Generation and evaluation of vaccine candidates against *Chlamydophila pneumoniae*

Ph. D. Thesis

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Publications related to the thesis

I. Faludi I, Burian K, Csanadi A, Miczak A, Lu X, Kakkar VV, Gonczol E, Endresz V. Adjuvant modulation of the immune response of mice against the LcrE protein of *Chlamydophila pneumoniae*. Int J Med Microbiol. 2009; 299(7):520-8.

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II. Faludi I, Csanádi A, Szabó AM, Burián K, Endrész V, Miczák A. Production and purification of low calcium response protein H of *Chlamydophila pneumoniae*. Acta Microbiol Immunol Hung. 2009; 56(4):389-97.

III. Csanadi A, Faludi I, Miczak A. MSMEG_4626 ribonuclease from *Mycobacterium smegmatis*. Mol Biol Rep. 2009; 36(8): 2341-4.

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Abbreviations

- **BCG** *M. bovis* bacillus Calmette-Guérin
- **BD** below detectable levels
- **bp** base pair
- ***C. pneumoniae*** *Chlamydophila pneumoniae*
- **CAD** coronary artery disease
- **CFU** colony forming unit
- **CMI** cell-mediated immunity
- **CopN** chlamydia outer protein N
- **DAB** diaminobenzidine tetrahydrochloride
- **DNA** deoxyribonucleic acid
- ***E. coli*** *Escherichia coli*
- **EB** elementary body
- **ELISA** enzyme-linked immunosorbent assay
- **ELISPOT** enzyme-linked immunosorbent spot
- **FCA** Freund's complete adjuvant
- **Fig.** Figure
- **FITC** fluorescein isothiocyanate
- **GM** geometric mean
- **HIV** human immunodeficiency virus
- **HRP** horseradish-peroxidase
- **Hsp60** heat shock protein 60
- **ICC** immunocytochemistry
- **IFN-γ** interferon γ
- **IFU** inclusion forming units
- **Ig** immunoglobulin
- **IL** interleukin
- **KC** keratinocyte-derived chemokine
- **kDa** kilodalton
- **LB** Luria–Bertani
- **LcrE** low-calcium response E protein
- **LcrH** low-calcium response H protein
- **LPS** lipopolysaccharide

- ***M. smegmatis*** - *Mycobacterium smegmatis*
- **MALDI-TOF MS** matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
- **MHC** major histocompatibility complex
- **MIP-2** macrophage-inflammatory protein-2
- **MOMP** major outer membrane protein
- **MoPn** Mouse pneumonitis; *Chlamydia trachomatis* biovar
- **MSMEG_4626** gene coding Rne protein
- **MTT** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
- **MW** molecular weight
- **OD** optical density
- **OMP** outer membrane protein
- **PAGE** polyacrylamide gel electrophoresis
- **PBS** phosphate buffered saline
- **PCR** polymerase chain reaction
- **PI** proliferation index
- **RB** reticulate body
- **RNA** ribonucleic acid
- **RNase** ribonuclease
- **Rne** ribonuclease E protein = RNase E
- **rRNA** ribosomal RNA
- **S.D.** standard deviation
- **SDS** sodium dodecyl sulphate
- **SFC** spot-forming cells
- **SPG** sucrose-phosphate-glutamic acid buffer
- **Th** T helper
- **TNF-α** tumor necrosis factor α
- **TTSS** type III secretion system
- **Yop** *Yersinia* outer-membrane protein

Summary

Chlamydophila pneumoniae is an obligate intracellular human pathogen, which causes respiratory tract infections, i.e. pneumonia, bronchitis, pharingitis and sinusitis. Seroepidemiological studies show that *C. pneumoniae* is widespread and nearly everybody becomes infected with it during his life. *C. pneumoniae* can also cause chronic infections, which might lead to atherosclerosis and heart disease. It is suspected to have a role in the pathogenesis of Alzheimer disease and multiple sclerosis. These are the reasons why it is important to develop vaccine against *C. pneumoniae* infection.

C. pneumoniae possesses a type III secretion system (TTSS), which allows them to secrete effector molecules into the inclusion membrane and the host cell cytosol. Low calcium response proteins E (LcrE) and H (LcrH) are parts of TTSS. The LcrE is surface exposed in *C. pneumoniae* elementary body (EB) and capable of eliciting protective antibodies in infected hosts, and therefore has potential as a candidate vaccine to prevent infection with this significant human pathogen. LcrH is a TTSS chaperone protein, expressed in the middle to late stages of the developmental cycle. 6His-tagged LcrE and LcrH were cloned from *C. pneumoniae* CWL029, expressed in *Escherichia coli* (*E. coli*) and purified from the supernatant using the HIS-select TALON CellThru Resin.

The immunogenicity and protective effect of recombinant LcrE protein combined either with Freund's or Alum adjuvants were investigated in mice. The immunization with both protocols resulted in a significant reduction of the number of viable *C. pneumoniae* in the lungs after challenge. Lower IgG2a/IgG1 ratio in Alum-immunized mice suggested a shift towards Th2 type immune response, but the presence of LcrE-specific IFN-γ producing cells in LcrE-Alum-immunized mice indicates Th1 type response also. LcrE-specific IgA level was higher in both the sera and the lungs after using Freund's adjuvant. Phenotype of LcrE-specific IFN-γ producing cells was CD4+ in Alum- and Freund's-immunized mice, but CD8+ cells were also detected in Freund's-immunized mice.

These results confirm that LcrE induces protective immunity in mice. The results also show that Alum is able to activate the CD4+ cell-based cellular immunity, thus it can be regarded as an alternative adjuvant during vaccine screening and a useful adjuvant in a potential subunit protein vaccine against *C. pneumoniae* infection.

The immunogenicity and protective effect of recombinant LcrH protein mixed with Freund's was investigated in mice. The immunization resulted in LcrH-specific IgG production without a significant reduction of the number of viable *C. pneumoniae* in the lungs after challenge.

Mycobacterium smegmatis is a species of rapidly growing saprophyte with a number of properties that make it an effective vaccine vector. Recombinant *M. smegmatis* expressing protective antigens of different pathogens and molecules modulating the immune responses offers some potential for reduction of the burden of tuberculosis, HIV and hepatitis B infections. Recombinant *M. smegmatis* expressing chlamydial proteins can be used as a vaccine vector. For this reason we studied homologues and heterologous protein over-expression and RNA metabolism in *M. smegmatis*.

Introduction

Because of the acute and chronic infections caused by *C. pneumoniae* and the fact that nearly everybody becomes infected with it during his life it is important to develop immunization against *C. pneumoniae*.

For possible vaccine candidates we were cloning certain genes of type III secretion system (which allows the bacteria to secrete effector molecules into the inclusion membrane and into the cytosol of the host cell) from *C. pneumoniae*. The genes for these vaccine candidate proteins were cloned either into *E. coli* expression vector or into *E. coli*-mycobacterium shuttle plasmid and *E. coli* and *M. smegmatis* were transformed with them. BALB/c mice were immunized either with the purified proteins or with recombinant *M. smegmatis* bacterium. Different immunization methods were applied.

Chlamydiae

Chlamydiae are common pathogens that infect humans and animals. They have diverged from other bacteria very early in bacterial evolution, and their genome degradation suggests that they adapted to intracellular lifestyle a long time ago. The obligatory intracellular lifestyle first suggested them to be protozoa or viruses, in late 1960's they were proved to belong to bacteria [1]. Chlamydiae are Gram-negative bacteria. Their envelope consists of an inner membrane, a periplasmic space, and an outer membrane (Fig. 1). Chlamydial envelope structure differs from that of other Gram-negative bacteria. Chlamydiae have no peptidoglycan in the periplasmic space of its envelope. Instead, the rigidity of the envelope derives from the cysteine-rich proteins that are integrated into the chlamydial outer membrane. Also, the chlamydial truncated LPS differs from the LPS of other Gram-negative organisms by its lower endotoxicity. Chlamydial lipid A contains five fatty acid chains while there are six fatty acid chains in the lipid A structure of the highly active endotoxin of enterobacteria. The fatty acid chains are longer in chlamydial lipid A than in enterobacterial lipid A [2].

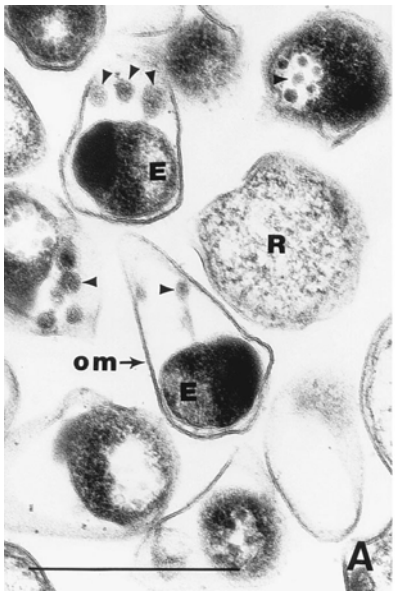


Fig. 1. Electron micrograph of *C. pneumoniae*. E, elementary body; R, reticulate body; om, outer membrane. Arrowheads indicate small electron-dense bodies (minibodies). Bar = 0.5 μm. Reprinted from reference [3].

Chlamydiae have been placed in their own order Chlamydiales. Characteristic to this bacterial order is their unique biphasic developmental cycle. At the beginning of the cycle, the small (Ø 0.3μm), infectious form of chlamydia, EB, attaches to the host cell and enters the cell within membrane-bound vesicle. Inside the vesicle, the bacterium is able to inhibit the phagolysosomal fusion by modifying the nascent inclusion membrane. The chlamydia-containing inclusions become fusogenic with sphingomyelin and cholesterol containing exocytic vesicles, and thereby they are dissociated from the endocytic pathway. Within the intact vesicle, *Chlamydia* EBs rapidly differentiates to metabolically active form, reticulate bodies (RB, Ø 1μm) and start replication by binary fission. After multiple divisions inside the vacuole, RBs transform to infective EB forms. The genomic DNA condensates with the aid of histone-like proteins and disulfide bridges of bacterial cysteine-rich outer membrane proteins are cross-linked [4]. At the end of this cycle, EBs are released from the host cell in order to attach and infect new host cells. The duration of chlamydial developmental cycle in cultured cell lines is approximately 48-72 hours (Fig. 2).

