Vaccination against chlamydial and mycobacterial infections

Ph.D. Thesis

Ágnes Míra Szabó

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Ágnes Míra Szabó

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

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Publications related to the thesis

I. Szabó AM, Endrész V, Somogyvári F, Miczák A, Faludi I. Isocitrate lyase encoding plasmids in BCG cause increased survival in ApoB100-only LDLR-/- mice. *Mol Biol Rep.* 2013; 40(8):4721-5. **Impact factor: 2,929**

II. Szabó AM, Sipák Z, Miczák A, Faludi I. ABC transporter ATPase of *Chlamydophila pneumoniae* as a potential vaccine candidate. *Acta Microbiol Immunol Hung.* 2013; 60(1):11-20. **Impact factor: 0,787**

III. Faludi I, Szabó AM. Vaccination with DNA vector expressing chlamydial low calcium response protein E (LcrE) against *Chlamydophila pneumoniae* infection. *Acta Microbiol Immunol Hung.* 2011; 58(2):123-34. **Impact factor: 0,787**

IV. Faludi I, Szabó AM, Burián K, Endrész V, Miczák A. Recombinant *Mycobacterium smegmatis* vaccine candidates. *Acta Microbiol Immunol Hung.* 2011; 58(1):13-22. **Impact factor: 0,787**

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Abbreviations

ABC ATP binding casette

ACP ABC transporter ATPase encoded by *CpB0255* gene of *Chlamydophila pneumoniae*

AG arabinogalactan

Alum Aluminum hydroxide gel

ATB ABC transporter ATPase encoded by *Rv0986* gene of *M. tuberculosis*

ATPase adenosine triphosphatase

BCG bacillus Calmette-Guérin

bp base pair

C. pneumoniae *Chlamydophila pneumoniae*

CAD coronary artery disease

CFU colony-forming units

DAB diaminobenzidine tetrahydrochloride

DC dendritic cell

DNA deoxyribonucleic acid

E. coli *Escherichia coli*

EB elementary body

ELISA enzyme-linked immunosorbent assay

Fig. Figure

HIV human immunodeficiency virus

HRP horseradish-peroxidase

hVps human vacuolar protein sorting

Icl isocitrate lyase

IFN interferon

IFU inclusion forming unit

Ig immunoglobulin

IL interleukin

Inc inclusion membrane protein

kDa kilodalton

LAM lipoarabinomannan

LB Luria-Bertani

LcrE low-calcium response E protein

LcrH low-calcium response H protein

LDL low density lipoprotein

LM lipomannan

M. bovis *Mycobacterium bovis*

M. smegmatis *Mycobacterium smegmatis*

M. tuberculosis *Mycobacterium tuberculosis*

MDR Multidrug-resistant

MW molecular weight

NK cells natural killer cells

OD optical density

PBS phosphate buffered saline

PCR polymerase chain reaction

PIMs phosphatidylinositol mannosides

pΔRC eukaryotic expression vector

RB reticulate body

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TB tuberculosis

TDM trehalose dimycolate

Th T helper

TTSS type III secretion system

WHO World Health Organization

XDR Extensively drug-resistant

Yop Yersinia outer-membrane protein

1. Introduction

Metabolism and pathogenicity of intracellular bacteria (mycobacteria, *Chlamydophila pneumoniae* were studied in the laboratory when I started my Ph.D. studentship. Possible vaccine targets were identified, RNA metabolism, glyoxylate cycle, regulatory proteins were investigated. **My work focuses on recombinant BCG as an improved vaccine candidate, ABC transporter ATPase of *C. pneumoniae* and *Mycobacterium tuberculosis*, and low calcium response protein E as possible components of vaccines.** There is a big competition in finding new drugs and vaccines among biotechnology and pharmaceutical companies and there is a vast difference between our budget and theirs. The investigation of intracellular bacteria is a very important research area. The survival and spread of these bacteria depend on their ability to replicate inside the eukaryotic cell. The similar life habit might be realized through the homologous proteins.

More efficient vaccination against *C. pneumoniae* and *M. tuberculosis* would be a great step in the prevention and treatment of these infections. For possible vaccine candidates we cloned LcrE protein encoded by *lcrE* of type III secretion system from *C. pneumoniae*, as well as chlamydial ABC transporter ATPase protein. The genes for these vaccine candidate proteins were cloned into *Escherichia coli* expression vector and *E. coli* was transformed with them.

The survival of *M. tuberculosis* in infected macrophages requires the activity of isocitrate lyase (Icl), a key enzyme in the glyoxylate cycle. *icl* gene was cloned into *E. coli* and *E. coli*-mycobacterium shuttle plasmids and *E. coli* and *Mycobacterium bovis* BCG were transformed with them.

BALB/c and ApoB100-only LDLR-/- (B6;129S-ApoBtm2SgyLdlrtm1Her/J) mice were immunized either with the purified proteins or with recombinant *M. bovis* BCG bacterium and a eukaryotic expression vector.

1.1 The pathogens and the diseases

Tuberculosis (TB) has troubled humankind throughout history. It has been a leading cause of death, and still is in low-and middle-income countries. TB is a classic example of airborne infection. The causative agent, *M. tuberculosis*, is a very successful pathogen. One third of the human population is infected with this bacterium. WHO declared TB a public health emergency in 1993. The annual World TB Day on 24 March marks the day in 1882

when Robert Koch detected the TB bacillus [1]. TB, also known as “morbus hungaricus” in Hungary, was found in the spines and skulls of the over 4000-year-old Egyptian mummies [2]. Despite the earlier forecast, TB is still here and the aim of the StopTB Strategy is to radically reduce the global burden of TB by 2015, unfortunately, this expectation seems overly optimistic [3]. The genus *Mycobacterium* comprises more than 140 well-characterized species [4], most of them are non-pathogenic to human being. Several species are, however, associated with human disease such as tuberculosis or leprosy. The causative agent of TB is a fairly large, non-motile, rod-shaped bacterium, belongs to the obligate aerobe group and it is found mainly in the well-aerated upper lobes of the lungs. The rods are 2-4 microns in length and 0.2-0.5 microns in width. It can not be classified by Gram's staining method due to its resistance to acid-alcohol following coloration with phenicated fuchsin (Ziehl-Neelsen stain). The mycobacterial cell wall complex contains peptidoglycan and high concentration of lipid which assist to survive inside the macrophages, and cause resistance to many antibiotics, impermeability to stains and resistance to osmotic lysis [5]. The lipid complex which makes up over 60% of the cell wall, consists of three major components: mycolic acids, cord factor (trehalose dimycolate, TDM) and wax-D. This unique cell wall structure and its characteristics play an imperative role in the pathogenicity of mycobacteria and is one of the main reasons of the resistance to most antibiotics [6, 7]. The cell wall consists of two parts: an outer part and the core of the cell wall. The cell wall core is made up of peptidoglycan covalently linked to arabinogalactan (AG) and mycolic acids subsequently. The outer layer consists of free lipids, linked with fatty acids. Different cell wall components such as phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM) are also found in it. TDM inhibits the phagosome-lysosome fusion and plays a part in the maintenance of granulomatous response. LAM inhibits the phagosome maturation by inhibiting the Ca^{2+}/calmodulin phosphatidyl inositol-3-kinase hVps34 pathways [8]. Infection with *M. tuberculosis* in healthy people is generally asymptomatic, because of the “wall off” by the immune system. TB usually attacks the lungs but can also cause extrapulmonary symptoms. It is transmitted interpersonally via droplets from the throat and lungs of infected people. The symptoms of active TB are coughing, chest pain, weight loss, and fever. TB is either latent or active. While active TB causes symptoms, latent TB does not, nor can it spread. Development of active TB is only 5% to 10% of all infections.

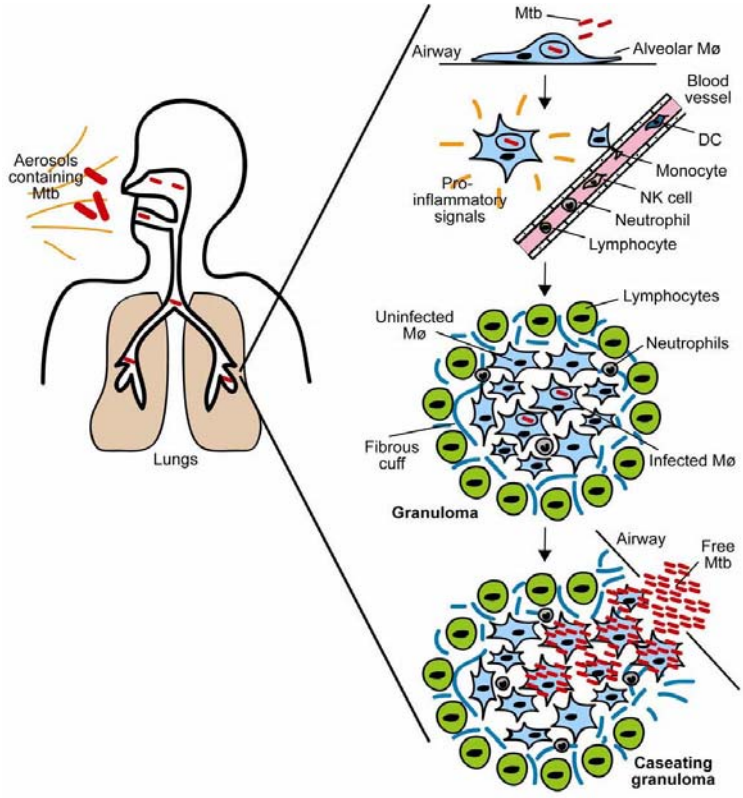

Fig. 1 The intracellular lifestyle of *M. tuberculosis* [9]

Figure 1 shows how bacteria, after inhalation, are phagocytosed by the alveolar macrophages. This eventuate leads to a pro-inflammatory response and the cells of the innate and adaptive immune systems will be recruited. The result is the formation of a granuloma, in which the bacteria are capable of living for an extended time. Deadening of the immune system assists the granuloma development and the reactivation of the bacteria.

According to the phylogenetic analysis of 16S and 23S DNA sequences, the family of *Chlamydiaceae* is divided into two genera, *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* consists of 3 species, *Chlamydia trachomatis*, *C. muridarum* and *C. suis*. In the genus *Chlamydophila* there are 6 species, *Chlamydophila pneumoniae*, *C. psittaci*, *C. pecorum*, *C. abortus*, *C. caviae and C. felis* [10]. *Chlamydiaceae* were first identified as a virus. The best known members of *Chlamydiaceae*, are the human genital and ocular pathogen *C. trachomatis* and the respiratory pathogen *C. pneumoniae*. According to the seroepidemiological studies, 50–70% of the adult population worldwide is seropositive to *C. pneumoniae* [11]. Respiratory infections caused by *C. pneumoniae* are often mild with no symptoms, or the only symptom of being persistent cough [12], and in most cases they probably remain undiagnosed. 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 chronic diseases. 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 association of the infection with other chronic diseases, such as asthma and chronic bronchitis, has been suggested [14]. The seroepidemiological data linking *C. pneumoniae* infection to coronary artery disease (CAD) have also been supported by detection of antigens by immunocytochemistry 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]. 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 [23, 24]. Therefore, vaccination has been suggested to be an effective strategy for prevention and also controlling the chronic chlamydial infection.

1. 2 Sheltered life

A large number of bacteria are able to live and survive in the eukaryotic cells. In order to adapt to this special lifestyle, they need to create a unique niche and try to avoid the attack of the humoral immune system [25]. In the usual scenario, after the invasion, macrophages ingest the bacteria and they are trapped by the phagosomes. After phagocytosis they are located in the membrane-bound vacuole (phagosome). In the absence of intracellular survival mechanisms, phagosomes-containing the bacteria-fuse with the lysosomal compartment thus creating the phagolysosome. This environment in the macrophages can be fatal for the bacteria. Intracellular bacteria are able to block this step and survive in the hostile environment. Some facultative and obligate intracellular bacteria possess special strategies to survive or avoid the hostile environment.

Yersinia species can prevent phagocytosis, *Salmonella* species are able to modify the phagocytosis. *Listeria* and *Shigella* can lyse the vacuolar membrane to escape into the cytosol, *Legionella* species block the phagolysosome fusion, *M. tuberculosis* blocks or attenuates phagolysosomes [26].

1.2.1 Survival strategy of *M. tuberculosis*

A survival of *M. tuberculosis* in infected macrophages requires the activity of isocitrate lyase (Icl), a key enzyme in the glyoxylate cycle. The glyoxylate shunt permits the effective utilization of two-carbon compounds, such as acetate (from the β-oxidation of fatty acids), to satisfy carbon requirements [27]. The glyoxylate cycle is required for the virulence of other bacteria and fungi [28, 29]. In mycobacteria, Icl has a dual role in the metabolism of fatty acids. It participates in the methylcitrate cycle too [30]. β-oxidation of odd-chain or branched-chain fatty acids results in the production of propionyl-CoA which is toxic for bacteria and fungi [31, 32]. Propionyl-CoA is metabolized in the methylcitrate cycle. The other way to use up proponyl-CoA is the methylmalonyl pathway. It requires a vitamin-B_{12}-derived cofactor [33].

A key to the intracellular survival of *M. tuberculosis* is its ability to prevent the fusion of phagosomes containing the internalized bacterium with the lysosomal system of the host cell. The addition of certain exogenous fatty acids modulates phagosome maturation, resulting in the killing of pathogenic bacteria, whereas other fatty acids stimulate pathogen growth [34]. Thus the role of lipids is multifaceted.

1.2.2 Survival strategy of *C. pneumoniae* – the *Chlamydia* inclusion

The members of the family *Chlamydiaceae* have a unique two-phased developmental cycle. It is initiated by endocytosis of an EB, the infectious but metabolically inactive form of chlamydia by a eukaryotic host cell. Inside the vesicle, the bacterium is able to inhibit the phagolysosomal fusion. At about 8 h after infection, EB begins to changes in its morphology and differentiate into RB, the metabolically active form of chlamydia and start to multiply by binary fission. 36 h after infection, RB reorganize into EB, which are able to lysis of the host cell (Fig. 2)

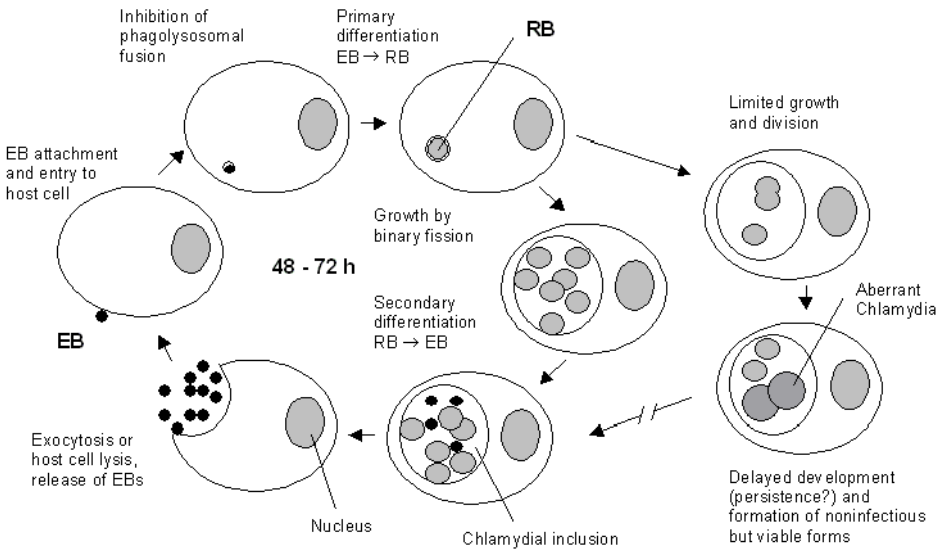

Fig. 2 The developmental cycle of *C. pneumoniae* [35]

Like many other human pathogens such as *Yersinia*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa* and pathogenic *E. coli*, *C. pneumoniae* also posseses a TTSS, enabling the bacteria to secrete effector proteins into the cytosol of the infected cell [36, 37]. This system is activated during the intracellular phase of the chlamydial replicative cycle and is responsible for both 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 modulates the cell response to ongoing chlamydial replication. The virulent elementary body is protected against the environmental effects [38]. There is a persistent phase where chlamydiae are able to remain alive for a long time. Supposedly, the persistent phase may have an important role in chronic infection [39].

