCL9. 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. 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/CXCL11 *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. 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. monocytogenes,* and Gram-negative bacteria, including *Salmonella enterica serovar* Paratyphi, *E. coli,* and *Helicobacter pylori* .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.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 anti-chlamydial activity at a 10 μg/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 monocytogenes*) bacteria, including human/animal and plant pathogens . Furthermore, AMPs were effective against *Staphylococcus epidermidis* in *in vivo* mouse model, and they also displayed anti-inflammatory activity.

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. 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. Several reports have indicated that molecular chaperones produced by pathogenic bacteria, can function as intracellular, cell surface, or extracellular signals in the course of infection processes. 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. The high degree of antigenicity of GroEL in patients implies that the protein is easily accessible to the immune system, perhaps because it 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. 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

We found that MIG/CXCL9 showed dose-dependent anti-chlamydial activity in *in vitro C. pneumoniae* infection. During our experiments it was revealed that the 60 kDa chlamydial proteins were responsible for the binding of MIG/CXCL9. Seven of the NCR peptides examined showed dose dependent anti-chlamydial effect in *in vitro C. trachomatis* infection. We found the chlamydial GroEL protein to be responsible for the binding of NCR247 peptide.

5. 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. 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-γ (MIG/CXCL9), the IFN-induced protein of 10 kDa (IP-10/CXCL10), and the IFN-inducible T-cell α-chemoattractant (ITAC/CXCL11), 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/CXCL11. 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-γ 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-γ resulted in strong increases in MIG/CXCL9 production. Recombinant 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*.

7. Acknowledgements

I would like to thank my tutor, **Katalin Burián** for guiding me through the maze of microbiology, and for always helping me with every question and problem I had.
I owe many thanks to my colleagues, especially to **Tímea Mosolygó** and **Valéria Endrész**, for their help over the years. For the excellent technical assistance I should express my gratitude to **Tilda (Istvánné Lévai)** and **Gizike (Gizella Paraginé Zsótér).**

This thesis is dedicated to my parents, who I owe the most.

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

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Impact factor: 0.646
ΣIF = 4.819

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