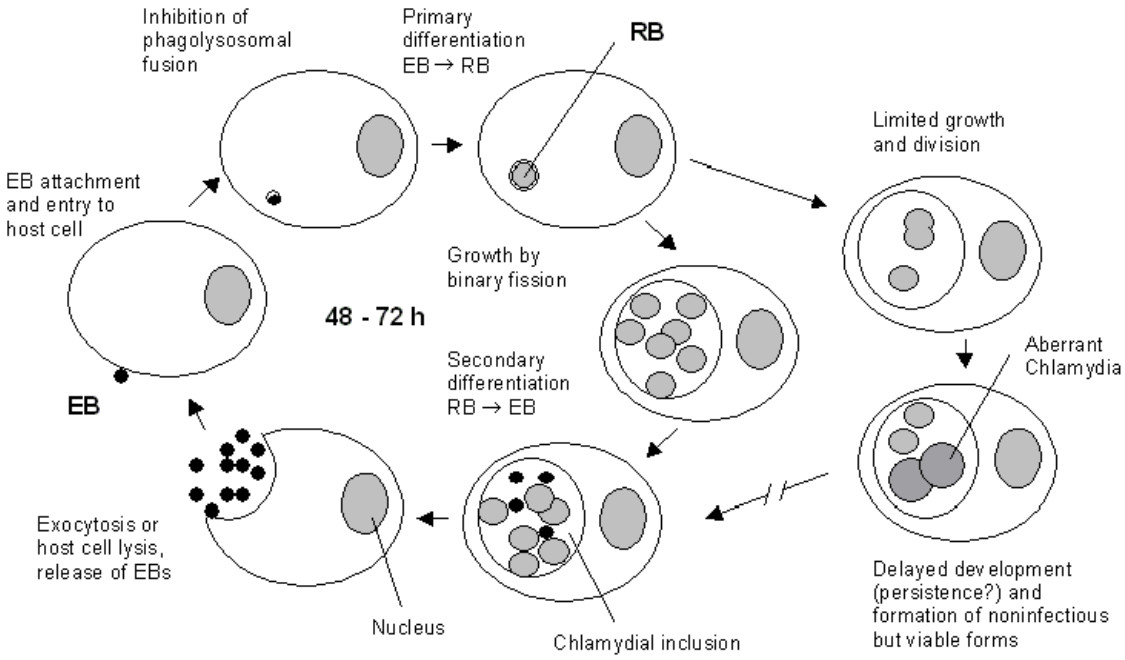


Fig. 2. Developmental cycle of *Chlamydia* (modified after reference [5])

The taxonomy retains currently known chlamydia strains with >90% 16S rRNA identity in the family *Chlamydiaceae* and separates other chlamydia-like organisms that have 80-90% 16S rRNA relatedness to the *Chlamydiaceae* into new families. According to the phylogenetic analysis of 16S and 23S DNA sequences, the family of *Chlamydiaceae* had been divided in two genera, *Chlamydia* and *Chlamydophila*, instead of one genus, *Chlamydia*. Two new species, *Chlamydia muridarum* and *Chlamydia suis* join *Chlamydia trachomatis* in the emended genus *Chlamydia*. *Chlamydophila* assimilates the current species, *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia psittaci*, to form *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*. Three new *Chlamydophila* species are derived from *Chlamydia psittaci*: *Chlamydophila abortus, Chlamydophila caviae* and *Chlamydophila felis* [6]. The most well known members of chlamydiae are human genital and ocular pathogen *C. trachomatis* and respiratory pathogen *C. pneumoniae*.

C. pneumoniae was first isolated in 1965 from the conjunctiva of a Taiwanese child participating in a trachoma vaccine trial [7]. The isolation was in the yolk sac of an embryonated chicken egg, the only method then available for growth of chlamydiae. In 1971, when cell culture methods became available, the organism (TW-183) was observed to form round, dense inclusions in host cells in cell culture which were more similar in morphology to those of *C. psittaci* than to those of *C. trachomatis*. An

organism cultured from the eye of a child in Iran in 1968 and isolated in a chicken egg yolk sac (IOL-207) has also proven to be *C. pneumoniae* [8]. Despite the conjunctival source of these two isolates, serologic studies suggested that the organism was not related to eye disease. The organism's role as a human pathogen was not defined until 1983, when the first respiratory isolate (AR-39) was obtained in Seattle, Washington, from a university student with pharyngitis [9]. Saikku *et al.* [10] described that the orphan TW-183 organism was associated with pneumonia. The strain name TWAR was derived from the laboratory designation of the first conjunctival and respiratory isolates (TW-183 and AR-39). In 1989, TWAR was established as a third species of *Chlamydia*, *C. pneumoniae* [3]. Since only one strain or serovar of *C. pneumoniae* has been identified, at this time the strain name, TWAR, is synonymous with the designation *C. pneumoniae.*

Acute infections caused by C. pneumoniae

C. pneumoniae is one of the most common human pathogens that cause acute infections like pneumonia, bronchitis, and pharyngitis. According to the serological studies, 50-70% of adult population worldwide is seropositive to *C. pneumoniae* [11]. Respiratory infections caused by *C. pneumoniae* are also often mild with no symptoms or only symptom of extended cough [12] and in most cases they probably remain undiagnosed.

Chronic diseases associated with C. pneumoniae infection

An asymptomatic and thereby untreated chlamydial infection may become persistent and lead to chronic conditions. Also, incomplete immunity after natural infection facilitates repeated infections that may exacerbate pathology and lead to chronic conditions. The association of *C. pneumoniae* with chronic human diseases was first shown in seroepidemiological studies, which demonstrated the association of antibodies to *C. pneumoniae* with acute myocardial infarction [13]. Later, the seroepidemiological association of the infection with other chronic conditions, such as asthma and chronic bronchitis, has been suggested [14]. The seroepidemiological association of *C. pneumoniae* with coronary artery disease (CAD) has also been supported by detection of antigens by immunocytochemistry (ICC) or nucleic acids of the organism by PCR in the affected tissues [15, 16, 17]. Yet, the timing of *C. pneumoniae* infection and development of arteriosclerosis is poorly understood [18, 19, 20, 21, 22]. The diagnosis of an acute *C. pneumoniae* infection is demanding, but it is

even more challenging in the case of diagnosing chronic condition. In chronic infections, the amount of bacteria in the infected tissue may be very low, or the site of infection is unreachable for sampling (e.g. vessel wall). A combination of two different tests, PCR and serological tests is often adapted to the diagnosis of chronic chlamydial infections. Usually, inadequate samples of the site of the infection or the difficulty to interpret the meaning of steady-state levels of *C. pneumoniae*-specific antibodies hamper the reliable diagnosis [23]. Although acute chlamydial infections are curable with antibiotics, the antimicrobial therapy, used in treatment of acute infections, may not be effective in resolving the infection in the associated chronic conditions [24, 25]. Therefore, vaccination has been suggested to be an effective strategy for prevention and also controlling the chronic chlamydial infection.

Vaccines against intracellular microbes

Vaccination has a major impact on reduction of morbidity and mortality caused by several bacterial and viral infections. However, there are still diseases like HIV and malaria, for which effective vaccines are not available [26]. The requirements for protective immunity in these infections are obviously different from the responses that are induced by the current vaccines [27]. Vaccines in clinical use usually consist of either killed or attenuated microbes (influenza, polio, measles, tuberculosis) or subunits of the organisms (*Haemophilus influenzae* type B, Meningococcus A and C, Hepatitis B) [28]. These types of vaccines readily induce long-lived humoral immune response as well as cell-mediated response and production of protective antibodies has indeed been sufficient against most of the aforementioned infections. However, in infections like HIV and malaria, as well as in many other infections caused by intracellular microbes, protective immunity depends strongly on the action of cell-mediated immunity; i.e. on T lymphocytes that are able to mediate the destruction of infected cells and/or the infectious agent residing inside the cell. So far, the induction of protective and long-lasting cell-mediated immunity has proven to be a difficult task despite of advances in understanding of the nature of immune responses.

Recombinant M. smegmatis as a vaccine candidate

M. smegmatis, first identified in 1884, is a saprophyte; it is a species of rapidly growing mycobacteria. It grows fast and can propagate one generation every 1–3 h. It is non-pathogenic and commensal in humans [29, 30], and it is powerful cell immunity adjuvant. Unlike other mycobacterial species, such as *M. tuberculosis*, that survive in

host cells by inhibiting phagosome maturation, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells [31, 32]. *M. smegmatis* has a number of properties that may make it an effective vaccine vector. This fast-growing mycobacterium is unable to arrest phagolysosome maturation and cannot evade intracellular killing [33, 34, 35, 36]. Moreover, its rapid clearance by the host differs from *M. tuberculosis* or even from the vaccine strain *M. bovis* bacillus Calmette-Guérin (BCG) [32]. Therefore, *M. smegmatis* has been used as vaccine vector, because it activates dendritic cells and induces CD8-mediated immune responses [37, 38, 39, 40]. Recombinant *M. smegmatis* has been used as vector in defence against tuberculosis [41, 42, 43, 44, 45], HIV [39, 40, 46, 47], Hepatitis B virus [48] and *Helicobacter pylori* [49].

Type III Secretion System components as potential vaccine proteins

Like in many other human pathogens such as *Yersinia*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa* and pathogenic *E. coli*, *C. pneumoniae* possess a TTSS, enabling the bacteria to secrete effector proteins into the cytosol of the infected cell [50, 51]. This system is active during the intracellular phase of the chlamydial replicative cycle and is responsible both for the insertion of chlamydial proteins into the inclusion membrane that separates the growing chlamydial microcolony from the host cell cytoplasm, and also for secretion of proteins into the host cell cytoplasm which modulate the cell response to ongoing chlamydial replication.

The protein encoded by *lcrE* is homologous to *Yersinia* YopN, a surface protein [52] thought to be a TTSS response regulator, which senses either a host cell contact *in vivo* or Ca^{2+} concentration *in vitro*. It is located at the outermost position in the TTSS structure [53]. The fact that LcrE protein exposed in the EB suggests that the TTSS apparatus may also be fully assembled in extracellular chlamydiae, possibly to be used in early events of cell infection, e.g., in order to assist the entry of chlamydiae into the host cell and the successful establishment of the early chlamydial inclusion vacuole [54].

The *lcrH* encoding a chaperone, unlike other TTSS systems, is not clustered with other known homologues of TTSS genes. The *lcrH* gene cluster is expressed late in the developmental cycle, at the time when RBs are redifferentiating to EBs. Maximal expression occurs at 48 h postinfection and is similar to that of the known late gene *omcB*, which encodes a cysteine-rich outer envelope structural protein in EBs [55, 56].