In the case of the pathogenic vacuole, Rab GTPases (Rab4, 11, 1, 6 and 10) are able to associate with the inclusion membrane which permit interaction with the ER and Golgi-derived vesicles. Lipid droplets and neutral lipid storage organelles are issued from the host cytoplasm by translocation across the inclusion membrane. The modification of the inclusion membrane is ensured by the integral membrane proteins. Incs interact with the Rab GTPases and participate in the recruitment of Rab GTPases to the inclusion membrane, which play a key role in the inclusion biogenesis [40].

1.3 Vaccination against intracellular bacteria

Vaccination against human pathogens is one of the best ways to put an end to the serious effects of certain diseases. In clinical use, vaccines consist of either killed or attenuated microbes such as influenza, polio, measles, tuberculosis or subunits of the organisms (*Haemophilus influenzae* type B, Meningococcus A and C, Hepatitis B) [41]. Long-lived immune responses (cell-mediated response and production of protective antibodies) are induced by this type of vaccines. Most current vaccines are able to induce the immune response and effective for diseases mentioned above. In addition, cell-mediated immunity (mediated by T lymphocytes) is crucial in the immune protection of intracellular microbes. Antigen-presenting cells (macrophages or dentritic cells) produce IL-12, which is the inducer of the functional Th1 response and the protection mediator is the effector cytokine, IFN-γ. At the same time, for diseases such as AIDS, malaria and other infections caused by intracellular pathogens, effective vaccines are not available [42].

1.3.1 BCG vaccine versus new vaccines for tuberculosis

Although *M. bovis* BCG (Bacille Calmette-Guérin) is the only approved vaccine used for the prevention of TB in humans, the protective efficacy of the BCG vaccine varies widely in different parts of the world, reaching a maximum of 78 % [43]. The vaccine contains live attenuated strain of *M. bovis* which was attenuated between 1908 and 1920 by 231 serial passages. During 13 years in vitro passage, the virulent strain was seen to be less virulent for animals. BCG vaccine was first used in humans against tuberculosis in 1921. Since that time, BCG is the most widely used vaccine all over the world, but its protective value as anti-TB vaccine is questionable. BCG can protect children against severe forms of the disease, particularly meningitis, but it seems to be much less effective, or even ineffective against the most prevalent form, pulmonary tuberculosis in adults. To reduce the global burden of TB, new vaccination strategies are needed, not only in newborns, but also in adolescents and adults.

Most current efforts to improve the level of protective immunity provided by BCG include the development of recombinant BCG vaccines expressing different antigens [44]. A number of vaccines, diagnostic technologies, and drugs are in clinical development. *Mycobacterium smegmatis*, first identified in 1884, is a rapidly growing saprophyte, it can propagate one generation every 1-3 h. It is non-pathogenic and commensal in humans [45,

46], and it acts as a 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 [47, 48]. *M. smegmatis* has a number of properties that can make it an effective vaccine vector. This fast-growing mycobacterium is unable to arrest phagolysosome maturation and cannot evade intracellular killing [49, 50, 51, 52]. Moreover, its rapid clearance by the host differs from that of the *M. tuberculosis* or even the vaccine strain *M. bovis* bacillus Calmette-Guérin [48]. Furthermore, *M. smegmatis* has been used as a vaccine vector because it activates dendritic cells and induces CD8-mediated immune responses [53, 54, 55, 56]. Finally, killed *M. smegmatis* has been shown to provide the same adjuvant activity as *M. tuberculosis* during the induction of experimental autoimmune encephalomyelitis [57].

1.4 Possible vaccine candidates

1.4.1 ABC transporter ATPase

ABC transporter ATPase encoded by *Rv0986* gene of *M. tuberculosis* H37Rv strain, is involved in cell adhesion and entry [58], and plays a part in the inhibition of phagosome-lysosome fusion [59]. The expression of ATPase protein gene is up-regulated by at least 20-fold in mycobacterium during infection [60]. These are the reasons why it can be a suitable vaccine candidate. The proteins which are involved in bacterial cell invasion might be similar in different intracellular bacteria. *C. pneumoniae* has a homologous ATPase protein, encoded by *CpB0255* gene. The purpose of this study was the cloning, over-expression and purification of chlamydial ABC transporter ATPase protein; examination of the immunogenicity and the protective ability of subcutaneous recombinant protein vaccination with chlamydial ABC transporter ATPase against *C. pneumoniae* infection in mice.

1.4.2 LcrE protein

The protein encoded by *lcrE* is homologous to *Yersinia* YopN, a surface protein [61] 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 [62]. The fact that LcrE protein is 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 early chlamydial inclusion vacuole. [63]. Herrmann *et al*. [64] 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*. [65] described a protective effect of recombinant LcrE protein mixed with Freund's adjuvant given subcutaneously against *C. pneumoniae* challenge in a hamster model. The protective effect of immunization with LcrE protein mixed with Freund's or Alum adjuvant against experimental *C. pneumoniae* infection in mice was earlier described by our group [66]. Studies have shown that cell-mediated immunity is necessary for protection against *C. pneumoniae* in mice. In immunization models DNA has been used to induce Th1 type immunity against *C. pneumoniae*. In several studies, a partial protection against the infection has been observed in mice vaccinated with naked DNA carrying genes for major outer membrane protein, 60 kDa heat shock protein or ADP/ATP translocase [67, 68, 69].

The purpose of our study was to test the immunogenicity and the protective ability of intramuscular *lcrE*-DNA immunization and *lcrE*-DNA priming/recombinant LcrE protein booster immunization regime against *C. pneumoniae* infection in mice.

1.5 Aims

The purpose of this study was:

- The over-expression and purification of chlamydial LcrE and ABC transporter ATPase proteins in large quantities
- To clone the genes for these vaccine candidate proteins into *Escherichia coli* expression vector and to transform *E. coli* with them
- The effect of Icl:
    - *icl* gene was cloned into *E. coli* and *E. coli*-mycobacterium shuttle plasmids and *E. coli* and *Mycobacterium bovis* BCG were transformed with them.
- Comparison the immunogenicity and the ability of protein vaccination, BALB/c and ApoB100-only LDLR-/- (B6;129S-ApoBtm2SgyLdlrtm1Her/J) mice were immunized either with the purified proteins or with recombinant *M. bovis* BCG bacterium and a eukaryotic expression vector.

2. Materials and methods

2.1 Bacterial strains and growth conditions

M. bovis BCG, *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. bovis* BCG was grown in Middlebrook 7H9 broth or 7H10 agar (Difco Laboratories, Detroit, MI, USA) enriched with 10% Middlebrook OADC (oleic acid/albumin/dextrose/catalase) (Difco) and 0.05% Tween 80 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with the appropriate antibiotic. *C. pneumoniae* was propagated in HEp-2 cells.

2. 2 Plasmid constructions

2.2.1 LcrE plasmids

A 1200 bp fragment containing the *lcrE* (GeneID: 895078; Locus tag: CPn0324) gene was 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 ligation to prokaryotic expression vector (p6HisF-11d), the resulting plasmid was pLCRE. E3 5'-GCT AAG CTT ATG GCA GCA TCA GGA-3' and E4 5'-TTA GGG CCC GTA TTG GTT TTG CAT GGC-3' for ligation to eukaryotic expression vector (pΔRC) [70]. *C. pneumoniae* CWL029 DNA was used as template. Primers were planned by using the sequence of CWL029 [71].

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 DNA for eukaryotic expression vector (pΔRC) was digested with *Hind*III and *Apa*I and inserted into the pΔRC by digesting it with the same enzymes. The resulting plasmid was pΔRCLcrE. For DNA immunization we purified recombinant pΔRCLcrE and pΔRC plasmids in large scale by GenElute™ HP Endotoxin-Free Plasmid Megaprep Kit from Sigma.

2.2.2 ABC transporter ATPase plasmid

A 681 bp fragment containing the gene of ABC (ACP) (GeneID:33241335; Locus tag:CpB0255) gene from *C. pneumoniae* was amplified by PCR, using the following primers: AB3 5'-GGG CAT ATG TCC TTA CTT ATA GAA-3' and AB4 5'-AAC GGA TCC AGA GGG AGT GTT TTC-3' and *C. pneumoniae* CWL029 DNA as template. Primers were planned by using the sequence of CWL029 [71]. This fragment was digested with *Nde*I and *Bam*HI and inserted into p6HisF-11d(icl) by digesting with the same enzymes and replacing the *icl* gene. The resulted plasmid was pACP.

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

2.2.3 pMV262(icl) plasmid

We constructed pMV262(icl), which carries *icl* after the mycobacterial *icl* promoter (GenBank accession no. CAE55284.1) in pMV262 [72]. I1 5'-ACT ATC TAG ATC CGC AGG ACG TCG A-3' and I2 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3' primers were used to synthetize *icl* with *M. tuberculosis* H37Rv chromosomal DNA as template. Primers were planned by using the sequence of H37Rv [73]. The amplified DNA was cut with *Xba*I and *Hind*III and inserted into pMV262 opened with the same enzymes.

PCR was performed in a GeneAmp II thermocycler (Applied Biosystem, 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.

Table 1. Summary of genes, primers and PCR reaction conditions

Genes	Primers	PCR reaction conditions
lcrE	**E1** 5'-GGA GGC ATA TGG CAG CAT CA-3' **E2** 5'-CAC AGG ATC CGT ATT GGT TTT GCA TGG C-3'	94 º C 1 94 º C 20" 55 º C 20" } 30x 68 º C 2' 68 º C 3' 4 º C ∞
ABC transporter ATPase	**AB3** 5'-GGG CAT ATG TCC TTA CTT ATA GAA-3' **AB4 5'**-AAC GGA TCC AGA GGG AGT GTT TTC-3'	
icl	**I1** 5'-ACT ATC TAG ATC CGC AGG ACG TCG A-3' **I2** 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3'	

2.3 Gene copy number determination

The High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany; Cat. No.: 1796828) was used for DNA Extraction. Each DNA sample was analysed in triplicate. The reaction volume was 15 µl, containing 3 µl of DNA, 1 µmol/l of each of the primers (2µl), 7.5 µl of reaction buffer (IQ^{TM} 2X Supermix, Bio-Rad Laboratories, Hercules, CA, USA) 0.6 µl of EVAGreen (20x EVAGreen^{TM} Biotium Inc., Hayward, CA, USA) and 1.9 µl of distilled water. We used the forward primer 5'-AGC GCA TAT GTC TGT CGT CGG-3' and reverse primer 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3' for *icl*. The reference gene (GeneBank accession no. O53899) primer set was 5'-TGG CAT ATG AAC CGG CAA CCT ATC-3' and 5'-GAG GAT CCT CAT TCA TAG GAC GTG-3'. The primer sets have very similar efficiencies. A BIO-RAD CFX 96 instrument (Bio-Rad, Hercules, CA, USA) was used for quantitation. The PCR conditions were initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation (94 °C for 20 s), annealing (55 °C for 30s) and extension (68 °C for 45s). The emitted fluorescence was measured after the extension step.

Quantitation was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The gene copy number was calculated by the ΔCt method with Bio-Rad CFX Manager 1.1 Gene Expression software.

Table 2. Summary of primers and conditions of RT-PCR

Genes	**Primers**	**Conditions**
icl	5'-AGC GCA TAT GTC TGT CGT CGG-3' 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3'	95 °C 10 min 94 °C 20 s 55 °C 30 s 68 °C 45 s } 35x 4 ºC ∞
Reference gene (GeneBank accession no. O53899)	5'-TGG CAT ATG AAC CGG CAA CCT ATC-3' 5'-GAG GAT CCT CAT TCA TAG GAC GTG-3'	

2.4 Protein analysis techniques

2.4.1 Expression of LcrE and ACP

For over-expression, *E. coli* HB101(pGP1-4) cells carrying either pLCRE or pACP plasmids were grown and treated according to the method of Tabor and Richardson [74]. Briefly, cells containing the plasmids were grown at 32 °C in LB medium in the presence of the required antibiotics (ampicillin, 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 30 min or longer, cells were harvested by centrifugation and cell pellets were frozen.

2.4.2 Purification of LcrE and ACP

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, either LcrE or ACP proteins were purified from the supernatant using TALON CellThru Resin (Clonetech), following the vendor's instructions.

2.4.3 Protein detection

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [75]. Gels were stained with Coomassie brillant blue [76].

2.4.4 Electroporation of *M. bovis* BCG

Competent *M. bovis* BCG prepared in 10% glycerol was transformed with pMV262 or pMV262(icl) by electroporation with a Gene Pulser (Bio-Rad, München, Germany) set at 2.5 kV and 25 µF, and with the pulse controller resistance set at 1,000 Ω. Transformed BCG was selected on Middlebrook 7H10 agar plates supplemented with 30 µg/ml kanamycin.

2.5 Immunological methods and toxicity testing

2.5.1 Immunization method of LcrE

Specific-pathogen-free 13-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 10 were immunized: (Group 1) with 50 µg pΔRC plasmid intramuscularly once and 4 weeks later with the purified LcrE protein diluted in PBS at a dose of 20 µg mixed with 25 µl Alum (Aluminium hydroxide Gel, Sigma) in 150 µl subcutaneously into the tail base; or (Group 2) with 50 µg pΔRCLcrE plasmid intramuscularly two times with 4-week interval, or (Group 3) with 50 µg pΔRCLcrE plasmid intramuscularly once and 4 weeks later with 20 µg LcrE protein combined with Alum adjuvant. As control we used Group 4; mice were immunized with 50 µg pΔRC plasmid intramuscularly two times with 4-week interval. Two

weeks after the last immunization blood was collected in heparinized capillaries from the retro-orbital plexus. All experiments complied with the University of Szeged guidelines for the use of laboratory animals.

2.5.2 Mouse experiments to study the survival of the recombinant BCG

We used ApoB100-only LDLR-/- (B6;129S-ApoBtm2SgyLdlrtm1Her/J) mice,16 females per group and their weight ranged from 25-30 g with the genetic background of a mixture of C57BL/6 and a particular 129 strain from Jackson Laboratories (Bar Harbor, Maine, USA). These mice express full-length ApoB-100 in their LDL particles and have 3-fold higher plasma levels of ApoB100 than those in LDLR−/− mice. The mice were maintained under standard husbandry conditions and were supplied with food and water ad libitum. Intraperitoneal infection of the KO mice was started at 12-14 weeks of age and consisted of 100 µl of PBS containing 10^6 colony-forming units (CFU) of either BCGpMV262 or BCGpMV262(icl). Four mice from each group were sacrificed on days 10, 14, 21 and 35 post-infection. The mice were euthanized and their spleens and lungs were excised.

2.5.3 Culturing of BCG from the lungs and the spleen

The dissected spleens were homogenized by pressing them through nylon mesh into PBS (1 ml) containing 0.05% Tween 20. The lungs were removed and homogenized mechanically in 1 ml of PBS containing 0.05% Tween 20. 100 µl of tissue suspension was used. Ten fold dilutions of these suspensions were plated on Middlebrook 7H10 agar for cultivation of the bacteria. CFUs were determined after 21 days of incubation at 37°C.

2.5.4 Western blot

Cell lysates of *E. coli* over-expressing ACP, LcrE, Icl and purified control (Chlamydial LcrH), ACP and LcrE proteins and concentrated *C. pneumoniae* elementary bodies were heated to 95°C for 5 min in sample buffer and separated by SDS-10% PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 overnight at 4°C.

In the case of Icl, membranes were probed with BCGpMV262(icl) immunized and control (BCGpMV262) mouse homogenized lungs (1:10 dilution in 5% skim milk and 0.05% Tween 20 containing PBS).

In the case of ACP, membranes were probed with protein-immunized and control (alum only) mouse sera (1:50 dilution in 5% skim milk and 0.05% Tween 20 containing PBS). After washings, the filter was incubated with horseradish-peroxidase (HRP)-conjugated anti-mouse IgG (α-mouse IgG-HRP, Sigma) and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma) with hydrogen peroxide in 10 mM Tris pH 7.5.

2.5.5 ELISA test

LcrE/ACP-specific antibodies in serial two-fold dilutions of the sera were detected by ELISA, using plates coated with recombinant LcrE/ACP protein (100 ng/well), and HRP-conjugated secondary antibody was used for detection. The titres were determined at dilutions demonstrating an optical density (OD) higher than 0.1.

2.5.6 Immunization with ACP protein

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 and were given food and water *ad libitum*. Mice in groups of 5 were immunized subcutaneously at the tail base either with the purified ACP protein diluted in phosphate buffered saline (PBS) at a dose of 20 μg mixed with 25 μl Alum (Aluminum hydroxide Gel, Sigma) or with Alum only (control) in 150 μl volume 3 times at 3-week intervals. Mice were sacrificed 14 days after the last immunization. Blood was collected in heparinized capillaries from the retro-orbital plexus.