The protein product of the *lcrH* is expressed when EBs are forming and protein can be detected in the EBs [54, 57]. LcrH is one of the specialized chaperones which function to stabilize and assure efficient secretion of translocator proteins and also to regulate expression of some of the TTSS genes [58]. It is homologous to *Yersinia lcrH*, which codes for a small acidic cytoplasmic protein (19 kDa) with a predicted pI of 4.5 which is thought to act as a chaperone for the translocator apparatus proteins, YopB and YopD, and is thought to have an additional regulatory role for *yop* expression [51].

Herrmann *et al.* [59] detected increasing expression of LcrE towards the end of the *C. pneumoniae* infection cycle. The fact that LcrE appears to be presented to antibodies on the surface of EB makes this protein a possible vaccine candidate. Sambri *et al.* [60] described a protective effect of recombinant LcrE protein mixed with Freund's adjuvant given subcutaneously against *C. pneumoniae* challenge in a hamster model. Since Freund's complete adjuvant (FCA) was originally described by Freund *et al.* [61], it has been the most widely utilized and effective adjuvant for experimental antibody production. FCA induces Th1 response and acute-phase reactants and inflammatory cytokines (IL-6 and IL-1β but not TNF-α). Unfortunately, the use of FCA has been associated with a variety of lesions, including localized injection site granulomas, distant subpleural, hepatic, and renal granuloma formation, necrotizing dermatitis, and spinal cord compression from an injection site granuloma [62]. FCA has been suggested to be replaced by other adjuvants in animals when possible. Aluminum-adjuvanted vaccines, with an excellent safety record, have been used in humans for many years. Aluminum-adjuvanted antigens are rapidly cleared after injection, which leads to peak antibody titres 3 to 4 weeks after injection with a rapid decline, although repeated injections can lead to prolonged antibody responses [63]. Aluminum salts induce antibodies and Th2 responses; this adjuvant is responsible for priming of splenic B cells and for the accumulation of IL-4-producing cell population in the spleen [64]. As *C. pneumoniae* is an obligate intracellular bacterium, a cell-mediated immune response is crucial for protection against infection. In BALB/c mice the Th1 response is most likely important for clearance of infection in both primary infection and re-infection with high IFN-γ levels and low IL-10 levels [65]. In human the immune response during primary infection is similar with high IFN-γ levels and low IL-10 response [66]. Tammiruusu *et al.* [67] detected that T lymphocytes, especially CD8+ T cells, and IFN-γ are important in the protection against intranasal *C. pneumoniae* infection in mice.

Aim

The purpose of this study was:

- the over-expression and purification of chlamydial LcrE and LcrH proteins in large quantities;
- comparison the immunogenicity and the protective ability of subcutaneous recombinant protein vaccination with LcrE in combination with Freund's or Alum adjuvants against *C. pneumoniae* infection in mice;
- creation of recombinant *M. smegmatis* expressing chlamydial proteins which can be used as vaccine:
    - for this purpose we studied protein over-expression in *M. smegmatis*, its Rne/Rng family protein was cloned and expressed and some of its associated proteins were identified;
    - furthermore chlamydial proteins were cloned into *M. smegmatis*.

Materials and methods

Bacterial strains and growth conditions

M. smegmatis MC2 155, *E. coli* DH5α, *E. coli* HB101 and *C. pneumoniae* CWL029 (ATCC) strains were used. *E. coli* strains were routinely grown in LB (Luria–Bertani) medium supplemented with the appropriate antibiotic(s). *M. smegmatis* MC2 155 was grown in LB medium or in Middlebrook 7H9 broth or 7H11 agar (Difco) supplemented with 10% Middlebrook OADC (oleic acid/albumin/dextrose/catalase) enrichment (Difco) and 0.05% Tween 80 (Sigma).

Plasmid constructions

A 3.1 kb DNA fragment containing the MSMEG_4626 gene (Gene ID: 4531291; Locus tag: MSMEG_4626) encoding the ribonuclease (RNase) Rne/Rng family protein was amplified by PCR, using the following oligonucleotide primers: S1 5'-GTG CAT ATG GCC GAA GAT GCC CAT-3' and S2 5'-ACC GGA TCC GTG ATG CTC GTC TAG-3' and *M. smegmatis* MC2 155 DNA as template.

A 1200 bp and a 700 bp fragments containing the *lcrE* (GeneID: 895078; Locus tag: CPn0324) and *lcrH* (GeneID: 894648; Locus tag: CPn0811) genes respectively were amplified by PCR, using the following primers: E1 5'-GGA GGC ATA TGG CAG CAT CA-3' and E2 5'-CAC AGG ATC CGT ATT GGT TTT GCA TGG C-3' for LcrE; and: H1 5'- TCT CAT ATG AGC AAG CCC TCT C -3' and H2 5'- TCT GGA TCC CTC CTT AGA ATC TTA CTA ACG -3' for LcrH, with *C. pneumoniae* CWL029 DNA as template.

PCR was performed in a GeneAmp II thermocycler (Applied Biosystems, Foster City, CA, USA) with Advantage GC cDNA polymerase (Clontech, Mountain View, CA, USA) and the amplification conditions were set as recommended by the manufacturer.

The amplified DNAs were digested with NdeI and BamHI and inserted into the plasmid p6HisF-11d(icl) [71] by digesting it with the same enzymes and replacing the *icl* gene, the resulting plasmids were pSRNE1, pLCRE1 and pLCRH. p6HisF11d(icl) is a pET-11d (Novagen) based plasmid carrying the 6His and FLAG tags in the N-terminal of the cloned insert.

Since pET plasmids can not be used in mycobacteria we constructed pSRNE2 as well as pLCRE2 which carries RNase E, and LcrE respectively after the mycobacterial

icl promoter (Rv0467), 6His and FLAG tags in pMV262 [72]. I1 5'-ACT ATC TAG ATC CGC AGG ACG TCG A-30 and I2 50-GAC AGC CAT GGA CAA CTC CTT A-3' primers were used to synthesize the *icl* promoter with *M. tuberculosis* H37Rv chromosomal DNA as template. The amplified DNA was cut with XbaI and NcoI and inserted into pSRNE1 and pLCRE1 opened with the same enzymes. The XbaI–BamHI fragment from pSRNE1 and from pLCRE1 were inserted to pMV262, the resulting plasmids were pSRNE2 and pLCRE2.

Expression of Rne, LcrE and LcrH

For over-expression, *E. coli* HB101(pGP1-4) cells carrying either the pSRNE1, pLCRE1 or the pLCRH plasmids were grown and treated according to the method of Tabor and Richardson [73]. Briefly, cells containing the plasmids were grown at 32°C in LB medium in the presence of the required antibiotics (ampicillin and kanamycin). Over-expression of proteins was induced by shifting the temperature to 42°C for 20 min. After induction, the temperature was shifted down to 37°C for an additional 90 min or longer, cells were harvested by centrifugation and cell pellets were frozen.

Purification of Rne, LcrE and LcrH proteins

Cell lysates were prepared by resuspending the frozen cell pellets in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7,0) containing protease inhibitor cocktail (Sigma) and lysozime (Sigma) (0,75 mg/ml). Bacteria were opened by sonication. After centrifugation Rne, LcrE and LcrH proteins were purified from the supernatant using the TALON CellThru Resin (Clontech), following the vendor's instructions.

Protein detection

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [74]. Gels were stained with Coomassie brillant blue [75].

Generation of recombinant *M. smegmatis* expressing either RNase E or LcrE

Competent *M. smegmatis* MC2 155 prepared in 10% glycerol was transformed with either the pSRNE2 or the pLCRE2 plasmids by electroporation with a Gene Pulsar (Bio-Rad) set at 2.5 kV and 25 μF and with the pulse controller resistance set at 1,000 Ω [76, 77]. Transformed *M. smegmatis* was selected on Middlebrook 7H10 (Difco,

Sparks, MD) agar plates supplemented with 30 μg/ml kanamycin. To monitor the expression of RNase E or LcrE, individual colonies of recombinant *M. smegmatis* were grown in Middlebrook 7H9-OADC-Tween broth in the presence of 30 μg/ml of kanamycin and were harvested by centrifugation. After a rinse with sterile phosphate-buffered saline (PBS), mycobacterial cells were lysed by using a MiniBeadbeater-8 cell disrupter (BioSpec Products, Inc, Barlesville, Oklahoma), with glass beads (106 μm, Sigma) and adding a protease inhibitor cocktail (Sigma). Cell lysates were cleared by centrifugation. The lysate of recombinant *M. smegmatis* was separated by 10% SDS-PAGE and the expression of the LcrE protein was checked with Western blot.

Western blot

Purified LcrE and LcrH proteins and concentrated *C. pneumoniae* elementary bodies (prepared as described earlier [78]) were heated to 95°C for 5 min in sample buffer, and the proteins were separated by 10% SDS-PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked overnight at 4°C with PBS containing 5% skim milk and 0.05% Tween 20. Membranes were probed with LcrE or LcrH immunized and non-immunized mouse sera (1:50 dilution in PBS containing 5% skim milk and 0.05% Tween 20). After washings, the filter was incubated with HRP-conjugated anti-mouse IgG, and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) with hydrogen peroxide in 10 mM Tris pH 7.5.

Identification of proteins by mass spectrometry

The gel slices containing the corresponding polypeptides copurified with LcrH from *E. coli* and with RNase E from *M. smegmatis* were cut from the gel and analysed by mass spectrometry. Briefly, the proteins were digested with trypsin directly in the gel slices, and the resulting products of digestion were eluted and analysed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. The resulting peptide mass lists were subjected to a database search. Post source decay analysis was used to confirm the MALDI-TOF results. Homology search was carried out with the computer programs of Altschul *et al.* [79]. Protein parameters were calculated by the ExPASY Server [80].

Immunization either with LcrE or with LcrH proteins or with recombinant and wild type *M. smegmatis*

Specific-pathogen-free 6-8-week-old, female BALB/c mice were obtained from Charles River Laboratories UK (Kent England). Mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged and were given food and water *ad libitum*. The mice in groups of 25 were immunized subcutaneously into the tail base with the purified LcrE protein diluted in PBS at a dose of 20 μg mixed with 25 μl Alum (Aluminum hydroxide Gel, Sigma) or 75 μl Freund's adjuvants (FCA) (Chemicon International, Temecula, CA, USA; 1st inoculation with complete and 2nd and 3rd inoculations with incomplete Freund's adjuvant) in 150 μl volume 3 times at 3-week intervals.

Mice in groups of 5 were immunized subcutaneously at the tail base either with the purified LcrH protein diluted in PBS at a dose of 20 μg in 75 μl mixed with 75 μl Freund's adjuvant (1st inoculation with complete; 2nd and 3rd inoculations with incomplete Freund's adjuvant) 3 times at 3-week intervals.

Mice in groups of 5 were immunized intraperitoneally either with the recombinant *M. smegmatis* or with wild type *M. smegmatis* (10^8 colony forming unit (CFU) in a 100-μl total injection volume). Four weeks after the first immunization, mice were boosted with the same quantity of the same mycobacteria. Mice were sacrificed 14 days after last immunization. Blood was collected in heparinized capillaries from the retro-orbital plexus.

Challenging the LcrE and LcrH immunized and non-immunized mice

Two weeks after the last immunization with LcrE the immunized and non-immunized mice (absolute naive animals) were challenged with $4x10^5$ inclusion forming units (IFU) of *C. pneumoniae* (CWL029, ATCC) in 25 μl PBS intranasally under pentobarbital sodium anaesthesia. At this time point serum samples were collected in heparinised capillaries by retro-orbital bleeding for testing the antibody production and groups of 5 mice were euthanized and spleens were dissected and homogenized by pressing through nylon mesh into complete growth medium containing RPMI 1640 (Sigma), 10% foetal calf serum, 10 mM HEPES (Sigma), L-glutamine (0.3 mg/ml; Sigma), gentamicin (60 μg/ml, Sanofi Aventis, Hungary) and 50 μM 2-mercaptoethanol (Sigma) for testing cell mediated immunity. The remaining mice were

sacrificed 7 days after infection. By heart puncture blood was collected in heparin. The lungs were removed and homogenized mechanically in 2 ml of sucrose-phosphate-glutamic acid buffer (SPG) for cultivation of bacteria and LcrE-specific IgA antibody detection.

LcrH-immunized mice were challenged with $4x10^5$ inclusion forming units (IFU) of *C. pneumoniae* (CWL029, ATCC) in 25 µl PBS intranasally under pentobarbital sodium anaesthesia. At this time point serum samples were collected in heparinised capillaries by retro-orbital bleeding for testing the antibody production. At 7 days after infection the mice were sacrificed. The lungs were removed and homogenized mechanically in 2 ml of SPG for cultivation of bacteria.
All animal experiments complied with the University of Szeged Guidelines for the Use of Laboratory Animals.

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

C. pneumoniae was propagated on HEp-2 cells (ATCC) as described earlier [81]. The partially purified and concentrated EBs were aliquoted and stored at –80 °C until use. A mock preparation was prepared from uninfected HEp-2 cell monolayer processed in the same way as the infected cells. The titre of the infectious EBs was determined by inoculation of serial dilutions of the EB preparation onto HEp-2 monolayers, and after 48 h culture cells were fixed with acetone and stained with monoclonal anti-*C. pneumoniae* antibody (DAKO Ltd. Ely, UK) and FITC-labelled anti-mouse IgG (Sigma). The number of *C. pneumoniae* inclusions was counted under a UV microscope, and the titre was expressed as IFU/ml. Lung homogenates from individual mice were centrifuged (10 min, 400 g), serial dilutions of the supernatants were inoculated onto HEp-2 cell monolayers and the titre (IFU/ml) of *C. pneumoniae* was determined as described for the titration of the *C. pneumoniae* inoculum.

ELISA tests

LcrE-specific antibodies were detected by ELISA, using plates coated with recombinant LcrE protein (100 ng/well), and horseradish-peroxidase (HRP)-conjugated secondary antibodies (α-mouse IgG-HRP; α-mouse IgA-HRP, Sigma and α-mouse IgG1 and IgG2a-HRP, Biosource) were used for detection. Purified mouse IgG1 and IgG2a (Cappel, Laboratories, Downington, PA, USA)-coated plates were used for determination of the corresponding secondary antibody dilutions that gave quantitatively similar reactivity in the ELISA assay. The titres were determined at

dilutions demonstrating an optical density (OD) higher than 0.1. Cytokines (IL-4, IL-6, IL-10, IFN-γ – BD OptEIA set, BD Biosciences Pharmingen, San Diego, CA, USA; KC, MIP-2 – R&D Systems Minneapolis, MN, USA) were determined in the lung homogenates by ELISA according to the manufacturers instructions.

Lymphocyte proliferation assay

Proliferation of spleen cells *in vitro* stimulated with LcrE and *C. pneumoniae* was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) method [82]. Single-cell suspensions from two spleens were pooled and resuspended in complete growth medium used during homogenization of spleen cells. The proliferative response of $5x10^5$ splenocytes in three parallel wells to 2 μg/ml concentration of LcrE recombinant protein, purified formalin-inactivated *C. pneumoniae* EBs (1 μg/ml) [65] and HEp-2 mock preparation treated similarly were detected after incubation for 3 days. The proliferation was determined by MTT assay according to the manufacturer's instructions (Boehringer Mannheim Biochemica, Mannheim, Germany). Proliferation index (PI) was calculated according to the following formula: ODs obtained in the presence of the test antigen divided by the ODs obtained in the presence of culture medium alone.

ELISPOT assay for the determination of IFN-γ producing cells

ELISPOT assay was performed to define the number and the phenotype of the spleen cells producing IFN-γ after *in vitro* re-stimulation with LcrE protein or with viable *C. pneumoniae* at a multiplicity of infection of 0.2 IFU, or with an equivalent amount of HEp-2 mock preparation. To determine the phenotype of IFN-γ producing cells, the spleen cell suspensions were depleted of CD4+ and CD8+ cells, respectively by using micro-beads coated with the respective antibody [CD4 (L3T4), CD8a (Ly-2), Miltenyi Biotec, Bergisch, Germany] and applying the magnetic cell sorting (MACS) system of Miltenyi Biotec. The outcome of the procedure was controlled by FACS analysis after direct staining of the depleted cells by α-CD4-TC and α-CD8-rPE antibodies (Caltag Laboratories, Burlingame, CA, USA). Ninety-six-well filter plates with a cellulose ester membrane (MAHAS4510, Millipore Corporation, Bedford, MA, USA) were coated overnight at 4 °C with the capture antibody of the mouse IFN-γ ELISPOT set (BD Biosciences). The stimulated spleen cells ($4x10^5$) were distributed into each well in triplicate and incubated. After 20 h, the plates were washed, and

biotinylated detection antibody, HRP-streptavidin and substrate solution were then added, as suggested by the protocol. The mean number of spots counted in triplicate wells under a dissecting microscope was used to calculate the number of spot-forming cells (SFC) per 1 million (1M) spleen cells.

Statistical analysis

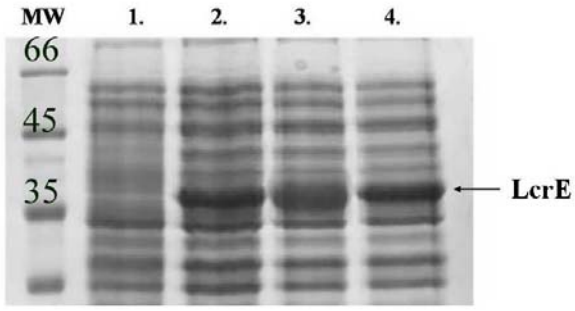
Statistical analysis of the data was carried out by GraphPad Prism 5 software using Wilcoxon-Mann-Whitney two-sample test. Differences were considered significant at $P<0.05$.

Results

Over-expression of LcrE and LcrH proteins in *E. coli*

LcrE is a protein consisting of 399 amino acids. Its calculated molecular mass is 43 kDa; because of the 6His and FLAG tags, our fusion protein is 4 kDa larger. LcrH protein is smaller, it contains 230 amino acids. Its calculated molecular mass is 26 kDa; it also contains the 6His and FLAG tags. Fig. 3. depicts *E. coli* cell lysates separated by SDS-PAGE after over-expression. Upon heat-induction a strong band appears at the indicated position (Fig. 3A-B., lane 2, 3, 4). Since these proteins carry the 6His and FLAG tags, both tags can be exploited during purification. Fig. 3 shows that after heat-induction the strong over-expression occurred after 90 minutes in case of LcrE (Fig. 3A lane 3) and after 150 minutes in case of LcrH (Fig. 3B lane 4).

A.



B.

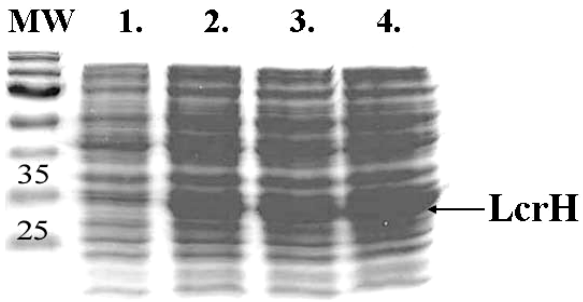


Fig. 3: 10% SDS-PAGE analysis of *E. coli* extracts with cloned *lcrE* gene (A) and with cloned *lcrH* gene (B). Lanes 1 show extracts of *E. coli* cultures without heat-induction and lanes 2, 3 and 4 show extracts of *E. coli* preparation with heat-induction. Samples were taken: A. at 60' (lane 2), 90' (lane 3) and 120' (lane 4); B. at 45' (lane 2), 90' (lane 3) and 150' (lane 4) after heat-induction.

Chlamydial protein purification and identification by MALDI-TOF MS

The purifications presented in Fig. 4 were carried out with the TALON CellThru Resin. In case of the crude extract of LcrE-expressing *E .coli* the SDS-PAGE analysis demonstrates a strong band at the indicated position (around MW 45 kDa) in lane 1 (Fig. 4A). In case of the purified LcrE strong bands are visible at the same positions in the eluted fractions 2 and 3 (Fig. 4A. lane 5, 6). Despite the use of protease inhibitors, the LcrE molecule seems to be unstable. LcrH-*E. coli* crude extract and the eluted fractions 3 and 4 show strong bands below MW 35 kDa marker (Fig. 4B. lane 1 and lanes 6, 7, respectively). With this purification method we are able to purify up to 20 mg proteins from 3-L bacterial culture.