2.5.7 Challenging the immunized and control mice

The 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 sucrose-phosphate-glutamic acid buffer for cultivation of bacteria.

2.5.8 Culturing of *C. pneumoniae* from the lungs

Lung homogenates from individual mice were centrifuged (10 min, 400 g), serial dilutions of the supernatants were inoculated onto HEp-2 cell 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.

2.5.9 Determination of the toxicity of propionate

BCG bacteria carrying pMV262(icl) or the control plasmid (pMV262) were grown at 37 °C in Middlebrook 7H9 (Difco) broth supplemented with OADC. Sodium proprionate (0.1%) and/or vitamin B_{12} (10μg/ml) were used where indicated. Optical density (600nm) was measured.

2.6 Statistical analysis

Statistical analysis of the data was carried out with GraphPad Prism 5 software, using the Student t test. Differences were considered significant at $p<0.05$.

3. Results

3.1 ACP as a possible new target molecule for immunization

3.1.1 Over-expression of ATB/ACP proteins

Fig. 3 shows the over-expression of ATB and ACP proteins of *M. tuberculosis* and *C. pneumoniae* respectively. ATB is a protein consisting of 248 amino acids. Its calculated molecular mass is 28 kDa; because of the 6His and FLAG tags, our fusion protein is 4 kDa larger. ACP protein is smaller, it contains 226 amino acids. Its calculated molecular mass is 25 kDa; it also contains the 6His and FLAG tags. The other protein parameters are summarized in Table 1. It can be seen that upon heat-induction a strong band appears at the indicated position (Fig. 3, lanes 2, 3, 5, 6). 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 60 minutes in case of both proteins (Fig. 3, lane 3-ATB; lane 6-ACP).

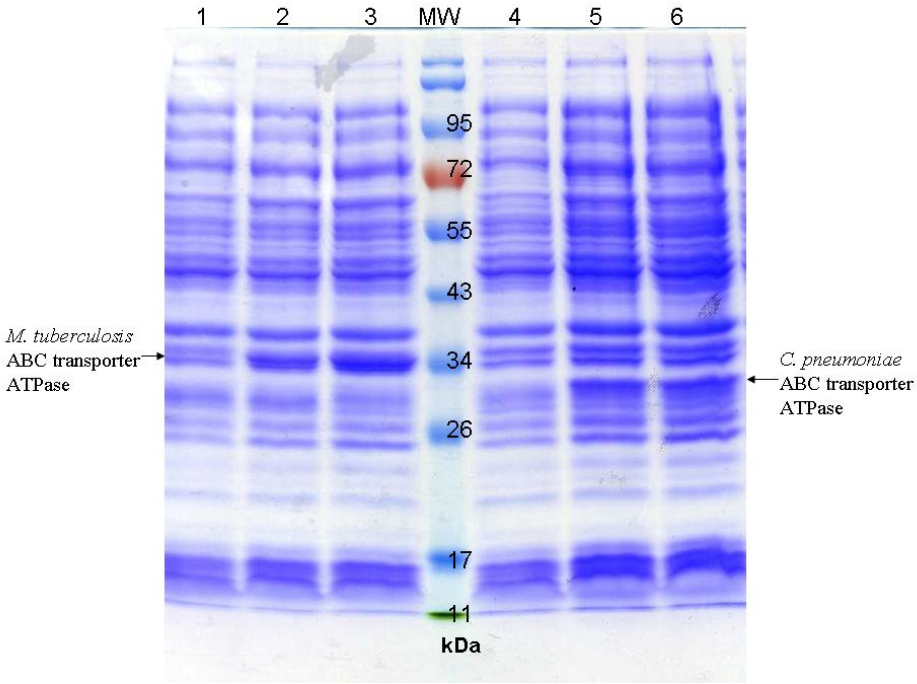

Fig. 3 10% SDS-PAGE analysis of *E. coli* extracts containing the overexpressed proteins Lanes 1, 4 show extract of *E. coli* culture without heat induction and lanes 2, 3 and 5, 6 show extracts of *E. coli* cultures with heat induction. Samples were taken at 30' (lanes 2, 5) and 60' (lanes 3, 6) after heat induction

Table 3. *In silico* comparison [77] of mycobacterial and chlamydial proteins

	CpB0255	**Rv0986**
Number of amino acids:	226	248
Molecular weight:	24814.4	27373.1
Theoretical pI:	6.65	5.63
Total number of negatively charged residues (Asp + Glu):	24	30
Total number of positively charged residues (Arg + Lys):	23	26
Formula:	$C_{1092}H_{1790}N_{310}O_{337}S_5$	$C_{1200}H_{1954}N_{346}O_{374}S_5$
Total number of atoms:	3534	3879
The estimated half-life is (*E. coli*, in vivo):	>10 hours	>10 hours

3.1.2 Purification of ACP protein

The purification results are presented in Fig. 4. The purification was carried out with the TALON CellThru Resin. ACP in the crude extract of heat induced *E. coli* and in the eluted fractions 3 and 4 appears as a strong band around MW 30 kDa position (Fig. 4, lanes 3, 4).

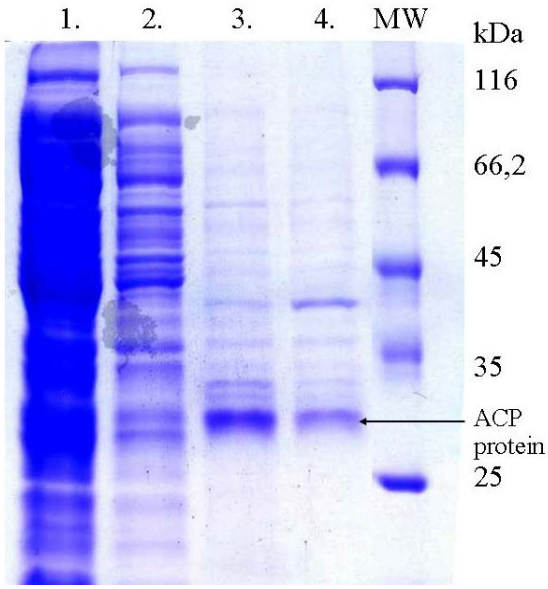

Fig. 4 10% SDS-PAGE analysis of the purification process of ACP

Lanes: 1 – the supernatant; 2 – wash buffer; 3–4 elution samples; MW – molecular weight marker

3.1.3. Level of ACP-specific IgG in the sera of immunized mice

Mice immunized with ACP protein mixed with Alum adjuvant responded with specific antibody production. ACP-specific IgG was detected by ELISA with a mean titre of 44512.

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

In order to find out the protective effect of ACP-specific immunity ACP-immunized mice were infected with *C. pneumoniae.* The mean number of recoverable bacteria from the lungs shows 30% reduction compared to the controls (data not shown).

3.1.5 Western blot analysis

The immunogenicity of the purified protein in mice and the chlamydia–specificity of the produced antibodies were confirmed by Western blot (Fig. 5). After immunization with ACP protein, the mouse sera reacted only with a protein at the appropriate position in the concentrated *C. pneumoniae* preparation, with the purified ACP (Fig. 5B) and with ATB protein (Fig. 5A). This cross reactivity confirmed the epitope similarity between ACP and ATB proteins. These proteins are virulence factors for intracellular bacteria; they are immunogenic and might be eligible components of a multi-subunit chlamydia/mycobacterium vaccine. The role of immune response needs further investigation.

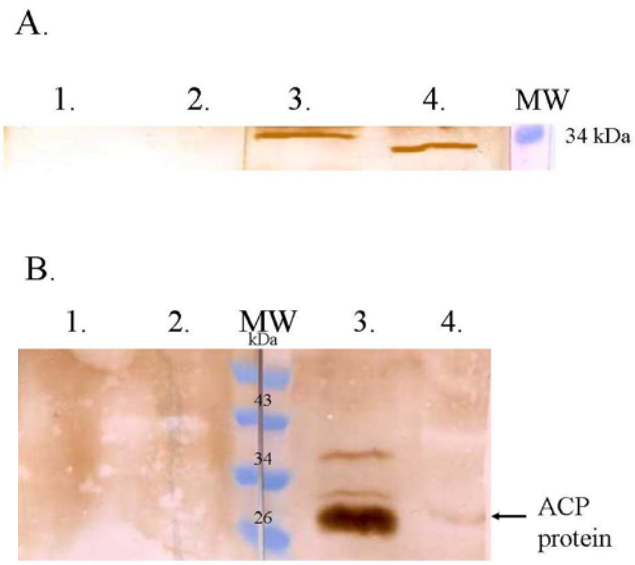

Fig. 5 ACP-immunized and control mouse sera were tested in Western blot assay

A. Overexpressed ATB (lanes 1, 3) and ACP (lanes 2, 4) containing cell lysates were probed with control mouse sera (lanes 1, 2) and with sera of ACP+Alum immunized mice (lanes 3, 4)

B. Purified ACP protein (lanes 1, 3) and concentrated *C. pneumoniae* preparation (lanes 2, 4) were probed with control mouse sera (lanes 1, 2) and with sera of ACP+Alum immunized mice (lanes 3, 4)

3.2 Modified BCG vaccine may improve the efficacy

The copy number of the pMV262(icl) in the BCG strain used for infection was 29. Fig. 6a shows the spleen weights of mice infected with BCG carrying Icl plasmids and the control. Twenty one days after infection, the spleens from mice that were infected with bacteria that carried the additional *icl* genes were three to four times larger than those of the controls (Fig. 6b).

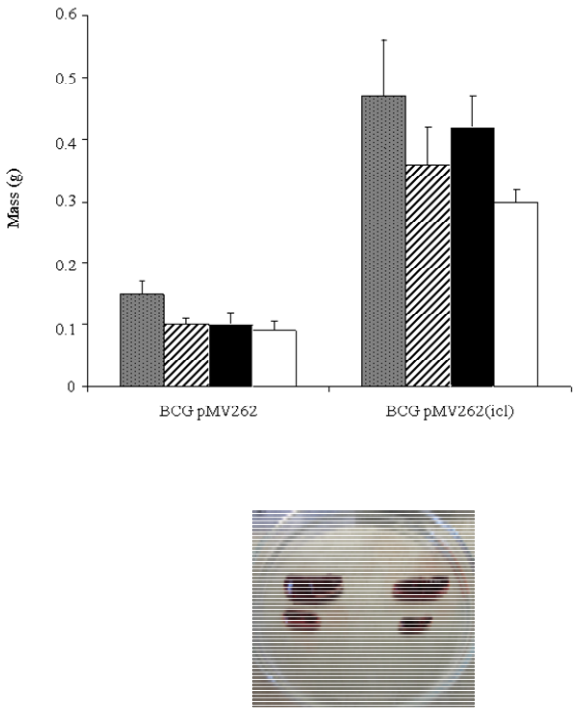

Fig. 6 Spleen masses (g) after 10 (■), 14 (▨), 21 (■) and 35 (□) days of infection. The spleen weights were significantly higher in case of BCGpMV262(icl) than in BCGpMV262-infected mice (p\0.05) (**a**). Differences in spleen size. Spleens were removed from mice 21 days after infection with BCGpMV262 (lower) or BCGpMV262(icl) (upper) (**b**)

Bacteria with one copy of *icl* (the chromosomal gene) appeared in the spleen earlier and their clearance was faster than that from the lungs (Fig. 7). In the spleen, very few bacteria were found 21 days after infection and they had disappeared by the end of the experiment (35 days), whereas some bacteria were still detected in the lungs at this time. Bacteria containing the plasmid with the *icl* gene survived much longer and their yield was 8-250 times higher. The CFUs were significantly higher in the samples from BCGpMV262(icl)-infected mice than the control ($p<0.05$) (Fig. 7).

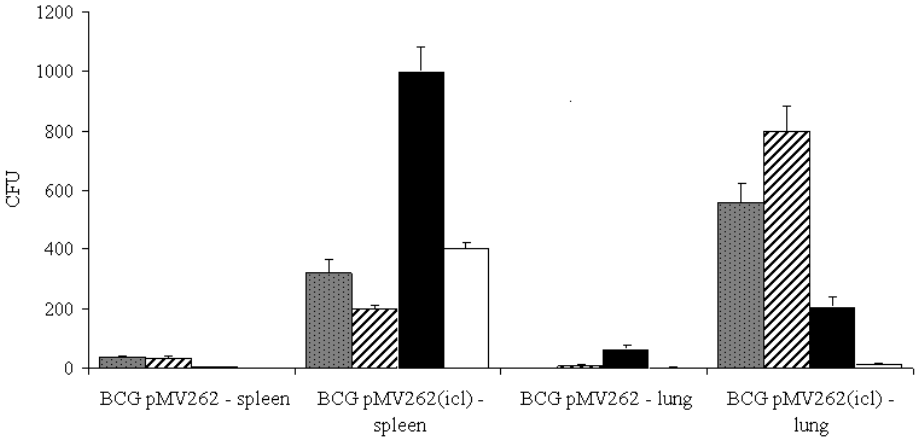

Fig. 7 Colony-forming units in 100 µl of tissue suspension from the spleen and lungs of infected animals after 10 (■), 14 (▨), 21 (■) and 35 (□) days of infection. The CFUs were significantly higher in the samples from BCGpMV262(icl) than in those from BCGpMV262-infected mice (p\0.005)

Lungs from the BCG pMV262(icl)-immunized mice had increased antibody level against Icl as shown in Fig. 8.

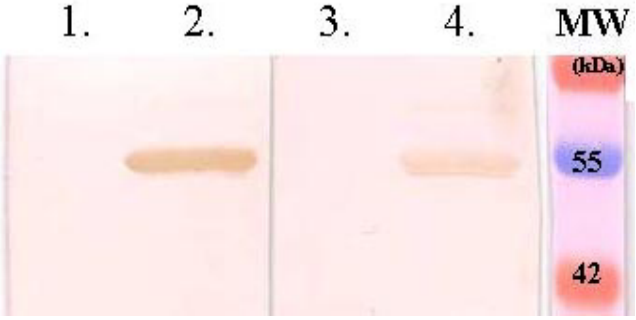

Fig. 8 BCGpMV262(icl) immunized and control mouse homogenized lungs were tested in Western blot assay. Purified control protein (chlamydial LcrH) (lanes 1, 3) and cell lysates from Icl-expressing *E. coli* (lanes 2, 4) were probed with BCGpMV262(icl) immunized (lanes 1, 2) and with BCGpMV262 immunized mouse homogenized lungs in 1:10 dilution (lanes 3, 4)

We determined the toxic effect of propionate produced from the β-oxidation of odd-chain or branched-chain fatty acids. Fig. 9 shows that propionate was less toxic for bacteria carrying icl plasmids in vitro. Although vitamin-B_{12} increased the growth rate of both strains BCG pMV262(icl) multiplied much faster.

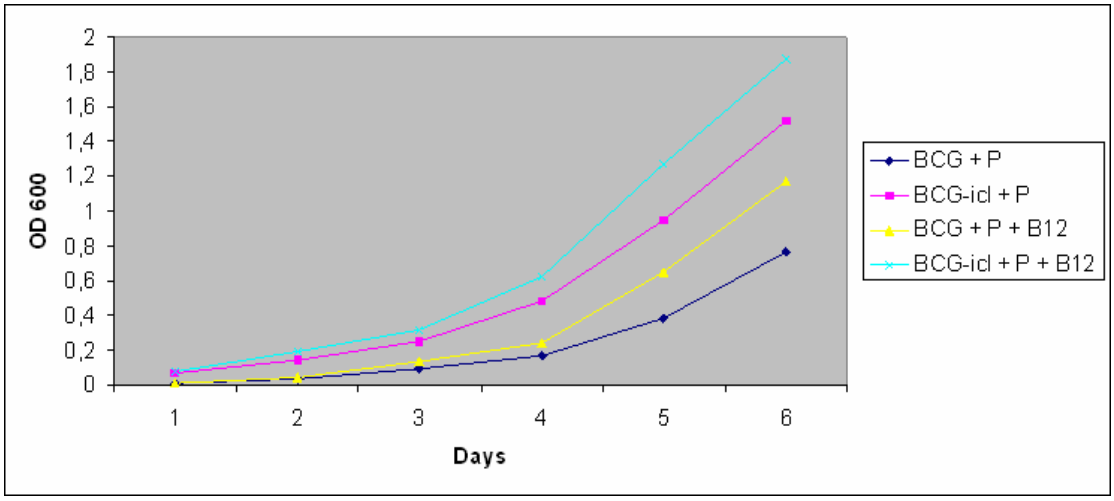

Fig. 9 Growth curves in the presence of sodium propionate (0.1 %) and with vitamin-B_{12} addition (10 µg/ml). Growth was monitored by measuring the optical density of cultures using at least two independent experiments

3.3 LcrE experiments

3.3.1 Level of LcrE specific IgG in the sera in the immunized mice

All of the mice immunized with LcrE protein mixed with Alum adjuvant, and most of the (7/10) mice immunized with pΔRCLcrE responded with LcrE specific antibody production. LcrE-specific IgG was detected by ELISA. There was a tendency for higher LcrE-specific IgG level (p= 0.0625) in pΔRCLcrE-primed and LcrE protein boosted (Group 3) mice compared to non-primed mice (Group 1) (Fig. 10).