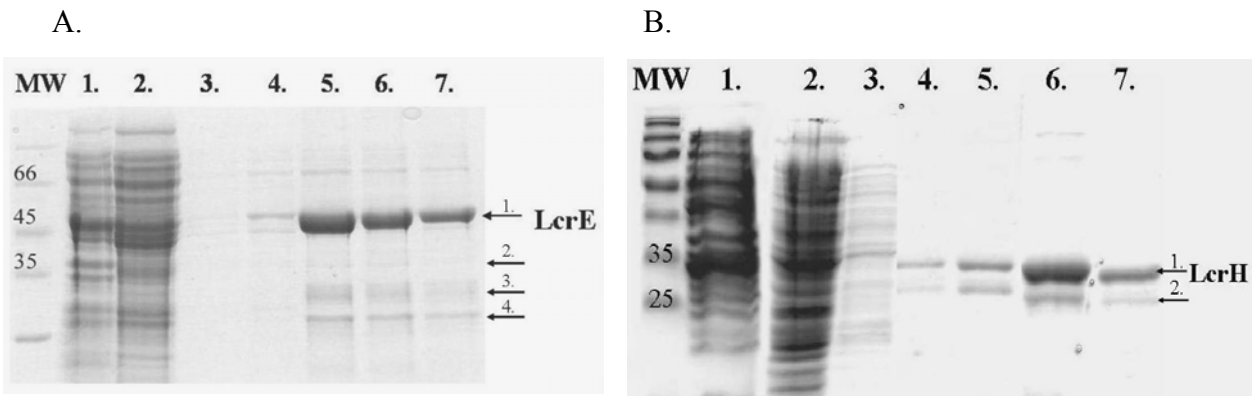


Fig. 4. 10% SDS-PAGE analysis of the purification process of LcrE (A) and LcrH (B). Crude extracts and purified proteins were analyzed under reducing conditions with ß-mercaptoethanol. The gels were stained with Coomassie blue. Lane MW - molecular weight marker; lane 1 - the original crude extract; lane 2 - the supernatant; lane 3 - wash buffer; lanes 4-7 - elution samples. (Table 1A shows the proteins co-purified with LcrE; table 1B shows the proteins co-purified with LcrH protein.) The identified proteins are labelled by arrows.

The eluted fractions containing LcrE were concentrated 10 times using concentrators with 5 000 MW cut-off and then subjected to 10% SDS–PAGE. After Coomassie blue staining, only 4 protein bands were identified. In the purified LcrH two protein bands were detected by SDS-PAGE. Gel slices containing the proteins of LcrE and LcrH samples were cut from the gel. The proteins extracted from the gel fragments were analysed by MALDI-TOF MS (Fig. 4). The identified proteins are listed in Table 1. The results suggested that a large proportion of the identified proteins were *C.*

pneumoniae proteins. The majority of the associated proteins identified in our study were *E. coli* ribosomal proteins, other co-purified proteins were those involved in stress response, which was presumably evoked by the heat-induction.

Table 1. MALDI-TOF MS analysis of proteins co-purified with LcrE (A) and LcrH (B) identified by SDS–PAGE fractionation. Numbers refer to gel bands shown in Fig. 4. MW is calculated for the full sequence listed in the database and may not reflect the actual size of the protein.

A.

Band	Name	Accession no.	MW (Da)	Coverage (%)
1	*Chlamydophila pneumoniae* low calcium response protein E	15618244	43306.7	39
2	*Chlamydophila pneumoniae* low calcium response protein E	15618244	43306.7	25
	E. coli cyclic AMP receptor protein	15803871	23640.6	23
	E. coli 50S ribosomal protein L2	15803844	29860.6	14
3	*Chlamydophila pneumoniae* low calcium response protein E	15618244	43306.7	9
	E. coli universal stress protein UP 12	16128590	15935.3	28
	E. coli 50S ribosomal protein L 14	15803837	13541.1	13
4	*E. coli* 30S ribosomal protein S 19	15803843	10430.3	32

B.

Band	Name	Accession no.	MW (Da)	Coverage (%)
1	*Chlamydophila pneumoniae* low calcium response protein H	15618720	26101	26
	E. coli protein L12	223571	29825	21
2	*E. coli* 30S ribosomal protein S3	15803841	26021	40
	E. coli 30S ribosomal protein S4	15803823	23512	33
	E. coli 50S ribosomal protein L3	15803847	22230	26
	Chlamydophila pneumoniae low calcium response protein H	15618720	26101	19

Western blot analysis of LcrE and LcrH proteins

The immunogenicity of the purified proteins in mice and the *Chlamydia*–specificity of the produced antibodies were confirmed by Western blot (Fig. 5). The proteins were used for immunization of mice, and sera reacted only with the proteins in the appropriate positions in the concentrated *C. pneumoniae* preparation.

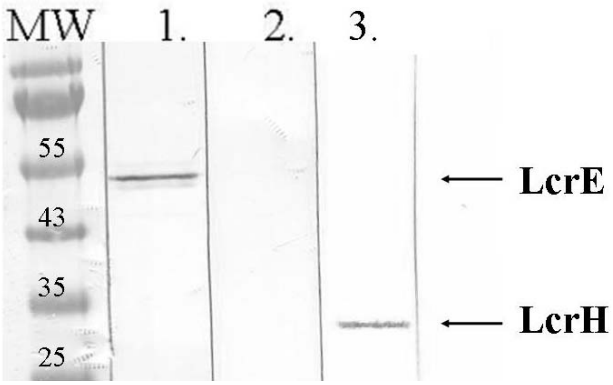


Fig. 5. LcrE and LcrH-immunized and non-immunized mouse sera were tested in Western blot assay. Concentrated *C. pneumoniae* was probed with sera of LcrE-Freund's immunized mice (lane 1), with non-immunized mouse sera (lane 2) and with sera of LcrH-Freund's immunized mice (lane 3)

Level of LcrE and LcrH-specific IgG in the sera and isotype of LcrE-specific IgG antibodies in immunized mice

High-titre LcrE-specific IgG was detected by ELISA in the mouse sera at the time of *C. pneumoniae* challenge, with no significant difference between the antibody levels induced by the different adjuvants (titres as geometric mean: LcrE-Freund's: 172216; LcrE-Alum: 164540). LcrE and chlamydia–specificity of IgG produced in immunized mice was confirmed by Western blot. Both Alum and Freund's immunized sera reacted with the band corresponding to LcrE protein in the purified LcrE preparation used for immunization and also with LcrE in purified chlamydia elementary body. Sera of mice infected 3 times with *C. pneumoniae* reacted with proteins of EB but did not recognize the LcrE protein band in Western blot, although the production of LcrE-specific antibodies during the infection was clearly detectable by ELISA using LcrE protein as antigen (mean OD of 5 mouse sera = 0.479, with a range of OD 0.222–2.469 at a dilution of 1:50). (Fig. 6).

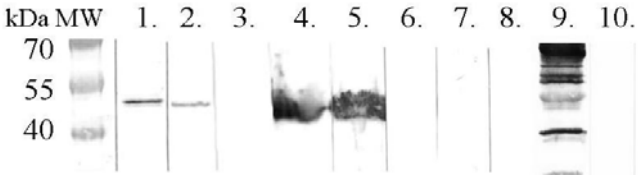


Fig. 6. Specificity of IgG in sera of LcrE-immunized and *C. pneumoniae*-challenged mice as tested in Western blot assay. *C. pneumoniae* EB was probed with sera of LcrE-Alum-immunized mice (lane 1), with sera of LcrE-Freund's-immunized mice (lane 2) and non-immunized mouse sera (lane 3). Purified LcrE protein was probed with sera of LcrE-Alum-immunized mice (lane 4), with sera of LcrE-Freund's-immunized mice (lane 5), with sera of non-immunized mice (lane 6), with a pool sera of 5 mice infected three times with *C. pneumoniae* (lane 7), and with a pool sera of mock-infected mice (lane 8). *C. pneumoniae* EB was probed with sera of mice infected three times with *C. pneumoniae* (lane 9) and with sera of mock-infected mice (lane 10).

Irrespective of the applied adjuvant high IgG1 level was detected in the sera, however the IgG2a titres were higher in Freund's adjuvant-immunized mice (Fig. 7). Both adjuvants induced a mixed Th1/Th2 isotype pattern; a higher relative IgG2a level indicates a Th1-biased response in the case of Freund's adjuvant-immunized mice.

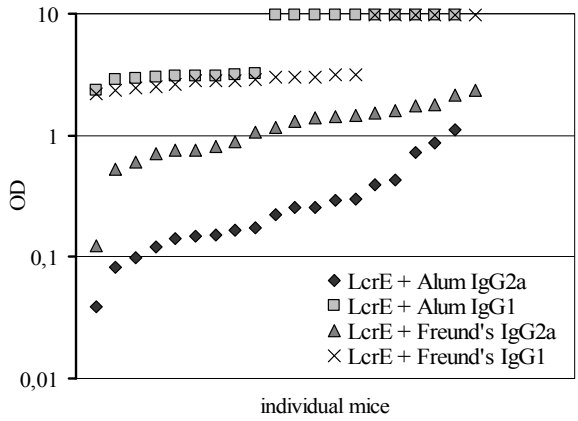


Fig. 7. LcrE-specific IgG1 and IgG2a levels in LcrE-immunized and *C. pneumoniae* challenged mouse sera. Isotype of LcrE-specific IgG in the sera of immunized mice was measured by ELISA. Symbols represent OD values measured with sera of individual mice. Sera were tested at a dilution of 1:102400 for IgG1, and 1:6400 for IgG2a.

LcrE-specific IgA was found in the sera and in the lungs. Freund's adjuvant was more effective in inducing LcrE-specific IgA at both sites; however IgA levels in the lungs showed high variation in Freund's adjuvant-immunized mice (Fig. 8).

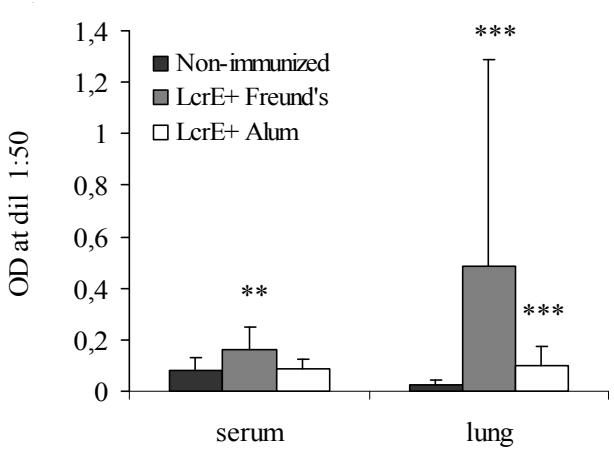


Fig. 8. LcrE-specific IgA in the sera and lungs of LcrE-immunized and *C. pneumoniae* challenged mice. Bars denote OD values (mean + S.D.) measured from sera and supernatants of lung homogenates of 20 mice at a dilution of 1:50. The LcrE-specific IgA level of immunized mice was significantly higher than in non-immunized mice (In the serum $P<0.007$ ** for Freund's adjuvant; in the lung $P< 0.0002$ *** for Freund's adjuvant, $P< 0.0003$ *** for Alum adjuvant).

Mice immunized with LcrH protein mixed with Freund's adjuvant responded with specific antibody production. LcrH-specific IgG was detected by ELISA with a mean titre of 5300.

The local cytokine response

To investigate whether the immunization affects the local cellular reaction in response to the infection, the cytokine production was analyzed in the supernatant of the lung homogenates of mice after *C. pneumoniae* challenge. IL-4 was present at marginal concentration and IL-10 levels were similarly increased in the lungs of all groups of mice. The IL-6 and IFN-γ content of the lungs was slightly diminished in the LcrE-Freund's immunized mice which might suggest a lower level of inflammation in these animals (Fig. 9). Suppression of pro-inflammatory cytokines, keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein-2 (MIP-2) in immunized compared to the non-immunized mice were also observed (Fig. 10).

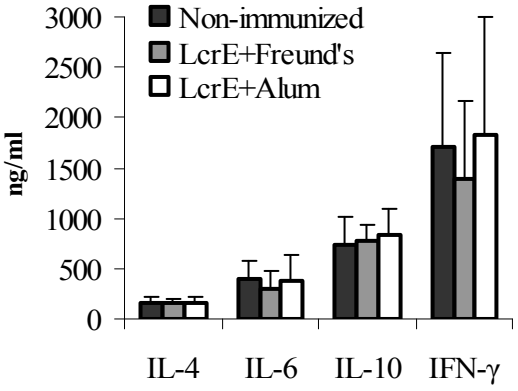


Fig. 9. Cytokine levels in the lungs of LcrE-immunized mice after *C. pneumoniae* infection. Cytokines in the supernatants of lung preparations of LcrE-immunized mice and non-immunized mice after *C. pneumoniae* infection were assayed by the respective cytokine OptEIA sets. Bars represent mean + S.D. cytokine concentrations of 20 samples.

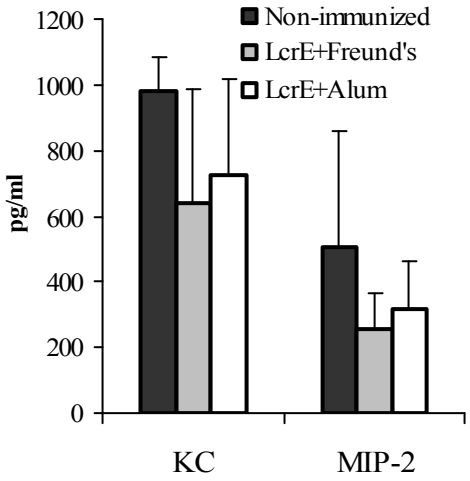


Fig. 10. KC and MIP-2 level in the lungs of LcrE-immunized and non- immunized mice after *C. pneumoniae* infection. KC and MIP-2 were determined in the lung homogenates by ELISA. Bars represent mean + S.D. cytokine concentrations of 20 samples.

Cellular immune response

For comparison and quantitation of the antigen-specific T-cell reactivity following immunization a lymphoproliferation assay was performed. The spleen cells of LcrE immunized mice collected 2 weeks after the last immunization were re-stimulated *in vitro* with LcrE protein, *C. pneumoniae* or mock antigens. Proliferation indices were determined. Proliferative responses were detected after both immunization methods

upon stimulation with LcrE protein and also with *C. pneumoniae* antigen (Fig. 11). Stimulation with LcrE protein induced proliferation of spleen cells from non-immunized but *C. pneumoniae* infected mice (data not shown).

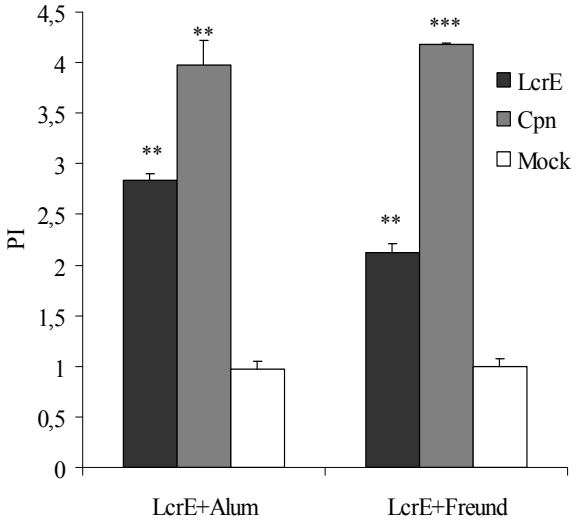


Fig. 11. Proliferation of spleen cells of LcrE-immunized and *C. pneumoniae* challenged mice. The pooled spleen cells of 2 LcrE immunized mice were stimulated *in vitro* with LcrE protein, *C. pneumoniae* antigen and mock antigen, and the proliferation was determined by MTT assay. Bars denote mean proliferation indices (PI) calculated in two independent experiments. Proliferation indices were significantly higher after stimulation with LcrE protein or *C. pneumoniae* antigen than mock antigen [$P<0.005$ ** for Alum adjuvant; $P<0.005$ ** (LcrE protein), $P<0.0005$ *** (*C. pneumoniae* antigen) for Freund's adjuvant].

To estimate the number of Th1 subset of T cells activated by LcrE-immunization, IFN-γ producing cells were enumerated in the spleens by ELISPOT assay. The number of LcrE-specific IFN-γ producing cells was higher in LcrE-immunized mice than in non-immunized mice and more IFN-γ producing cells were counted after LcrE-Alum immunization (Fig. 12).

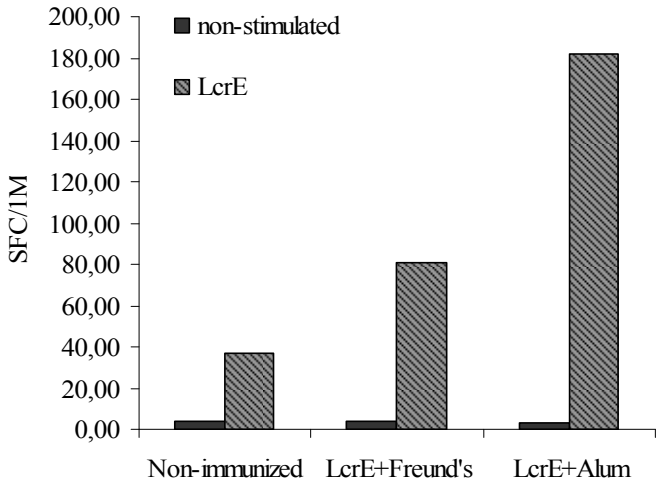


Fig. 12. Number of IFN-γ producing cells in the spleen of LcrE-immunized mice after *C. pneumoniae* infection. Pooled spleen cells of 2 mice were tested following restimulation with LcrE protein in the ELISPOT assay, using IFN-γ ELISPOT set. Bars indicate mean numbers of SFC/1 million (1M) spleen cells calculated in two independent experiments.

In order to define the phenotype of T cells which release IFN-γ ELISPOT assay was carried out with the spleen cells of LcrE immunized mice before *C. pneumoniae* challenge and depleted of CD4+ or CD8+ cells. Depletion of CD4+ cells resulted in a major reduction in the number of SFCs in LcrE and *C. pneumoniae* stimulated spleen cell cultures. In the case of LcrE-Freund's immunized mice the depletion of CD8+ cells also caused a measurable decrease in the number of IFN-γ secreting cells (Fig. 13).

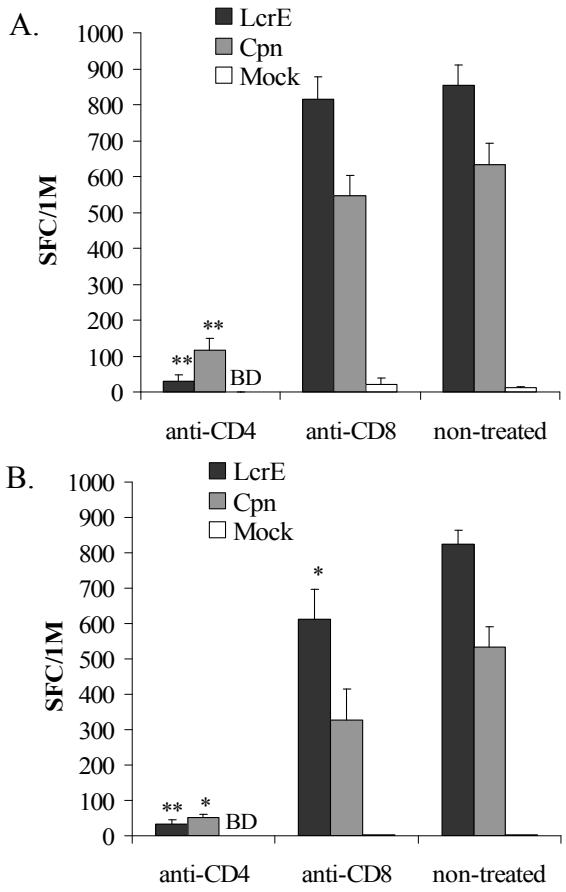


Fig. 13. The number and the phenotype of IFN-γ producing cells in mice immunized with LcrE-Alum (A) and with LcrE-Freund's (B). Pooled spleen cells of five mice were depleted of CD4+ or CD8+ cells using CD4 and CD8-specific antibodies and Miltenyi MACS system. The depleted and non-depleted cells were tested after *in vitro* re-stimulation with LcrE protein, *C. pneumoniae* antigen or mock preparation in the ELISPOT assay, using IFN-γ ELISPOT set. Bars indicate mean numbers of SFC/1 million (1M) spleen cells counted in triplicate wells. Number of SFC was significantly lower after stimulation with LcrE protein or *C. pneumoniae* antigen in CD4 depleted cells than in non-depleted cells (P<0,005** for Alum adjuvant; P<0.001 **, P<0.05 * for Freund's adjuvant); number of SFC was significantly lower after stimulation with LcrE protein in CD8 depleted cells than in non-depleted cells of Freund's-immunized mice P<0.05 *. (BD= below detectable level)

Protection against *C. pneumoniae* infection as measured by culturing of *C. pneumoniae* from the lungs

To find out whether LcrE protein applied in combination either with Freund's or Alum adjuvants can induce protective immune response against *C. pneumoniae* infection, *C. pneumoniae* titres in the lungs of immunized and non-immunized mice were compared. The reduction in *C. pneumoniae* burden in the lungs calculated as

geometric mean (GM) was 60% when Freund's adjuvant was used and 63% when Alum adjuvant was used compared to non-immunized mice (Fig. 14). The protection was significant (LcrE-Freund's: $P<0.0007$; LcrE-Alum: $P<0.0003$) and showed no difference irrespective of the used adjuvant. The immunological correlates of the protection are described below.