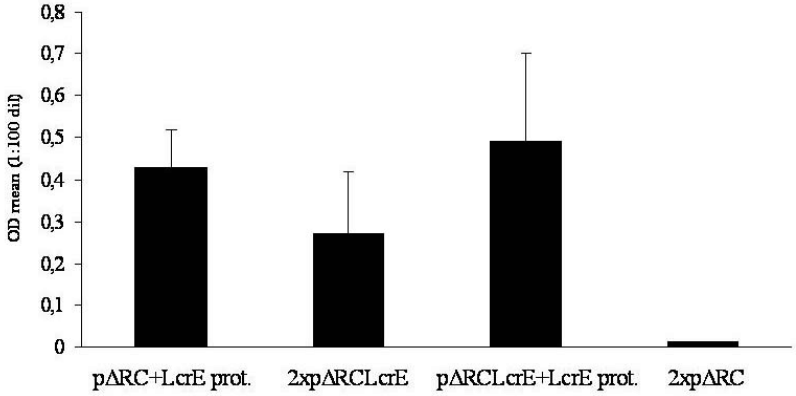

Fig. 10 Two weeks after the last immunization sera were tested by LcrE-specific ELISA at dilution of 1:100. LcrE-specific IgG was detected in pΔRC+LcrE protein immunized, in 2 × pΔRCLcrE-immunized and in pΔRCLcrE-primed and LcrE protein boosted mice in contrast to the 2 × pΔRC-immunized mice

Significant LcrE-specific IgG1 production was detected in the sera of LcrE protein immunized mice, however, the sera IgG2a level was low in mice without pΔRCLcrE priming (Fig. 11). Mice immunized with pΔRCLcrE plasmid only produced relatively higher level of LcrE-specific IgG2a antibody. Boosting the immune response in pΔRCLcrE-primed mice with LcrE protein raised not only the IgG1 but also the IgG2a level. The higher relative IgG2a level indicates a Th1-biased response in the case of DNA immunized mice irrespective of the nature of the booster inoculation.

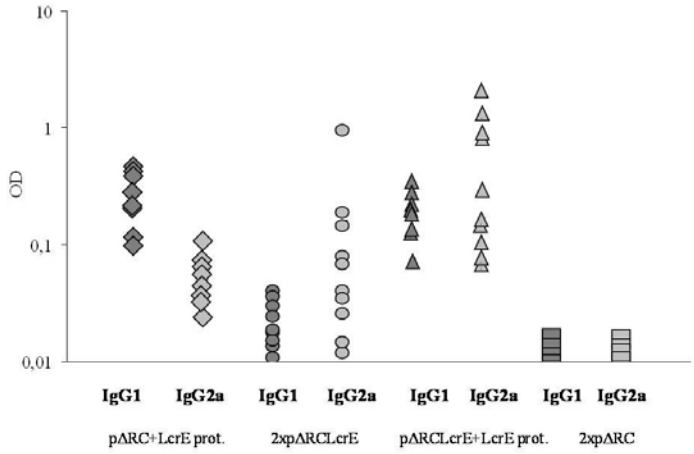

Fig. 11 LcrE-specific IgG1 and IgG2a levels in the sera of immunized and *C. pneumoniae* challenged mice. Isotype of LcrE-specific IgG in the sera of immunized mice was measured by ELISA. Symbols represent OD values measured with sera of individual mice. Sera were tested at a dilution of 1:800 for IgG1, and 1:100 for IgG2a

3.3.2 Western blot analysis of pΔRCLcrE-induced antibodies

The immunogenicity of the eukaryotic expression vector (pΔRCLcrE) in mice and the LcrE-specificity of the produced antibodies were confirmed by Western blot (Fig. 12) using PAGE-separated purified LcrE protein as antigen. Identity of purified LcrE protein was confirmed by MALDI-TOF mass spectrometry previously [38]. Sera of mice from Group 3 (immunized 2 × pΔRCLcrE) reacted with the band at the position corresponding to the molecular weight of LcrE protein similarly to the sera of LcrE protein immunized-mice of the earlier published experiment [19].

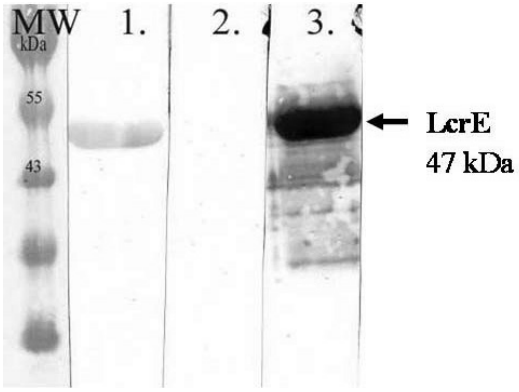

Fig. 12 Sera from immunized mice were tested in Western blot assay. Purified LcrE protein was probed with sera of 2 × pΔRCLcrE-immunized mice (lane 1), 2 × pΔRC-immunized mice (lane 2) and 3 × LcrE protein+Alum immunized mice (lane 3) [66]

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

To find out whether lcrE applied in DNA vaccine can induce protective immune response against *C. pneumoniae* infection, *C. pneumoniae* titres in the lungs of immunized and control mice were compared. The reduction in *C. pneumonia* burden in the lungs was 37% in mice immunized with LcrE protein mixed with Alum adjuvant (Group 1); 55% in pΔRCLcrE-immunized mice (Group 2) and nearly 72% in pΔRCLcrE-primed and LcrE protein boosted mice (Group 3) when compared to pΔRC-immunized controls (Group 4) (Fig.13). This latter comparison showed significant difference with a p value of 0.01.

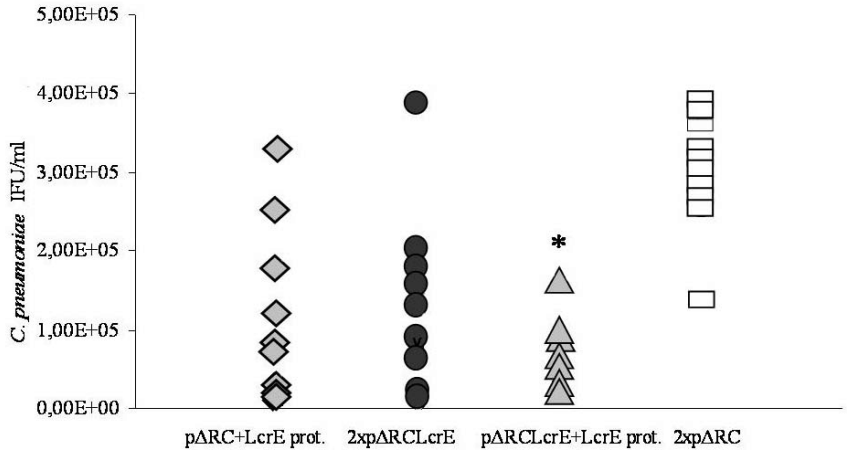

Fig. 13 *C. pneumoniae* titres in the lungs of LcrE-immunized mice after *C. pneumoniae* challenge. Lung homogenates were inoculated onto HEp-2 cell monolayers and chlamydial inclusions were detected by indirect immunofluorescence, using MOMP-specific monoclonal and FITC-labelled secondary antibodies. The titre of *C. pneumoniae* in the lungs of pΔRCLcrE+LcrE protein immunized mice was significantly (* $p = 0.01$) lower than in lungs of control (2 × pΔRC-immunized) mice

4. Discussion

4.1 ABC transporter ATPases can be useful components of vaccines

As shown, we have chosen an important virulence factor of *M. tuberculosis* and its homologous protein from chlamydia, for cloning and expression. The immunogenicity of purified ACP protein and *Chlamydia*-specificity of the produced antibody were proved by Western blot. Low level of protection (albeit not a statistically significant) against *C. pneumoniae* infection was observed by culturing of *C. pneumoniae* from the lungs of ACP immunized mice.

The methods we applied are suitable for cloning, expression and purification of mycobacterial and chlamydial proteins, enabling immunological studies to identify vaccine candidates that would be eligible components of a multi-antigen vaccine which can induce an optimal protective immune response against these infections.

4.2 Isocitrate lyase encoding plasmids in BCG cause increased survival in mice

Several vaccines against tuberculosis are currently undergoing clinical trials [78]. Some of them are subunit vaccines. Another approach is the use of recombinant BCG expressing antigens from *M. tuberculosis*. A recombinant *M. smegmatis* was recently shown to induce potent bactericidal immunity against *M. tuberculosis*. The Rv0467 (CAE55284.1) gene codes for Icl in *M. tuberculosis* H37Rv. In *M. bovis* BCG, the *icl* gene (GenBank accession no. CAL70492) differs in only one base and the amino acid composition is identical. The avirulent strain (*M. tuberculosis* H37Ra) has the same protein (GenBank accession no. ABQ72194). Sequence search was done using NCBI. Although we cloned *icl* from H37Rv, because of the identity the extended survival we observed should be due only to the increased copy number. The tricarboxylic acid cycle and the glyoxylate shunt together supply bacteria with both energy and precursors for carbohydrate synthesis from fatty acids. Icl has an important role in the methylcitrate cycle too where the processing of the toxic propionate occurs [79]. Thanks to the suggestion of an anonymous reviewer we determined the growth rate of the bacteria in the presence of propionate in vitro. Bacteria with increased *icl* copy multiplied much faster which can contribute to the increased number and survival in mice. Methycitrate cycle and the vitamin B_{12}- dependent methylmalonyl pathway contribute to mycobacterial pathogenesis by the proper metabolism of propionate during growth on fatty

acids in the phagosome [80]. The,,lipid lunch'' [81] results in long term survival. Being an enzyme expressed in the later stages of infection by *M. tuberculosis*, Icl may induce long-term protection if over-expressed from multicopy plasmids during BCG vaccination. In addition, the extended survival of this recombinant BCG itself may increase the efficacy of the vaccine. Western blot shows increased antibody response. It would be interesting to know the protective effect of this recombinant BCG. Because of the lack of biosafety level 3 facilities, we cannot perform these types of experiments. Recent results indicate functions for Icl which extend beyond fat metabolism. A novel metabolic route for carbohydrate metabolism was identified in which Icl is a key enzyme [82]. Gengenbacher *et al*. [83] showed that a reduced but constant intracellular ATP level is necessary in the adaptation of *M. tuberculosis* to non-growing survival. Icl deficient nutrient-starved bacilli failed to reduce their intracellular ATP level and died. The mycobacterial cell wall is very rich in lipids: there are around 250 distinct enzymes involved in fatty acid metabolism, five times more than in *E. coli* [84]. We used ApoB100-only LDLR2/2 mice to gain an insight into the role of a high-fat diet on the course of infection, but no significant differences were observed with the different diets in our preliminary experiments (unpublished data). Further studies would be necessary to address this question. It was reported that high levels of cholesterol in the host diet significantly enhanced the *M. tuberculosis* in the lung [84, 85] and impaired immunity to this pathogen [86].

4.3 *lcrE*-containing DNA immunization

C. pneumoniae has generated huge attention during the last decade, not only as a respiratory pathogen but because of its possible association with a number of acute and chronic diseases, including atherosclerosis, Alzheimer's disease and multiple sclerosis [38, 87, 88, 89]. 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. Structural proteins of type III secretion system are expected to be surface exposed and required for infection of host cells,

thus, these are attractive vaccine candidates to investigate [37, 90, 91, 92]. Protective effect of LcrE-specific immunity against *C. pneumoniae* infection in animal models has been studied. The results of our published experiments [66] are in agreement with previous reports [65, 93] that LcrE protein is an antigen with considerable potential as a vaccine subunit and in this study we confirmed that this antigen has protective effect formulated as a DNA vaccine, too. In our present study we show that we cloned *lcrE* gene into a eukaryotic expression vector, and *lcrE*-DNA immunization induced protective response against *C. pneumoniae* infection similar to that evoked by a single LcrE protein immunization. Moreover, in a prime-boost regime, i.e. *lcrE* DNA/LcrE protein vaccination, the protective effect reached 72% in respect of lung bacterial burden. This level of protection is significant and comparable to that achieved by multiple subcutaneous injection of LcrE protein mixed with Alum adjuvant as published earlier [94]. Our findings are that pΔRCLcrE immunization induces an immune response shifted towards Th1 type pathway characterized by increased IgG2a production, and immunization with LcrE protein which generates an IgG1-dominated Th2 type response did not alter the DNA priming-polarized Th1 type response in DNA-primed and protein-boosted mice. Further experiments with improved immunization regimens or modified plasmid vectors are planned for inducing an increased level of antibody and/or cellular response potentially leading to better protection.

The following of our results are considered novel

- ABC transporter ATPases can be useful components of vaccines, we showed that chlamydial ATPase induces protective immunity in mice

- Isocitrate lyase encoding plasmids in BCG cause increased survival in mice, this recombinant BCG can be a more effective vaccine

- *lcrE*-containing DNA immunization given as a priming and followed by a protein booster significantly reduced the number of viable bacteria in the lungs of mice after challenge with *C. pneumoniae*

Summary

Tuberculosis (TB) has troubled humankind throughout history. It has been a leading cause of death, and still is in low- and middle-income countries. TB is a classic example of airborne infection. The causative agent, *Mycobacterium tuberculosis,* is a very successful pathogen. One third of the human population is infected with this bacterium. WHO declared TB a public health emergency in 1993. The available vaccine, bacillus Calmette-Guérin (BCG) has variable (generally low) efficacy in different parts of the world and no new drugs were introduced in the last 40 years. Multidrug- and extensively drug-resistant (MDR and XDR, respectively) strains have developed.

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.

Better vaccines and new therapeutic drugs could be a successful breakthrough against intracellular bacteria.

M. tuberculosis ABC transporter ATPase (Rv0986) as virulence factor, plays a role in mycobacterial pathogenesis by inhibiting phagosome-lysosome fusion. Thus, it could be a potential vaccine candidate. *C. pneumoniae* possesses a protein named CpB0255, which is homologous with the mycobacterial Rv0986. We cloned, over-expressed and purified these bacterial ABC transporter ATPase proteins to study their biological properties.The immunogenicity and protective effect of recombinant chlamydial ATPase protein combined with Alum adjuvant were investigated in mice. The immunization resulted in the reduction of the number of viable *C. pneumoniae* in the lungs after challenge. Our results confirm that chlamydial ATPase induces protective immunity in mice, and the produced antibody also recognized mycobacterial ATPase protein, which justifies the epitope homology between these proteins.

We studied the role of isocitrate lyase in the interaction between *Mycobacterium bovis* BCG and mice.

ApoB100-only LDLR-/- (B6;129S-ApoBtm2SgyLdlrtm1Her/J) mice were inoculated with *M. bovis* BCG harbouring plasmids carrying the gene for isocitrate lyase. The presence of ~29 times more copies of this gene resulted in a higher bacterial yield in the spleens and lungs of the infected mice. The spleen was 3-4 times heavier, and in the spleen the bacteria survived over 10 days longer than did the bacteria with the control plasmid. Propionate was less toxic for bacteria carrying icl plasmids in vitro. This recombinant BCG can be a possible vaccine candidate.

C. pneumoniae possess type III secretion system (TTSS), which allows them to secrete effector molecules into the inclusion membrane and the host cell cytosol. Low calcium response protein E (LcrE) is a part of TTSS. The gene of LcrE in a 6His-tagged form was cloned from *C. pneumoniae* CWL029, expressed and purified from *Escherichia coli* using the HIS-select TALON CellThru Resin, this gene was also cloned into a eukaryotic expression vector (pΔRC). One group of BALB/c mice received an intramuscular pΔRC inoculation then the mice were immunized with purified LcrE protein; the second group of mice was immunized two times with the recombinant plasmid (pΔRCLcrE), and the third group was primed with pΔRCLcrE inoculation then boosted with LcrE protein. LcrE-specific antibody response was induced by DNA immunization with a shift towards Th1 isotype pattern compared to protein-immunization, this shifting pattern was observed in plasmid primed then protein-boosted animals. DNA immunization given as a priming and followed by a protein booster significantly reduced the number of viable bacteria in the lungs after challenge with *C. pneumoniae.* These results confirm that immunization with pΔRCLcrE can be an effective part of a vaccination schedule against *C. pneumoniae*.