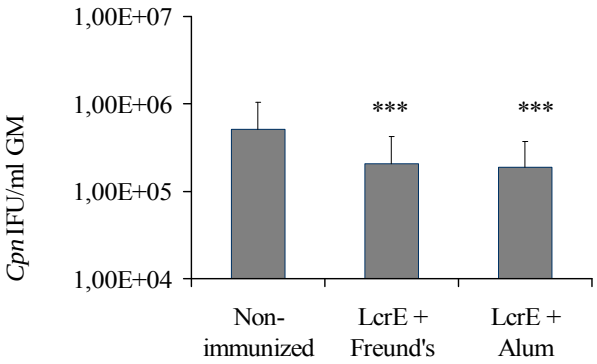


Fig. 14. *C. pneumoniae* titres in the lungs of LcrE-immunized mice after *C. pneumoniae* challenge. Lung homogenates were inoculated onto HEp-2 cell monolayers and chlamydial inclusions were detected by indirect immunofluorescence, using MOMP-specific monoclonal and FITC-labelled secondary antibodies. Bars denote geometric mean (GM) *C. pneumoniae* titres (IFU/ml) in the lung homogenates of 20 mice. The titre of *C. pneumoniae* in the lungs of LcrE-immunized mice was significantly lower than in that of non-immunized mice ($P<0.0007$ *** for Freund's adjuvant; $P<0.0003$ *** for Alum adjuvant).

In order to find out the protective effect of LcrH-specific immunity LcrH-immunized mice were infected with *C. pneumoniae.* The amount of recoverable *Chlamydia* from the lungs did not show significant reduction compared to the non-immunized controls (data not shown).

Protein over-expression and purification from *M. smegmatis*

Rne protein

Before cloning chlamydial proteins to pMV262 vector, preliminary experiments were carried out to check the conditions of the protein over-expression in *M. smegmatis.* Based on our previous experience, for this purpose an Rne family protein was chosen. The gene of Rne protein of *M. smegmatis* (MSMEG_4626) was cloned into pET (pSRNE1) or pMV262 (pSRNE2) and expressed in *E. coli* and *M. smegmatis*, respectively. The MSMEG_4626 gene product is a large protein consisting of 1,037 amino acids. Its calculated molecular weight is 112.7 kDa; because of the 6His and FLAG tags attached to it, our fusion protein is 4 kDa larger. In SDS-PAGE gels, it migrates as a 180 kDa protein (Fig.15).

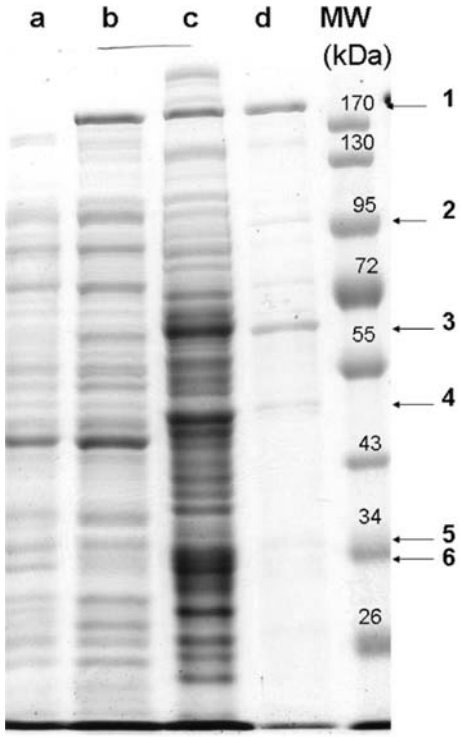


Fig. 15. SDS-polyacrylamide gel (10%) analysis of proteins from an *E. coli* carrying pSRNE1 and from a *M. smegmatis* carrying pSRNE2. Lane a. shows extract of *E. coli* culture without heat induction and lane b. with heat induction. Lane c. shows *M. smegmatis* extract and d. the affinity purified preparation from *M. smegmatis* extract [identified proteins are numbered (see Table 2)]. MW, molecular weight markers

M. smegmatis Rne is highly similar to Rne proteins from *Rhodococcus sp.*, *Streptomyces coelicolor*, *Frankia sp.* and *Corynebacterium diphtheriae*. The central portion of *M. smegmatis* Rne (amino acids 402–805) exhibits 37% identity and 59%

similarity to the amino terminal catalytic region of *E. coli* RNase G. In the case of *C. pneumoniae*, the corresponding values are 33% and 53%.

6His-tag labelled Rne from lysates of *M. smegmatis* were purified with Talon Metal Affinity Resin. The purified preparation separated by SDS-PAGE furnishes different protein bands besides RNase E (Fig. 15). These bands were cut out from the gel and the proteins were identified by mass spectrometry. Table 2 lists the identified proteins.

Table 2. Proteins identified from the purified RNase E preparation (see Figure 15.)

Band	Protein name	MW (kDa)	Locus tag
1	RNase E/G family protein	112.7	MSMEG_4626
2	GTP pyrophosphokinase	87.9	MSMEG_2965
	Negative regulator of genetic competence	93.5	MSMEG_6091
3	GroEL	56.2	MSMEG_1583
4	Translation elongation factor Tu	43.7	MSMEG_1401
5	Conserved hypothetical protein	35.2	MSMEG_3952
6	Dimethyladenosine transferase	32.8	MSMEG_5438
	50S Ribosomal protein L2	30.3	MSMEG_1439
	Universal stress protein family protein	31.4	MSMEG_5733

Proteolytic fragments of RNase E were found in all bands checked. GroEL, the major associated protein, is a chaperonin involved in the productive folding of proteins. In *M. smegmatis*, no heat shock treatment was applied during the expression of RNase E. The complexes are very stable; no proteins were detected in the flow-through material when 50 000 MW cut-off Vivaspin concentrators were used (data not shown). The universal stress protein is a small cytoplasmic protein whose expression is enhanced under stress conditions and increases cell survival. No visible differences were seen in the associated proteins when *M. smegmatis* was grown either in LB or in Middlebrook media.

LcrE protein

M. smegmatis was transformed with pLCRE2 which carries LcrE after the mycobacterial *icl* promoter. LcrE protein expression by the recombinant *M. smegmatis* was confirmed by Western blot (Fig. 16).

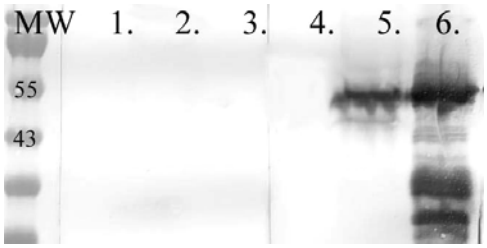


Fig.16. Western blot assay of wild type *M. smegmatis* and recombinant *M. smegmatis* extracts. Wild type *M. smegmatis* (lanes 1, 4), LcrE-recombinant *M. smegmatis* (lanes 2, 5) and LcrE protein (lane 3, 6) were probed with a pool of non-immunized mouse sera (1-3) and LcrE-immunized mouse sera (4-6).

The LcrE-immunized mouse sera reacted only with a band at the appropriate position in the concentrated LcrE-recombinant *M. smegmatis* preparation. Immunization with this recombinant *M. smegmatis* did not resulted in production of LcrE-specific antibodies by the inoculated mice (data not shown).

Discussion

C. pneumoniae has generated huge attention during the last decade, not only as a respiratory pathogen but because of its association with a number of acute and chronic diseases, including atherosclerosis, Alzheimer's disease and multiple sclerosis [83, 84, 85]. The true linkage and causality of *C. pneumoniae* infection in the development of chronic manifestations is poorly understood. Since antibiotics cannot fully inhibit chlamydial growth and because of the incomplete protection induced by natural infection, the development of an effective vaccine would be desirable to control the infections caused by this highly prevalent pathogen. With recent advances in chlamydial genomics and proteomics, a preference has developed for subunit vaccines. The multi-subunit approach to chlamydia vaccine will likely induce an effective long-lasting immunity. Proteins involved in translation, transcription, replication, recombination, and metabolic pathways are typically not high priority vaccine candidates, but exceptions to consider include proteins such as the type III secretion system proteins, Hsp60 and proteins with analogous function in other organisms that are antigenically distinct. Given the primary sequence similarity of several of the type III secretion system structural proteins that are expected to be surface exposed and required for infection of host cells, these are attractive candidates to investigate [86]. Examples in this category are the chlamydial outer membrane proteins, type III secretion system effector proteins [51], and inclusion membrane proteins [87, 88]. Each is an exceptionally important vaccine candidate either because of its presence on the surface of the organism or because of its presence in cytosolic compartments of the host cell and consequently may be expected to be presented by major histocompatibility complex (MHC) class I.

As shown, we have cloned, expressed and purified efficiently with high purity in large quantity the LcrE protein encoded by *lcrE*, which is a putative secreted effector and negative regulator of TTSS of *C. pneumoniae* [89] and the LcrH protein encoded by *lcrH* gene, which is one of the specialized chaperones with function to stabilize and assure efficient secretion of translocator proteins and also to regulate expression of some of the TTSS genes [58].

The mass spectrometry showed that full-length LcrE and LcrH were expressed.