Összefoglalás

A tuberkulózis (tbc) az emberiség egyik legrégebbi betegsége. A történelem során az egyik legtöbb halálesetet okozó betegség volt és továbbra is vezető halálok az elmaradott/ fejlődő országokban. A betegség okozója a *Mycobacterium tuberculosis* baktérium, mely cseppfertőzés útján jut az emberi szervezetbe. Földünk népességének 1/3-a tbc fertőzött. 1993-ban a WHO a tbc-t globális veszélyforrássá minősítette. Az egyetlen használatban lévő védőoltás, a bacillus Calmette-Guérin (BCG) vakcina, melynek hatékonysága kérdéses, elsősorban miliáris tuberkulózis és a meningitis tuberculosa visszaszorításában nyújt védelmet. Az utóbbi 40 évben nem sikerült új, hatékony gyógyszert kifejleszteni. További problémát jelent a multidrog-rezisztens (MDR) és extenzíven drog-rezisztens (XDR) baktériumok megjelenése.

Chlamydophila pneumoniae egy obligát intracelluláris humán patogén, mely a pneumoniák és más légúti fertőzések (bronchitis, pharingitis, sinusitis) gyakori kórokozója. Szeroepidemiológiai vizsgálatok rámutattak, hogy a baktérium világszerte elterjedt és majdnem mindenki átesik a fertőzésen élete során. Képes krónikus fertőzéseket is okozni, melyek szívinfarktushoz és atherosclerosishoz vezethetnek. Feltételezik szerepét, asthma bronchiale, Alzheimer-kór és sclerosis multiplex kórképek kialakulásában. Ezek a tények megerősítik a *C. pneumoniae* által okozott fertőzések elleni vakcinák mielőbbi kifejlesztésének szükségességét.

Hatékonyabb vakcinák és új terápiás gyógyszerek fejlesztése sikeres áttörést jelentene az intracelluláris baktériumok elleni küzdelemben.

A *M. tuberculosis* ABC transzporter ATPáz (Rv0986) virulencia faktorként szerepet játszik a pathogenezis folyamatában, a fagoszóma-lizoszóma fúzió megakadályozásával**.** Ennek következtében potenciális vakcina jelölt lehet. *C. pneumoniae* rendelkezik ezen fehérje homológjával (CpB0255). Munkánk során klónoztuk, kifejeztettük és tisztítottuk ezeket a bakteriális ABC transzporter ATPáz fehérjéket, biológiai tulajdonságuk tanulmányozása céljából.

Az immunogenitást és védőhatást, Alum adjuvánssal kombinált rekombináns chlamydiális ATPáz fehérjével vizsgáltuk egerekben. Az immunizálás eredményeként csökkent a tüdőből visszatenyésztett *C. pneumoniae* száma. Az eredmény azt bizonyítja, hogy a chlamydiális ATPáz immunvédelmet biztosít az egerekben és a termeltetett antitestek a mikobakteriális ATPáz fehérjét is felismerik, amely igazolja az epitóp homológiát ezen fehérjék között

Tanulmányoztuk az izocitrát liáz enzim szerepét *Mycobacterium bovis* BCG és egerek közötti kölcsönhatásban. ApoB100-only LDLR-/- (B6;129S-ApoBtm2SgyLdlrtm1Her/J) egereket oltottunk be *M. bovis* BCG baktériummal, mely plazmidon hordozza az Icl fehérjét kifejező génszakaszt. A 29-szer több kópiában jelenlevő gén magas baktérium hozamot eredményezett a fertőzött egerek tüdejében és lépében. A lépek 3-4-szer nagyobbak voltak, és az itt jelenlévő baktériumok túlélése, a kontroll plazmidot hordozó baktériumok túléléséhez képest, 10 nappal hosszabb volt. A propionát kevésbé volt toxikus azon baktériumok számára, melyek az *icl* gént hordozó plazmidot tartalmazták. Ez a rekombináns BCG lehetséges vakcina jelölt lehet a későbbiekben.

A *C. pneumoniae* hármas típusú szekréciós rendszerrel (TTSS) rendelkezik, mely lehetővé teszi a baktérium számára, hogy effektor fehérjéket juttasson a fertőzött sejt citoplazmájába. Az LcrE (Low calcium response protein E) fehérje ezen TTSS rendszer tagja. A 6His-tag-gel ellátott LcrE fehérje génjét, *C. pneumoniae* CWL029 DNS-ből klónoztuk. *Escherichia coli* baktériumban kifejeztettük. A fehérjéket HIS-select TALON CellThru Resin segítségével tisztítottuk. Az LcrE fehérjét kifejező gént, eukarióta expressziós vektorba (pΔRC) is klónoztuk. BALB/c egerek első csoportját intramuszkulárisan oltottuk pΔRC-vel, majd immunizáltuk tisztított LcrE fehérjével; az egerek második csoportját kétszer immunizáltuk a rekombináns plazmiddal (pΔRCLcrE), az egerek harmadik csoportját pedig pΔRCLcrE-vel „primingoltuk” és LcrE emlékeztető oltást adtunk.
A DNS immunizálás LcrE-specifikus ellenanyag termelést váltott ki, amely a Th1 típusú immunválasz felé tolódott el a fehérje immunizáláshoz képest. Ez a változás megfigyelhető volt abban a csoportban is, mely először DNS oltást kapott és utána fehérjét. A DNS immunizálás eredményeként, mely „primingból” és emlékeztető oltásból állt, csökkent a tüdőből visszatenyésztett *C. pneumoniae* száma. Ezek az eredmények megerősítik, hogy a pΔRCLcrE, rekombináns plazmiddal való immunizálás eredményes lehet a *C. pneumoniae* fertőzés ellen.

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Appendix

I.

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Isocitrate lyase encoding plasmids in BCG cause increased survival in ApoB100-only LDLR$^{-/-}$ mice

Agnes Mira Szabo · Valeria Endresz · Ferenc Somogyvari · Andras Miczak · Ildiko Faludi

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Abstract We studied the role of isocitrate lyase in the interaction between *Mycobacterium bovis* BCG and mice. ApoB100-only LDLR$^{-/-}$ (B6;129S-ApoB^{tm2Sgy}Ldlr^{tm1Her}/J) mice were inoculated with *M. bovis* BCG harbouring plasmids carrying the gene for isocitrate lyase. The presence of ~29 times more copies of this gene resulted in a higher bacterial yield in the spleens and lungs of the infected mice. The spleen was 3–4 times heavier, and in the spleen the bacteria survived over 10 days longer than did the bacteria with the control plasmid. Propionate was less toxic for bacteria carrying *icl* plasmids in vitro. This recombinant BCG can be a possible vaccine candidate.

Keywords BCG · Immunization · Isocitrate lyase · *M. tuberculosis*

Introduction

More than one-third of the world's population is infected with tuberculosis (TB) bacilli. *Mycobacterium tuberculosis* is a very successful pathogen. There were an estimated 9.4 million new cases of TB in 2009 and an estimated 1.7 million deaths (including 380 000 people with HIV), making this disease one of the world's most significant infectious killers. Millennium Development Goal 6 is that the incidence of TB should be falling by 2015 [1].

Although *Mycobacterium bovis* BCG is the only approved vaccine used for the prevention of TB in humans, the protective efficacy of the BCG vaccine varies widely in different parts of the world, reaching a maximum of 78 % [2]. Most current efforts to improve the level of protective immunity provided by BCG include the development of recombinant BCG vaccines expressing different antigens [3].

The survival of *M. tuberculosis* in infected macrophages requires the activity of isocitrate lyase (Icl), a key enzyme in the glyoxylate cycle. The glyoxylate shunt permits the effective utilization of two-carbon compounds, such as acetate (from the β-oxidation of fatty acids), to satisfy carbon requirements [4]. The glyoxylate cycle is required for the virulence of other bacteria and fungi [5, 6]. In mycobacteria, Icl has a dual role in the metabolism of fatty acids. It participates in the methylcitrate cycle too [7]. β-oxidation of odd-chain or branched-chain fatty acids results in the production of propionyl-CoA which is toxic for bacteria and fungi [8, 9]. Propionyl-CoA is metabolised in the methylcitrate cycle. The other way to use up propionyl-CoA is the methylmalonyl pathway. It requires a vitamin-B_{12}-derived cofactor [10].

A key to the intracellular survival of *M. tuberculosis* is its ability to prevent the fusion of phagosomes containing the internalized bacterium with the lysosomal system of the host cell. The addition of certain exogenous fatty acids modulates phagosome maturation, resulting in the killing of pathogenic bacteria, whereas other fatty acids stimulate pathogen growth [11]. Thus the role of lipids is multifaceted.

We used ApoB100-only LDLR$^{-/-}$ mice to determine the survival of *M. bovis* BCG carrying plasmids coding for Icl. This double-knock-out (KO) mouse strain produces only apolipoprotein-B-100 and is deficient in low-density lipoprotein (LDL) receptors. This mouse model has a lipid profile similar to that in most humans with atherosclerosis.

A. M. Szabo (✉) · V. Endresz · F. Somogyvari · A. Miczak · I. Faludi
Department of Medical Microbiology and Immunobiology, University of Szeged, Dóm tér 10, Szeged 6720, Hungary
e-mail: szabo.agnes.mira@gmail.com

Extended BCG survival is observed if the bacteria carry plasmids with the gene coding for Icl. We assume that this increased survival can result in more effective protection.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium bovis BCG was grown in Middlebrook 7H9 broth or 7H10 agar (Difco Laboratories, Detroit, MI, USA) enriched with 10 % Middlebrook OADC (oleic acid/albumin/dextrose/catalase) (Difco) and 0.05 % Tween 80 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with the appropriate antibiotic. *Escherichia coli* DH5α was used for plasmid preparation.

Plasmid construction

We constructed pMV262(icl), which carries *icl* after the mycobacterial *icl* promoter (GenBank accession no. CAE55284.1) in pMV262 [12]. I1 5′-ACT ATC TAG ATC CGC AGG ACG TCG A-3 and I2 5′-GTA AGC TTC AGA CTA GTG GAA CTG G-3′ primers were used to synthetize *icl* with *M. tuberculosis* H37Rv chromosomal DNA as template. Primers were planned by using the sequence of H37Rv [13]. The PCR amplification conditions were as recommended by the manufacturer, with a GeneAmp II (Applied Biosystems, Foster City, CA, USA) thermocycler with Advantage GC cDNA polymerase (BD Biosciences Clontech, Mountain View, CA, USA). The amplified DNA was cut with XbaI and HindIII and inserted into pMV262 opened with the same enzymes.

Gene copy number determination

The high pure PCR template preparation kit (Roche Diagnostic GmbH, Mannheim, Germany; Cat. No.: 1796828) was used for DNA extraction. Each DNA sample was analysed in triplicate. The reaction volume was 15 µl, containing 3 µl of DNA, 1 µmol/l of each of the primers (2 µl), 7.5 µl of reaction buffer (IQ^{TM} 2X Supermix, Bio-Rad Laboratories, Hercules, CA, USA) 0.6 µl of EVA-Green (20 × EVAGreen^{TM} Biotium Inc., Hayward, CA, USA) and 1.9 µl of distilled water. We used the forward primer 5′-AGC GCA TAT GTC TGT CGT CGG-3′ and reverse primer 5′-GTA AGC TTC AGA CTA GTG GAA CTG G-3′ for *icl*. The reference gene (GenBank accession no. O53899) primer set was 5′ TGG CAT ATG AAC CGG CAA CCT ATC 3′ and 5′ GAG GAT CCT CAT TCA TAG GAC GTG 3′. The primer sets have very similar efficiencies. A BIO-RAD CFX 96 instrument (Bio-Rad, Hercules, CA, USA) was used for quantitation. The PCR conditions were initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation (94 °C for 20 s), annealing (55 °C for 30 s) and extension (68 °C for 45 s). The emitted fluorescence was measured after the extension step.

Quantitation was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The gene copy number was calculated by the ΔC_t method with Bio-Rad CFX Manager 1.1 Gene Expression software.

Electroporation of *M. bovis* BCG

Competent *M. bovis* BCG prepared in 10 % glycerol was transformed with pMV262 or pMV262(icl) by electroporation with a Gene Pulsar (Bio-Rad, München, Germany) set at 2.5 kV and 25 µF, and with the pulse controller resistance set at 1,000 Ω [14, 15]. Transformed BCG was selected on Middlebrook 7H10 agar plates supplemented with 30 µg/ml kanamycin.

Mouse experiments

We used ApoB100-only LDLR$^{-/-}$ (B6;129S-ApoB^{tm2Sgy} Ldlr^{tm1Her}/J; 16 females per group and their weight ranged from 25 to 30 g) with the genetic background of a mixture of C57BL/6 and a particular 129 strain from Jackson Laboratories (Bar Harbor, Maine, USA). These mice express full-length ApoB-100 in their LDL particles and have threefold higher plasma levels of ApoB100 than those in LDLR$^{-/-}$ mice. The mice were maintained under standard husbandry conditions and were supplied with food and water ad libitum. Intraperitoneal infection of the KO mice was started at 12–14 weeks of age and consisted of 100 µl of PBS containing 10^6 colony-forming units (CFU) of either BCGpMV262 or BCGpMV262(icl). Four mice from each group were sacrificed on days 10, 14, 21 and 35 post-infection. The mice were euthanized and their spleens and lungs were excised.

All experiments complied with the University of Szeged guidelines for the use of laboratory animals.

Culturing of BCG from the lungs and the spleen

The dissected spleens were homogenized by pressing them through nylon mesh into PBS (1 ml) containing 0.05 % Tween 20. The lungs were removed and homogenized mechanically in 1 ml of PBS containing 0.05 % Tween 20. 100 µl of tissue suspension was used. Tenfold dilutions of these suspensions were plated on Middlebrook 7H10 agar for cultivation of the bacteria. CFUs were determined after 21 days of incubation at 37 °C.

Western blot

Cell lysates of *E. coli* over-expressing Icl and purified control (chlamydial LcrH) [16] protein were heated to 95 °C for 5 min in sample buffer and separated by SDS-10 % PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked with PBS containing 5 % skim milk and 0.05 % Tween 20 overnight at 4 °C. Membranes were probed with BCGpMV262(icl) immunized and control (BCG pMV262) mouse homogenized lungs (1:10 dilution in 5 % skim milk and 0.05 % Tween 20 containing PBS). After washings, the filter was incubated with HRP-conjugated anti-mouse IgG for 2 h, and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma) with hydrogen peroxide in 10 mM Tris pH 7.5 according to the manufacturer's instructions.

Statistical analysis

Statistical analysis of the data was carried out with GraphPad Prism 5 software, using the Student *t* test. Differences were considered significant at $P < 0.05$.

Determination of the toxicity of propionate

Bacteria carrying pMV262(icl) or the control plasmid (pMV262) were grown at 37 °C in Middlebrook 7H9 (Difco) broth supplemented with OADC. Sodium propionate (0.1 %) and/or vitamin B_{12} (10 µg/ml) were used where indicated. Optical density (600 nm) was measured. Results are representative of two experiments.

Results

The copy number of the pMV262(icl) in the BCG strain used for infection was 29. Figure 1a shows the spleen weights of mice infected with BCG carrying Icl plasmids and the control. Twenty one days after infection, the spleens from mice that were infected with bacteria that carried the additional *icl* genes were three to four times larger than those of the controls (Fig. 1b).