The immunogenicity of these proteins and *Chlamydia*-specificity of the immune response was proved by Western blot.

LcrE might be eligible components of a multi-subunit *Chlamydia* vaccine. The role of LcrH-specific immune response needs further investigation.

Protective effect of LcrE-specific immunity against *C. pneumoniae* infection in a mouse model has been studied. The results of our study are in agreement with previous reports [60, 67] that LcrE protein is an antigen with considerable potential as vaccine subunit. The relatively poor immunogenicity of subunit vaccines, however, requires effective delivery systems e.g. adjuvants. In connection with LcrE immunization, Tammiruusu *et al.* [67] applied the protein in a heat aggregated form and combined with *E. coli* heat-labile toxin in a BALB/c mouse model; Sambri *et al.* [60] delivered the protein together with FCA to hamsters. In our study, the type and the protective capacity of immune response induced by recombinant LcrE protein formulated with Alum was determined and compared to that induced by LcrE mixed with Freund's adjuvant. Aluminum hydroxide was chosen because it is the only adjuvant used widely with human and veterinary vaccines and it has excellent safety and adjuvanticity records [90]. Comparison was made with the effect of FCA, since it is still the gold standard for adjuvants producing excellent antibody response [62] and the T-cell response induced is mainly Th1 type in experimental animals [91]. Cell mediated immunity is crucial in host defense against intracellular pathogens such as *C. pneumoniae*. The correct mechanism of cell-mediated immune (CMI) response in this disease is not clear. One of the potential effector mechanisms is the inhibition of chlamydial growth by cytokines such as IFN-γ. Expression of this cytokine is a bench-mark of a Th1 response, while for Th2 it is IL-4, IL-6 and IL-10, these cytokines are more effective in stimulating B cells to produce antibody. Both CD8+ and CD4+ T cells were shown to be activated during *C. pneumoniae* primary infection in humans [66]. According to Rothfuchs *et al.* [92] CD4+ and CD8+ cells are each sufficient for protection against *C. pneumoniae* infection in mice through their IFN-γ production. Contribution of antibodies to defence against chlamydial infections is controversial. In this respect, Rodriguez *et al.* [93] showed that B cells have a role in protection, since B cell deficient mice and IgA-/- mice displayed increased susceptibility to *C. pneumoniae* infection.

The LcrE immunization with both protocols (i.e. adsorbed to Alum or mixed with Freund's adjuvat) resulted in a significant reduction in the number of *C. pneumoniae* cultured from the lungs after *C. pneumoniae* challenge compared to the

non-immunized mice. Since the adjuvants used are supposed to polarize the Th1/Th2 balance in different directions, we investigated the nature of the immune response evoked by these immunization methods, especially whether our antigen formulated with different adjuvants is recognized by CMI response.

The immunization was successful in respect of antibody response; our purified LcrE protein injected together with both adjuvants generated a high IgG level in mice. These IgG antibodies recognized LcrE protein in purified *C. pneumoniae* EB-s as demonstrated by Western blot assay. In agreement with earlier data [54], these results show the localization of LcrE protein in *C. pneumoniae* EBs. LcrE protein is exposed to the immune system during *C. pneumoniae* infection as showed by the sera of mice inoculated with *C. pneumoniae* and sera of persons positive in a *C. pneumoniae*-specific microimmunofluorescence assay [94] that contained antibodies reacting with LcrE in ELISA. Since the antibody isotype profile, namely the high IgG2a/IgG1 ratio closely correlates with Th1 type CMI response we determined the level of LcrE-specific IgG2a and IgG1 antibodies in the serum samples. High level of Th2 related IgG1 production was detected after FCA and even higher after Alum immunization, although increased IgG2a levels were detected after immunization using FCA, compared to Alum immunization. In Alum immunized mice, a marked dominance of IgG1 was observed, while more balanced IgG2a/IgG1 ratios were detectable in FCA-immunized animals. IgA is well known to protect mucosal surfaces during bacterial and viral infections. Here we report that LcrE protein immunization induces IgA production at mucosal surfaces. We found higher level of LcrE-specific IgA in both the sera and the lungs after using Freund's adjuvant than after Alum-immunization. Immunization with MOMP combined with CpG induces a strong Th1 response and also elevated IgA, and by elevating IgA induces a secretory IgA exerted protection against *C. muridarum* pulmonary infection and inflammatory pathology [95]. A protective effect of IgA induced by MOMP-DNA immunization was described in *C. trachomatis* MoPn-infected mice [96]. In these studies Th1 type immunity was associated with IgA production; similarly, in our studies, in LcrE-Freund's immunized mice, the Th1 skewed immune response coexisted with higher IgA production.

To gain a better understanding of the immunological factors that culminate in reduced bacterial burdens following intranasal *C. pneumoniae* challenge, we assessed the local cytokine levels in the lungs. We observed slight modulation of local cytokine expression in the lungs of mice immunized and subsequently challenged with

C. pneumoniae in comparison to non-immunized mice. Detection of IL-4 and IL-10 in Alum or Freund's immunized mice suggests induction of Th2 type immune response. The IL-6 level tends to be lower in mice immunized by using Freund's adjuvant, and lower levels of KC and MIP-2 pro-inflammatory cytokines were measured after both immunization protocols. Similar changes were reported in *Francisella tularensis* outer membrane protein (OMP) + Freund's adjuvant immunized and challenged mice by Huntley *et al.* [97].

Detection of LcrE-specific proliferation of splenocytes and IFN-γ producing spleen cells in mice proved that an induction of T-cell-mediated immune response had been elicited by both immunization methods. Interestingly, in several experiments stimulation with LcrE protein induced greater proliferative response of Alum immunized mice than that of Freund's immunized mice. The reason of these results is not clear and should be elucidated.

The detection of IFN-γ secreting cells is regarded as a surrogate marker of a protective effect of vaccination [98]. LcrE-specific IFN-γ secreting cells were readily detectable among the spleen cells of immunized and subsequently *C. pneumoniae* infected mice giving further evidence of activation of the cell mediated immunity. IFN-γ secreting cells were detected in non-immunized *C. pneumoniae* infected mice on LcrE stimulation, indicating that LcrE is presented to T cells during infection. The number of IFN-γ producing cells was higher in the spleens of LcrE-Alum immunized mice upon LcrE stimulation compared to non-immunized or LcrE-Freund's immunized mice. Although Alum is considered as an adjuvant that acts by augmenting mainly humoral immunity and activating Th2 type cells, there are studies reporting Th1 type responses induced by Alum formulated vaccines e.g. by subunit malaria [99] and influenza vaccines [100]. Depletion of CD4+ cells resulted in major reduction in the number of SFCs in LcrE and *C. pneumoniae* stimulated spleen cell cultures. In the case of LcrE-Freund's immunized mice depletion of CD8+ positive cells also caused measurable decrease in the number of IFN-γ secreting cells. These results indicate that the cellular immune response was mediated by CD4+ T cells in the case of Alum immunized mice, but in addition to CD4+ cells, LcrE-specific CD8+ cells were also activated by Freund's immunization. Similarly, when a *C. pneumoniae* protein (ID Cpn0029) was used for immunization in combination with FCA by Thorpe *et al*. [102], a highly protective immune response was achieved, and protein-specific CD4+ and also CD8+ IFN-γ

producing cells were identified in the spleen. However, after *C. pneumoniae* infection in mice, the majority of the whole EB-reactive IFN-γ -secreting splenocytes displayed CD4+ phenotype (101 and our unpublished observations). It was also reported that the polymorphic membrane protein 8 of *C. pneumoniae* stimulated only CD4+ splenocytes for IFN-γ production [101].

The recombinant *M. smegmatis* vaccines represent an attractive option for mass vaccination programmes against various infectious diseases.

M. smegmatis is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells [102, 32], facilitating rapid uptake of expressed antigens in *M. smegmatis* and cross-presentation of antigen. The recombinant *M. smegmatis* has been tested experimentally as a vaccine candidate for *M. tuberculosis* [103], as an alternative gene expression system to BCG or *M. tuberculosis* [104], and as an anticancer immunotherapy with cytokine-expressing *M. smegmatis* [105]. *M. smegmatis* vaccine strains expressing foreign antigens are a promising new generation of vaccines that induce remarkably strong and specific immune responses in the mammalian hosts when administered through mucosal immunization routes.

In this study we have evaluated whether *M. smegmatis* was a particularly effective vector for the delivery of homologous and heterologous antigens. Based on the results we got with Rne cloning and expression, we developed the recombinant *M. smegmatis* strain which expresses LcrE antigen in order to evaluate the capacity to reduce *C. pneumoniae* infection and induce immune responses after therapeutic immunization in mice.

Immunization with this recombinant *M. smegmatis* was not successful, immunized mice did not produce LcrE-specific antibodies. The presumable explanation is that the *M. smegmatis* vector expressed inadequate levels of LcrE protein to induce a detectable immune response. Barefoot *et al.* described similar results earlier. They compared different vaccine vectors expressing Cowpox virus B5 antigen. The vectors in that study were a non-replicating recombinant adenovirus, a highly attenuated replication competent vesicular stomatitis virus, *Venezuelan encephalitis* virus replicon particles, or recombinant *M. smegmatis*. *M. smegmatis* expressed very low levels of the B5 protein [106].

The following of our results are considered novel:

- We have cloned, expressed and purified in large quantity the LcrE protein encoded by *lcrE* and the LcrH protein encoded by *lcrH* gene of *C. pneumoniae.* The mass spectrometry showed that full-length LcrE and LcrH were expressed.
- The immunogenicity of these proteins and *Chlamydia*-specificity of the immune response was proved by Western blot.
- LcrE-immunization in combination with Alum adjuvant provided an equal level of protection against *C. pneumoniae* infection to LcrE-Freund's adjuvant immunization, in spite of the differences between the induced IgG isotypes, IgA levels and induction of IFN-γ secreting CD8+ cells.
- The protective effect of LcrE-immunization was shown as significantly decreased number of IFU in the lungs of immunized and *C. pneumoniae*-infected mice and induction of LcrE-specific IFN-γ secreting CD4+ T cells in the spleen.
- We have cloned, expressed and purified Rne protein encoded by MSMEG_4626 gene of *M. smegmatis.* The mass spectrometry showed that full-length Rne was expressed.
- LcrE protein of *C. pneumoniae* has been cloned into pMV262, expressed in *M. smegmatis* as it was proved by Western blot.

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Appendix