Bacteria with one copy of *icl* (the chromosomal gene) appeared in the spleen earlier and their clearance was faster than that from the lungs (Fig. 2). In the spleen, very few bacteria were found 21 days after infection and they had disappeared by the end of the experiment (35 days), whereas some bacteria were still detected in the lungs at this time. Bacteria containing the plasmid with the *icl* gene survived much longer and their yield was 8–250 times higher. The CFUs were significantly higher in the samples

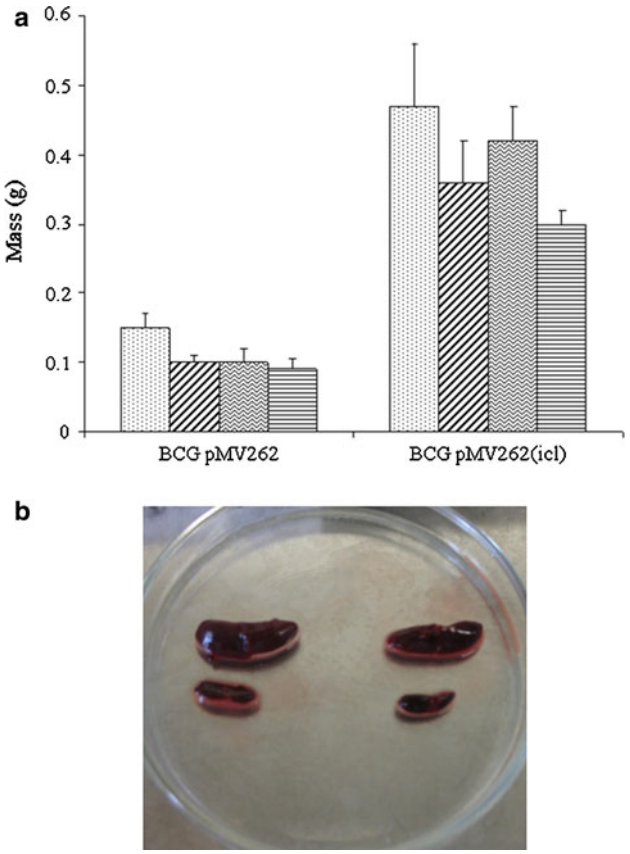

Fig. 1 Spleen masses (g) after 10 (□), 14 (▨), 21 (▩) and 35 (▤) days of infection. The spleen weights were significantly higher in case of BCGpMV262(icl) than in BCGpMV262-infected mice ($P < 0.05$). **a** Differences in spleen size. Spleens were removed from mice 21 days after infection with BCGpMV262 (*lower*) or BCGpMV262 (icl) (*upper*) (**b**)

from BCGpMV262(icl)-infected mice than the control ($P < 0.005$) (Fig. 2). Lungs from the BCG pMV262(icl) - immunized mice had increased antibody level against Icl as shown in Fig. 3.

We determined the toxic effect of propionate produced from the β-oxidation of odd-chain or branched-chain fatty acids. Figure 4 shows that propionate was less toxic for bacteria carrying *icl* plasmids in vitro. Although vitamin-B_{12} increased the growth rate of both strains BCG pMV262(icl) multiplied much faster.

Discussion

Several vaccines against tuberculosis are currently undergoing clinical trials [17]. Some of them are subunit vaccines. Another approach is the use of recombinant BCG expressing antigens from *M. tuberculosis*. A recombinant *M. smegmatis* was recently shown to induce potent bactericidal immunity against *M. tuberculosis* [18].

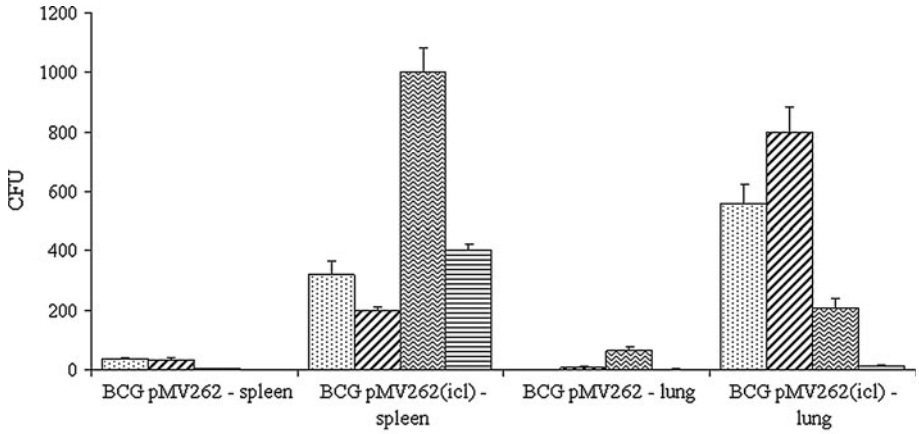

Fig. 2 Colony-forming units in 100 µl of tissue suspension from the spleen and lungs of infected animals after 10 (▭), 14 (▨), 21 (▩) and 35 (▤) days of infection. The CFUs were significantly higher in the samples from BCGpMV262(icl) than in those from BCGpMV262-infected mice ($P < 0.005$)

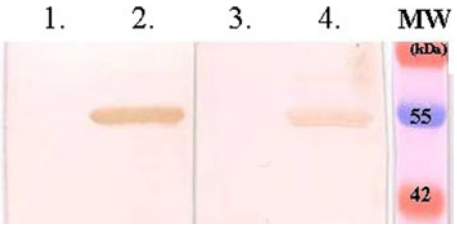

Fig. 3 BCGpMV262(icl) immunized and control mouse homogenized lungs were tested in Western blot assay. Purified control protein (chlamydial LcrH) (*lanes* 1, 3) and cell lysates from Icl expressing *E. coli* (*lanes* 2, 4) were probed with BCGpMV262(icl) immunized (*lanes* 1, 2) and with BCGpMV262 immunized mouse homogenized lungs in 1:10 dilution (*lanes* 3, 4)

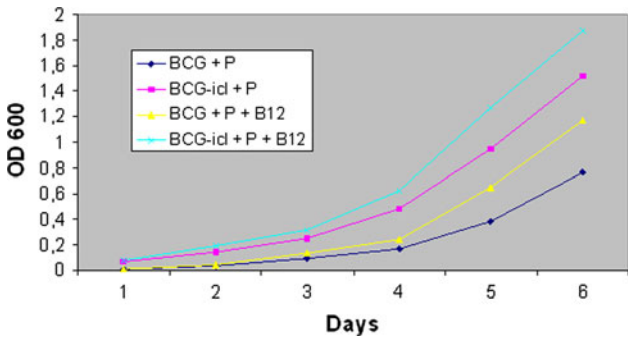

Fig. 4 Growth curves in the presence of sodium propionate (0.1 %) and with vitamin-B_{12} addition (10 µg/ml). Growth was monitored by measuring the optical density of cultures using at least two independent experiments

The Rv0467 (CAE55284.1) gene codes for Icl in *M. tuberculosis* H37Rv. In *M. bovis* BCG, the *icl* gene (GenBank accession no. CAL70492) differs in only one base and the amino acid composition is identical. The avirulent strain (*M. tuberculosis* H37Ra) has the same protein (GenBank accession no. ABQ72194). Sequence search was done using NCBI. Although we cloned *icl* from H37Rv, because of the identity the extended survival we observed should be due only to the increased copy number.

The tricarboxylic acid cycle and the glyoxylate shunt together supply bacteria with both energy and precursors for carbohydrate synthesis from fatty acids. Icl has an important role in the methylcitrate cycle too where the processing of the toxic propionate occurs [7]. Thanks to the suggestion of an anonymous reviewer we determined the growth rate of the bacteria in the presence of propionate in vitro. Bacteria with increased *icl* copy multiplied much faster which can contribute to the increased number and survival in mice. Methycitrate cycle and the vitamin B_{12}-dependent methylmalonyl pathway contribute to mycobacterial pathogenesis by the proper metabolism of propionate during growth on fatty acids in the phagosome [10]. The „lipid lunch" [19] results in long term survival. Being an enzyme expressed in the later stages of infection by *M. tuberculosis*, Icl may induce long-term protection if overexpressed from multicopy plasmids during BCG vaccination. In addition, the extended survival of this recombinant BCG itself may increase the efficacy of the vaccine. Western blot shows increased antibody response. It would be interesting to know the protective effect of this recombinant BCG. Because of the lack of biosafety level 3 facilities, we cannot perform these types of experiments.

Recent results indicate functions for Icl which extend beyond fat metabolism. A novel metabilc route for carbohydrate metabolism was identified in which Icl is a key enzyme [20]. Gengenbacher et al. [21] showed that a reduced but constant intracellular ATP level is necessary in the adaptation of *M. tuberculosis* to non-growing survival. Icl deficient nutrient-starved bacilli failed to reduce their intracellular ATP level and died.

The mycobacterial cell wall is very rich in lipids: there are around 250 distinct enzymes involved in fatty acid metabolism, five times more than in *E. coli* [13]. We used ApoB100-only $LDLR^{-/-}$ mice to gain an insight into the role of a high-fat diet on the course of infection, but no significant differences were observed with the different diets in our preliminary experiments (unpublished data).

Further studies would be necessary to address this question. It was reported that high levels of cholesterol in the host diet significantly enhanced the *M. tuberculosis* in the lung [22, 23] and impaired immunity to this pathogen [24].

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Conflict of interest The authors declare no competing interests

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II.

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ABC TRANSPORTER ATPase OF *CHLAMYDOPHILA PNEUMONIAE* AS A POTENTIAL VACCINE CANDIDATE

ÁGNES M. SZABÓ*, ZOLTÁN SIPÁK, ANDRÁS MICZÁK and ILDIKÓ FALUDI

Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary

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Better vaccines and new therapeutic drugs could be a successful breakthrough against intracellular bacteria. *M. tuberculosis* ABC transporter ATPase (Rv0986) plays a role in mycobacterial virulence by inhibiting phagosome-lysosome fusion. Thus, it could be a potential vaccine candidate. *C. pneumoniae* another important intracellular bacterium possesses a protein named CpB0255, which is homologous with the mycobacterial Rv0986. The aim of this study was the cloning, over-expression and purification of CpB0255 ABC transporter ATPase protein to study its biological properties. The immunogenicity and protective effect of recombinant chlamydial ATPase protein combined with Alum adjuvant were investigated in mice. The immunization resulted in the reduction of the number of viable *C. pneumoniae* in the lungs after challenge. Our results confirm that chlamydial ATPase induces protective immunity in mice.

Keywords: ABC transporter ATPase, CpB0255, over-expression

Introduction

The investigation of intracellular bacteria is a very important research area. The survival and spread of these bacteria depend on their ability to replicate inside the eukaryotic cell. The similar life habit might be realized through the homologous proteins. The family *Chlamydiaceae* includes obligate intracellular bacteria,

* Corresponding author; E-mail: szabo.agnes.mira@gmail.com

which have a unique two-phased developmental cycle. The virulent elementary body, which is protected against the environmental effects, is able to transform into metabolically active reticulate body [1]. There is a persistent phase where chlamydiae are able to remain alive for a long time. Supposedly, the persistent phase may have an important role in chronic infection [2].

The best known members of this family are the human genital and ocular pathogen *Chlamydia trachomatis* and the respiratory pathogen *Chlamydophila pneumoniae (C. pneumoniae)*. We examined the latter *C. pneumoniae*. This bacterium causes upper respiratory infections and pneumonia; and is associated with asthma, chronic bronchitis, atherosclerosis, acute myocardial infarction and Alzheimer's disease [3–7]. It has been shown that infections caused by intracellular bacteria could be challenging for microbiologists, moreover, the fight against tuberculosis (TB) could be a good example for failure of the conventional therapeutic approaches. TB, caused by *Mycobacterium tuberculosis (M. tuberculosis),* is still an enormous problem all over the world [8, 9]. According to the 2009 data of the WHO, 10 million new cases were detected and nearly 2 million victims are claimed by TB that year [8].

No new anti-TB drugs have been introduced in the past 40 years, nevertheless, their development is becoming increasingly important to face the new challenges posed by multidrug-resistant and extensively drug-resistant strains [10]. The efficiency of BCG vaccine is not adequate [11, 12].

These rankling numbers show that new effective drugs and immunization strategies are needed to overcome this problem. More efficient vaccination against *C. pneumoniae* and *M. tuberculosis* would be a great step in the prevention and treatment of these infections. *ABC transporter ATPase*, encoded by *Rv0986* gene of *M. tuberculosis* H37Rv strain, is involved in cell adhesion and entry [13], and plays a part in the inhibition of phagosome-lysosome fusion [14]. The expression of ATPase protein gene is up-regulated by at least 20-fold in mycobacterium during infection [15]. These are the reasons why it can be a suitable vaccine candidate. The proteins which are involved in bacterial cell invasion might be similar in different intracellular bacteria. *C. pneumoniae* has a homologous ATPase protein, encoded by *CpB0255* gene. The purpose of this study was the cloning, over-expression and purification of chlamydial ABC transporter ATPase protein; examination of the immunogenicity and the protective ability of subcutaneous recombinant protein vaccination with chlamydial ABC transporter ATPase against *C. pneumoniae* infection in mice.

Experimental procedures

Bacterial strains

C. pneumoniae CWL029 (ATCC), *Escherichia coli* DH5α and *E. coli* HB101 (pGP1-4)

Plasmid constructions

A 681 bp fragment containing the analogous *ABC transporter ATPase* (ACP) (GeneID: 33241335; Locus tag: CpB0255) gene from *C. pneumoniae* was amplified by PCR, using the following primers: AB3 5' GGG CAT ATG TCC TTA CTT ATA GAA G 3' and AB4 5' AAC GGA TCC AGA GGG AGT GTT TTC 3', and *C. pneumoniae* CWL029 DNA as template. PCR was performed in a GeneAmp II (AppliedBiosystems, Foster City, CA, USA) thermocycler with Advantage GC cDNA polymerase (Clontech, Mountain View, CA, USA) and the amplification conditions were set as recommended by the manufacturer. The amplified DNA was digested with *Nde*I and *Bam*HI and inserted into p6HisF-11d (*icl*) [16] by digesting it with the same enzymes and replacing the *icl* gene. The resulted plasmid was pATB and pACP.

Expression of ACP

For over-expression, *E. coli* HB101 (pGP1-4) cells carrying the pACP plasmids were grown and treated according to the method of Tabor and Richardson [17]. 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 30 min or longer, cells were harvested by centrifugation and cell pellets were frozen.

Purification of ACP protein

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, ACP protein was 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 [18]. Gels were stained with Coomassie brillant blue [19].

Western blot

Cell lysates of *E. coli* over-expressing ACP, purified ACP protein and concentrated *C. pneumoniae* elementary bodies (prepared as described earlier [20]) were heated to 95°C for 5 min in sample buffer, and the proteins were separated by SDS-10% PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 overnight at 4°C. Membranes were probed with protein-immunized and control (alum only) mouse sera (1:50 dilution in 5% skim milk and 0.05% Tween 20 containing PBS). After washings, the filter was incubated with HRP-conjugated anti-mouse IgG, and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma) with hydrogen peroxide in 10 mM Tris pH 7.5.

ELISA test

ACP-specific antibodies in serial twofold dilutions of the sera were detected by ELISA, using plates coated with recombinant ACP protein (100 ng/well), and horseradish-peroxidase (HRP)-conjugated secondary antibody (α-mouse IgG-HRP, Sigma) was used for detection. The titres were determined at dilutions demonstrating an optical density (OD) higher than 0.1.

Immunization and challenge

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 and were given food and water *ad libitum*. Mice in groups of 5 were immunized subcutaneously at the tail base either with the purified ACP protein diluted in phosphate buffered saline (PBS) at a dose of 20 µg mixed with 25 µl Alum (Aluminum hydroxide Gel, Sigma) or with Alum only (control) in 150 µl volume 3 times at 3-week intervals. Mice were sacrificed 14 days after the last immunization.

Blood was collected in heparinized capillaries from the retro-orbital plexus.

Challenging the immunized and control mice

The immunized mice were challenged with 4×10^5 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 sucrose-phosphate-glutamic acid buffer for cultivation of bacteria.

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

Culturing of C. pneumoniae *from the lungs*

Lung homogenates from individual mice were centrifuged (10 min, 400 g), serial dilutions of the supernatants were inoculated onto HEp-2 cell 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.

Results and Discussion

Protein over-expression

Figure 1 shows the over-expression of ACP protein of *C. pneumoniae*.

ACP protein contains 226 amino acids. Its calculated molecular mass is 25 kDa; it also contains the 6His and FLAG tags. It can be seen that upon heat-induction a strong band appears at the indicated position (Fig. 1, lanes 1, 2, 3). Since these proteins carry the 6His and FLAG tags, both tags can be exploited during purification. Figure 1 shows that after heat-induction the strong over-expression occurred after 60 minutes in case of the protein (Fig. 1, lane 3-ACP).

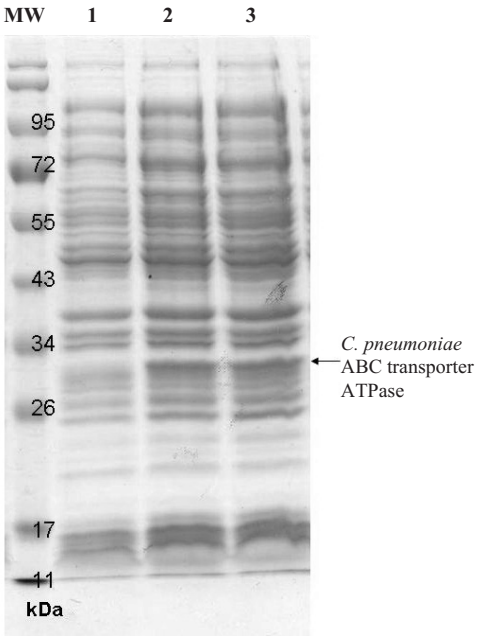

Figure 1. 10% SDS-PAGE analysis of *E. coli* extracts with cloned *ACP* gene
Lane 1 shows extract of *E. coli* culture without heat induction and lanes 2, 3 show extracts of *E. coli* cultures with heat induction. Samples were taken at 30' (lane 2) and 60' (lane 3) after heat induction

Purification of ACP protein

The purification results are presented in Figure 2. The purification was carried out with the TALON CellThru Resin. ACP in the crude extract of heat in-

duced *E. coli* and in the eluted fractions 3 and 4 appears as a strong band at MW 30 kDa position (Fig. 2, lanes 3, 4).

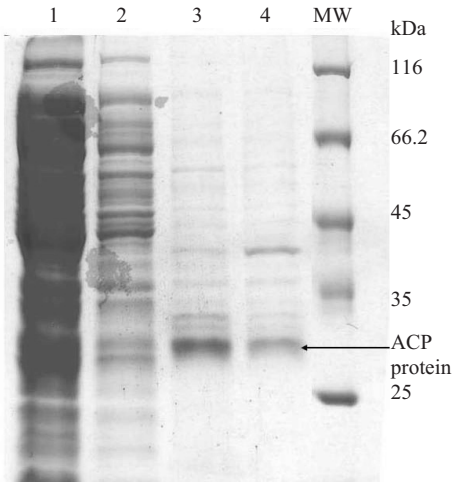

Figure 2. 10% SDS-PAGE analysis of the purification process of ACP
Lanes: 1 – the supernatant; 2 – wash buffer; 3–4 elution samples; MW – molecular weight marker

Level of ACP-specific IgG in the sera of immunized mice

Mice immunized with ACP protein mixed with Alum adjuvant responded with specific antibody production. ACP-specific IgG was detected by ELISA with a mean titre of 44512.

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

In order to find out the protective effect of ACP-specific immunity ACP-immunized mice were infected with *C. pneumoniae.* The mean number of recoverable bacteria from the lungs show 30% reduction compared to the controls (data not shown).

Western blot analysis

The immunogenicity of the purified protein in mice and the chlamydia-specificity of the produced antibodies were confirmed by Western blot (Fig. 3). After immunization with ACP protein, the mouse sera reacted only with a protein at the appropriate position in the concentrated *C. pneumoniae* preparation, with the purified ACP (Fig. 3B).

This protein is a virulence factor for intracellular bacteria; it is immunogenic and might be eligible components of a multi-subunit chlamydia vaccine. The role of immune response needs further investigation.

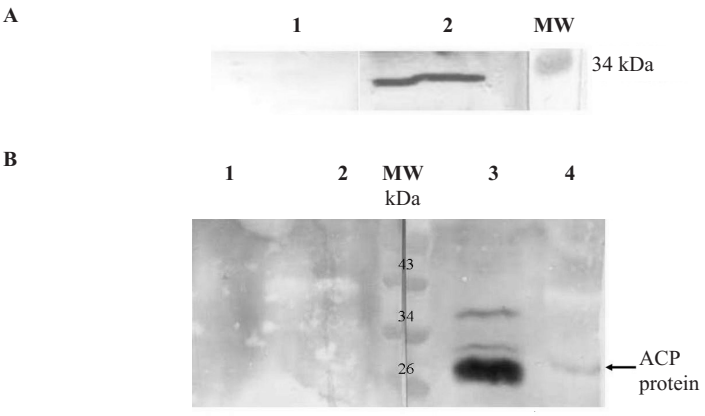

Figure 3. ACP-immunized and control mouse sera were tested in Western blot assay
A) Cell lysates from ACP-overexpressing *E. coli* were probed with control mouse sera (lane 1) and with sera of ACP+Alum immunized mice (lane 2)
B) Purified ACP protein (lanes 1, 3) and concentrated *C. pneumoniae* preparation (lanes 2, 4) were probed with control mouse sera (lanes 1, 2) and with sera of ACP+Alum immunized mice (lanes 3, 4)

Conclusion

As shown, we have chosen an important virulence factor of *C. pneumoniae* for cloning and expression. The immunogenicity of purified ACP protein and *Chlamydia*-specificity of the produced antibody were proved by Western blot. Low level of protection (albeit not a statistically significant) against *C. pneumoniae* infection was observed by culturing of *C. pneumoniae* from the lungs of ACP immunized mice.

The methods we applied are suitable for cloning, expression and purification of mycobacterial and chlamydial proteins, enabling immunological studies to identify vaccine candidates that would be eligible components of a multi-antigen vaccine which can induce an optimal protective immune response against these infections.

Acknowledgements

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VACCINATION WITH DNA VECTOR EXPRESSING CHLAMYDIAL LOW CALCIUM RESPONSE PROTEIN E (LcrE) AGAINST *CHLAMYDOPHILA PNEUMONIAE* INFECTION

ILDIKÓ FALUDI* and ÁGNES MÍRA SZABÓ

Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary

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Chlamydophila pneumoniae is an obligate intracellular human pathogen, which causes acute respiratory tract infections and can also cause chronic infections.

C. pneumoniae possess type III secretion system (TTSS), which allows them to secrete effector molecules into the inclusion membrane and the host cell cytosol. Low calcium response protein E (LcrE) is a part of TTSS. The gene of LcrE in a 6His-tagged form was cloned from *C. pneumoniae* CWL029, expressed and purified from *Escherichia coli* using the HIS-select TALON CellThru Resin, this gene was also cloned into a eukaryotic expression vector (pΔRC). One group of BALB/c mice received an intramuscular pΔRC inoculation then the mice were immunized with purified LcrE protein; the second group of mice was immunized two times with the recombinant plasmid (pΔRCLcrE), and the third group was primed with pΔRCLcrE inoculation then boosted with LcrE protein. LcrE-specific antibody response was induced by DNA immunization with a shift towards Th1 isotype pattern compared to protein-immunization, this shifting pattern was observed in plasmid primed then protein-boosted animals. DNA immunization given as a priming and followed by a protein booster significantly reduced the number of viable bacteria in the lungs after challenge with *C. pneumoniae.* These results confirm that immunization with pΔRCLcrE can be an effective part of a vaccination schedule against *C. pneumoniae*.

Keywords: LcrE protein, *Chlamydophila pneumoniae,* DNA immunization

* Corresponding author; E-mail: faludi.ildiko@gmail.com

Introduction

Chlamydophila pneumoniae (C. pneumoniae) is an obligate intracellular, Gram-negative bacterium, which is one of the most common human pathogens that cause acute infections like pneumonia, bronchitis and pharyngitis. According to the seroepidemiological studies, 50–70% of adult population worldwide is seropositive to *C. pneumoniae* [1]. Respiratory infections caused by *C. pneumoniae* are often mild with no symptoms, or only symptom of extended cough [2], and in most cases they probably remain undiagnosed. 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 chronic diseases. 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 [3]. Later, the association of the infection with other chronic diseases, such as asthma and chronic bronchitis, has been suggested [4]. The seroepidemiological data linking *C. pneumoniae* infection to coronary artery disease (CAD) have also been supported by detection of antigens by immunocytochemistry or nucleic acids of the organism by PCR in the affected tissues [5–7]. Yet, the timing of *C. pneumoniae* infection and development of arteriosclerosis is poorly understood [8–12]. 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 [13, 14]. Therefore, vaccination has been suggested to be an effective strategy for prevention and also controlling the chronic chlamydial infection.

Like in many other human pathogens such as *Yersinia, Salmonella, Shigella, Pseudomonas aeruginosa* and pathogenic *E. coli, C. pneumoniae* possesses a TTSS, enabling the bacteria to secrete effector proteins into the cytosol of the infected cell [15, 16]. This system is active during the intracellular phase of the chlamydial replicative cycle and is responsible for both 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 modulates the cell response to ongoing chlamydial replication. The protein encoded by *lcrE* is homologous to *Yersinia* YopN, a surface protein [17] 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 [18]. 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 [19].

Herrmann et al. [20] 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. [21] described a protective effect of recombinant LcrE protein mixed with Freund's adjuvant given subcutaneously against *C. pneumoniae* challenge in a hamster model. The protective effect of immunization with LcrE protein mixed with Freund's or Alum adjuvant against experimental *C. pneumoniae* infection in mice was earlier described by our group [22]. Studies have shown that cell-mediated immunity is necessary for protection against *C. pneumoniae* in mice. In immunization models DNA has been used to induce Th1 type immunity against *C. pneumoniae*. In several studies, a partial protection against the infection has been observed in mice vaccinated with naked DNA carrying genes for major outer membrane protein, 60 kDa heat shock protein or ADP/ATP translocase [23–25].

The purpose of this study was to test the immunogenicity and the protective ability of intramuscular *lcrE*-DNA immunization and *lcrE*-DNA priming/recombinant LcrE protein booster immunization regime against *C. pneumoniae* infection in mice.

Materials and Methods

Bacterial strains and growth conditions

E. coli DH5*a, 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) if they carry plasmids.

Plasmid constructions

A 1200 bp fragment containing the *lcrE* (GeneID: 895078; Locus tag: CPn0324) gene was 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 ligation to prokaryotic expression vector (as we described previously), and the resulting plasmid was pLCRE [22]); and: E3 5'- GCT AAG CTT ATG GCA GCA TCA GGA G -3' and E4 5'- TTA GGG CCC GTA TTG GTT TTG CAT GGC -3' for ligation to eukaryotic expression vector, *C. pneumoniae* CWL029 DNA was used 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 DNA for eukaryotic expression vector (pΔRC) [27] was digested with HindIII and ApaI and inserted into the pΔRC by digesting it with the same enzymes. The resulting plasmid was pΔRCLcrE. For DNA immunization we purified recombinant pΔRCLcrE and pΔRC plasmid in a large scale by GenElute™ HP Endotoxin – Free Plasmid Megaprep Kit from Sigma.

Expression and purification of LcrE

For over-expression, *E. coli* HB101(pGP1-4) cells carrying pLCRE plasmid were grown and treated according to the method of Tabor and Richardson [28] as it was described previously [22].

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

Immunization method

Specific-pathogen-free 13-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 10 were immunized: (Group 1) with 50 μg pΔRC plasmid intramuscularly once and 4 weeks later with the purified LcrE protein diluted in PBS at a dose of 20 μg mixed with 25 μl Alum (Aluminum hydroxide Gel, Sigma) in 150 μl subcutaneously into the tail base; or (Group 2) with 50 μg pΔRCLcrE plasmid intramuscularly two times with 4-week interval,

or (Group 3) with 50 μg pΔRCLcrE plasmid intramuscularly once and 4 weeks later with 20 μg LcrE protein combined with Alum adjuvant. As control we used Group 4; mice were immunized with 50 μg pΔRC plasmid intramuscularly two times with 4-week interval. Two weeks after the last immunization blood was collected in heparinized capillaries from the retro-orbital plexus.

Challenging the immunized mice

Two weeks after the last immunization mice were challenged with 4×10^5 inclusion forming units (IFU) of *C. pneumoniae* (CWL029) in 25 μl PBS intranasally under pentobarbital sodium anaesthesia. Seven days after infection the mice were sacrificed. 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.

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

Western blot

Purified LcrE protein was heated to 95°C for 5 min in sample buffer, and the protein was separated by 10% SDS-PAGE. The separated protein was 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 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.

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

C. pneumoniae was propagated in HEp-2 cells (ATCC) as described earlier [29]. The partially purified and concentrated EBs were aliquoted and stored at –80°C until use. 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 MOMP (major outer membrane protein of *C. pneumoniae*)-specific monoclonal antibody 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, Sigma, α-mouse IgG1 and IgG2a-HRP, Biosource) was used for detection.

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

Level of LcrE specific IgG in the sera in the immunized mice

All of the mice immunized with LcrE protein mixed with Alum adjuvant, and most of the (7/10) mice immunized with pΔRCLcrE responded with LcrE specific antibody production. LcrE-specific IgG was detected by ELISA. There was a tendency for higher LcrE-specific IgG level ($p = 0.0625$) in pΔRCLcrE-primed and LcrE protein boosted (Group 3) mice compared to non-primed mice (Group 1) (Figure 1).

Significant LcrE-specific IgG1 production was detected in the sera of LcrE protein immunized mice, however, the IgG2a level was low in mice without

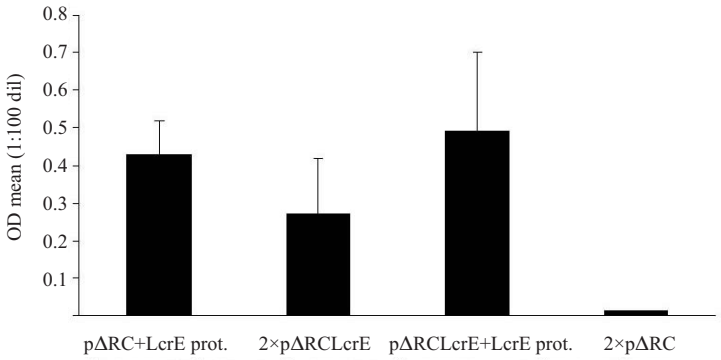

Figure 1. Two weeks after the last immunization sera were tested by LcrE-specific ELISA at dilution of 1:100. LcrE-specific IgG was detected in pΔRC+LcrE protein immunized, in 2 × pΔRCLcrE-immunized and in pΔRCLcrE-primed and LcrE protein boosted mice in contrast to the 2 × pΔRC-immunized mice

pΔRCLcrE priming (Figure 2). Mice immunized with pΔRCLcrE plasmid only produced relatively higher level of LcrE-specific IgG2a antibody. Boosting the immune response in pΔRCLcrE-primed mice with LcrE protein raised not only the IgG1 but also the IgG2a level. The higher relative IgG2a level indicates a Th1-biased response in the case of DNA immunized mice irrespective of the nature of the booster inoculation.

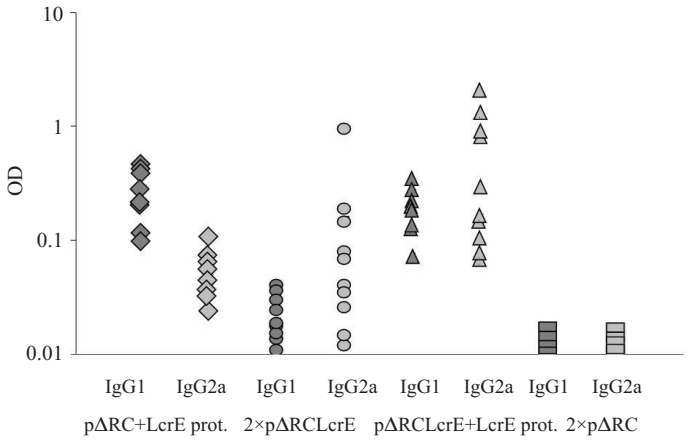

Figure 2. LcrE-specific IgG1 and IgG2a levels in the sera of immunized and *C. pneumoniae* challenged mice. Isotype of LcrE-specific IgG in the sera of immunized mice was measured by ELISA. Symbols represent OD values measured with sera of individual mice. Sera were tested at a dilution of 1:800 for IgG1, and 1:100 for IgG2a

Western blot analysis of pΔRCLcrE-induced antibodies

The immunogenicity of the eukaryotic expression vector (pΔRCLcrE) in mice and the LcrE-specificity of the produced antibodies were confirmed by Western blot (Figure 3) using PAGE-separated purified LcrE protein as antigen. Identity of purified LcrE protein was confirmed by MALDI-TOF mass spectrometry previously [30]. Sera of mice from Group 3 (immunized 2 × pΔRCLcrE) reacted with the band at the position corresponding to the molecular weight of LcrE protein similarly to the sera of LcrE protein immunized-mice of the earlier published experiment [22].

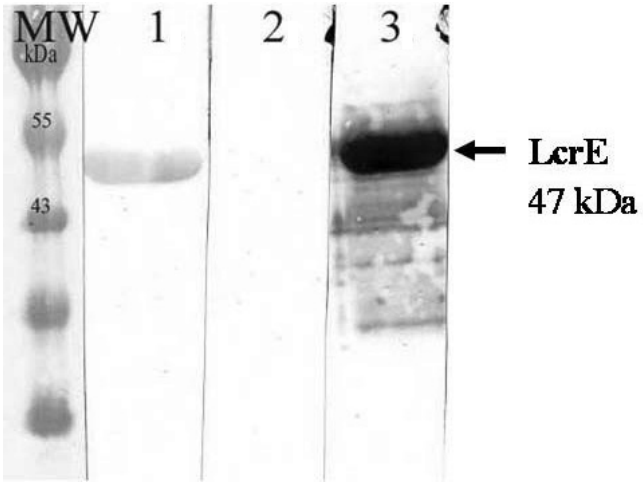

Figure 3. Sera from immunized mice were tested in Western blot assay. Purified LcrE protein was probed with sera of 2 × pΔRCLcrE-immunized mice (lane 1), 2 × pΔRC-immunized mice (lane 2) and 3 × LcrE protein+Alum immunized mice (lane 3) [22]

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

To find out whether *lcrE* applied in DNA vaccine can induce protective immune response against *C. pneumoniae* infection, *C. pneumoniae* titres in the lungs of immunized and control mice were compared. The reduction in *C. pneumoniae* burden in the lungs was 37% in mice immunized with LcrE protein mixed with Alum adjuvant (Group 1); 55% in pΔRCLcrE-immunized mice (Group 2) and nearly 72% in pΔRCLcrE-primed and LcrE protein boosted mice (Group 3) when

compared to pΔRC-immunized controls (Group 4) (Figure 4). This latter comparison showed significant difference with a P value of 0.01.

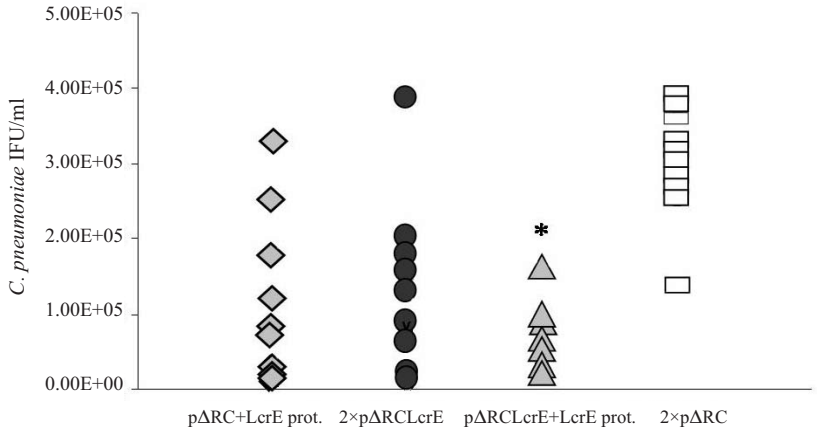

Figure 4. *C. pneumoniae* titres in the lungs of LcrE-immunized mice after *C. pneumoniae* challenge. Lung homogenates were inoculated onto HEp-2 cell monolayers and chlamydial inclusions were detected by indirect immunofluorescence, using MOMP-specific monoclonal and FITC-labelled secondary antibodies. The titre of *C. pneumoniae* in the lungs of pΔRCLcrE+LcrE protein immunized mice was significantly (* P = 0.01) lower than in lungs of control (2 × pΔRC-immunized) mice

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 [29–32]. 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. Structural proteins of type III secretion system are expected to be surface exposed and required for infection of host cells, thus, these are attractive vaccine candidates to investigate [16, 33–36].

Protective effect of LcrE-specific immunity against *C. pneumoniae* infection in animal models has been studied. The results of our published experiments [22] are in agreement with previous reports [21, 26] that LcrE protein is an antigen with considerable potential as a vaccine subunit and in this study we confirmed that this antigen has protective effect formulated as a DNA vaccine, too.

In our present study we show that we cloned *lcrE* gene into a eukaryotic expression vector, and *lcrE*-DNA immunization induced protective response against *C. pneumoniae* infection similar to that evoked by a single LcrE protein immunization. Moreover, in a prime-boost regime, i.e. *lcrE*-DNA/LcrE protein vaccination, the protective effect reached 72% in respect of lung bacterial burden. This level of protection is significant and comparable to that achieved by multiple subcutaneous injection of LcrE protein mixed with Alum adjuvant as published earlier [22]. Our findings are that pΔRCLcrE immunization induces an immune response shifted towards Th1 type pathway characterized by increased IgG2a production, and immunization with LcrE protein which generates an IgG1-dominated Th2 type response did not alter the DNA priming-polarized Th1 type response in DNA-primed and protein-boosted mice. Further experiments with improved immunization regimens or modified plasmid vectors are planned for inducing an increased level of antibody and/or cellular response potentially leading to better protection.

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RECOMBINANT *MYCOBACTERIUM SMEGMATIS* VACCINE CANDIDATES

ILDIKÓ FALUDI*, ÁGNES MÍRA SZABÓ, KATALIN BURIÁN, VALÉRIA ENDRÉSZ and A. MICZÁK

Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary

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Mycobacterium smegmatis is a species of rapidly growing saprophytes 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. This paper discusses the molecular methods used to generate recombinant *M. smegmatis* and the results obtained with some of these recombinants.

Keywords: *Mycobacterium smegmatis*, vaccination, recombinant technology

Introduction

The genus *Mycobacterium* comprises approximately 140 well-characterized species [1–4]. Throughout the centuries, two well-known species, *Mycobacterium tuberculosis* and *M. leprae,* have been frequent causes of immense human suffering. In 2009, 1.3–1.4 million patients of the HIV-negative and an additional 0.4 million of the HIV-positive people died from tuberculosis (TB). Furthermore 9.4 million new TB cases were recorded (including 3.3 million women) [5, 6]. Leprosy afflicts more than 10 million people, primarily in developing countries. *M. tuberculosis* and mycobacteria of the *M. avium-intracellulare-scrofulaceum*

* Corresponding author: E-mail: faludi.ildiko@gmail.com

group are pathogens of patients with acquired immune deficiency syndrome. However, the majority of mycobacteria are present in the environment as saprophytes.

M. smegmatis, first identified in 1884, is a rapidly growing saprophyte, it can propagate one generation every 1–3 h. It is non-pathogenic and commensal in humans [7, 8], and it acts as a 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 [9, 10]. *M. smegmatis* has a number of properties that can make it an effective vaccine vector. This fast-growing *Mycobacterium* is unable to arrest phagolysosome maturation and cannot evade intracellular killing [11–14]. Moreover, its rapid clearance by the host differs from that of the *M. tuberculosis* or even the vaccine strain *M. bovis* bacillus Calmette-Guérin [10]. Furthermore, *M. smegmatis* has been used as a vaccine vector because it activates dendritic cells and induces CD8-mediated immune responses [15–18]. Finally, killed *M. smegmatis* has been shown to provide the same adjuvant activity as *M. tuberculosis* during the induction of experimental autoimmune encephalomyelitis [19].

The role of *M. smegmatis* in immunotherapy

Structural components of mycobacteria have been studied for immune-potentiating effects since the 1970s. Ribi et al. [20] proved the antitumour activity of the cell walls of *M. tuberculosis, M. bovis, M. phlei* and *M. smegmatis*. Schwartz et al. [21] demonstrated inhibition of the growth of sarcoma cells in guinea pigs after infection with different species of mycobacteria. The effect was most pronounced at the time of the maximum biological activity of the applied mycobacterium. As a curiosity, mention should be made of the study of the synthesis of vitamin B12 by several mycobacterial strains, among them *M. smegmatis* [22].

Recombinant technology applied in *M. smegmatis*

Molecular mycobacteriology emerged in the 1980s. In 1985, different research groups demonstrated that genes from *M. tuberculosis, M. leprae* and *M. bovis* BCG could be cloned and expressed in *Escherichia coli* [23–26]. The basic tools for the mycobacterial genetic system were developed by Jacobs et al. in 1987

[27]. They devised the methodology for efficient gene transfer in mycobacteria by generating recombinant shuttle plasmids which are chimaeras containing mycobacteriophage DNA and an *E. coli* cosmid. These can replicate in *E. coli* as plasmids and in mycobacteria as phages and transfer DNA between both genera. These shuttle vectors for the first time permit the introduction of foreign DNA by electroporation into *M. smegmatis* and BCG. Through the expression of genes of protective antigens from a variety of pathogens, it became feasible to convert cultivable mycobacteria into useful multivaccine vehicles. The efficient plasmid transformation of *M. smegmatis* was elaborated. The identified high-efficiency (more than 10^5 transformants per microgram of plasmid DNA) transformable mutant strain of *M. smegmatis* (mc^2155) proved to be an extremely useful model system for the study of various aspects of mycobacterial physiology [28]. A method which allowed the stable integration of DNA fragments at a single site in the chromosome of *M. smegmatis* was developed by Martín et al. [29], who devised a vector containing an *E. coli* replicon, the kanamycin resistance gene for selection in mycobacteria and a fragment from *Streptomyces* plasmid pSAM2. However, the most used integrative plasmids are derived from L5 mycobacteriophage by Lee et al. [30]. The site-specific integration vectors transform mycobacteria with high efficiency to produce stable recombinants that contain a well-defined single-copy insert of the plasmid, these vectors can be used to efficiently insert large segments of DNA into mycobacteria.

The development of vectors and methodologies to introduce foreign DNA into members of the genus *Mycobacterium* has led to new approaches for the investigation of these important bacteria.

Recombinant *M. smegmatis* vectors in the defence against infectious diseases

IL-12/GLS (granulysin), Antigen 85B and ESAT6-CFP10 fusion protein

A recombinant *M. smegmatis* was constructed as a potential therapeutic vaccine against tuberculosis by Yi et al. [31]. Viable non-pathogenic *M. smegmatis* (ATCC607) carrying the pZM03 plasmid [a co-expression plasmid encoding human granulysin (GLS) and murine interleukin 12 (IL-12)] was used as a therapeutic vaccine to induce strong specific immunity against *M. tuberculosis in vivo*. Mice vaccinated with the therapeutic recombinant *M. smegmatis* (clone pZ607) displayed an enhanced immune response to *M. tuberculosis*-specific anti-

gen. This enhancement proved to be not less than (as assessed by the detection of IL-12 and IFN-gamma in the serum, the anti-purified protein derivative [PPD] sIgA in the broncho-alveolar lavage fluid, and the proliferation and IFN-gamma secretion by the splenocytes in response to PPD) or even higher than (as assessed via anti-PPD IgG2a in the serum) that in mice immunized with BCG. Since this therapeutic vaccine can induce strong specific Th1 responses against *M. tuberculosis* in BALB/c mice and causes no obvious harm to the host, the recombinant vaccine might be a candidate therapeutic vaccine against tuberculosis. Yang et al. [32] later reported that a recombinant *M. smegmatis* carrying pZM03 exerted combined anti-tuberculosis activity, i.e. immunotherapeutic effects with stimulation of the Th1 response, and the antibacterial activity of GLS.

Mycobacterial antigen 85B-induced immunogenicity is a strong correlate of vaccine-induced immunity against *M. tuberculosis* in mice. Wild-type *M. smegmatis* contains genes that encode the proteins of antigen 85B, but perhaps because of the secretion of these proteins is minimal, wild-type *M. smegmatis* is unable to induce protection against challenge with tuberculosis in mice. Lindsey et al. [33] over-expressed the immunodominant antigen 85B in *M. smegmatis*. Immunization with this recombinant mycobacteria induced protection against tuberculosis by enhanced T cell response in lungs.

Zhang et al. [34] evaluated a recombinant vaccine prepared from *M. smegmatis* that expresses a fusion of early secreted antigenic target 6-kDa antigen (ESAT6) and culture filtrate protein 10 (CFP10). These authors immunized mice with the recombinant *M. smegmatis* expressing the ESAT6-CFP10 fusion protein (rM.S-e6c10) or with BCG. The mice in the recombinant *M. smegmatis* group had a significantly higher titre of anti-ESAT6-CFP10 antibodies than did animals in control groups. Mice immunized with recombinant *M. smegmatis* produced significantly higher levels of IFN-γ and IL-2 than mice in the BCG-immunized group. Finally, immunization with recombinant *M. smegmatis* dramatically reduced the number of *M. tuberculosis* colony-forming units in the lungs of *M. tuberculosis* challenged mice, indicating its potential in clinical application.

Cowpox virus B5 (CPXV-B5)

Barefoot et al. [35] compared different vaccine vectors expressing a 35kDa secreted form of the Cowpox virus B5 antigen, which is known to be a strong inducer of the humoral immune response. B5 is conserved among poxviruses and has homologues among many mammalian orthopoxviruses, including variola and

vaccinia. The B5-specific immune response is of relevance as concerns the biodefence. The B5 protein was produced by *E. coli* carrying a plasmid and was used as a purified recombinant protein, or was expressed by different vectors such as 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 which could not be readily detected by Western blotting. All other vectors expressed robust levels of the B5 protein. While the expression of B5 by *M. smegmatis* was barely detectable by Western blotting, the presence of the gene was confirmed by sequencing. All vectors induced detectable serum antibody titres except *M. smegmatis*.

Human immunodeficiency virus proteins

M. smegmatis was engineered by Cayabyab et al. [16] as a vector expressing full-length HIV type 1 (HIV-1) HXBc2 envelope protein (gp120). Mice were inoculated twice with recombinant *M. smegmatis* expressing gp120, at an interval of 10 weeks. However, the peak responses after the two inoculations were not greater than those seen following a single inoculation. The responses in the boosted mice declined thereafter, but remained detectable even 1 year following the initial immunization. Hovav et al. [18] showed that a recombinant mycobacterial vector induced a cellular immune response that is biased toward memory cells and that can expand dramatically on re-exposure to an HIV-1 envelope antigen. Their observation that a small number of *M. smegmatis*-elicited p18-specific CD8+ T-cells can expand into large populations of functionally competent cytotoxic-T-lymphocytes following heterologous boosting with recombinant gp140-expressing adenovirus vector suggests that recombinant *M. smegmatis* may be useful as a priming vector in prime/boost vaccine regimens.

Yu et al. [36] described recombinant *M. smegmatis* vectors constructed to express the HIV-1 group M consensus *env* gene CON6 as either a surface, or an intracellular, or a secreted protein and determined the expression of the insert and the immunogenicity of these constructs. They demonstrated the ability of HIV-1 Env protein expressed by the recombinant *M. smegmatis* to induce mucosal T-cell immune responses after immunization. Recombinant *M. smegmatis* induced lower anti-HIV-1 T-cell responses than did a recombinant adenovirus (rAd)-recombinant vaccinia virus (rVV) prime/boost regimen in the spleen, but it induced IFN-gamma responses similar to those induced by rAd-rVV in the reproductive

tract and lungs of female mice, as detected by an enzyme-linked immunospot assay. These data demonstrate that recombinant *M. smegmatis* is immunogenic for the induction of HIV-1 T-cell responses at mucosal surfaces. The authors found that neither a prime nor a boost with recombinant *M. smegmatis* alone could induce any detectable anti-HIV-1 Env antibody. However, a single boost of the recombinant *M. smegmatis*-immunized mice with recombinant CON6 gp140CF protein induced higher anti-Env antibody titres than those observed in mice immunized with CON6 gp140CF protein alone or with empty recombinant *M. smegmatis* plus Env protein.

Mycobacteria are also regarded as promising vectors for DNA vaccine delivery. Mo et al. [37] characterized the ability of mycobacteria harbouring a plasmid encoding the fluorescence reporter gene under the control of a eukaryotic promoter to transfer the gene into mammalian cells, and the effects of various bacterial chromosomal mutations on the efficiency of transfer *in vivo* and *in vitro*. They observed that *M. smegmatis* strains (referred to as Rep^{High} *M. smegmatis*) that overexpressed replication proteins (Reps) responsible for initiation of the replication and copy number of mycobacterial plasmids increased the plasmid copy number up to 10-fold and transferred genes to the infected HeLa (human cervical adenocarcinoma) or J774 (murine macrophage) cells upon infection up to 3.5-fold more frequently than did a control *M. smegmatis* strain (Rep^{Wt} *M. smegmatis*). Vaccination with Rep^{High} *M. smegmatis* strains harbouring a plasmid encoding HIV type 1 gp120 under the control of a eukaryotic promoter generated gp120-specific CD8 T-cell responses among peripheral blood mononuclear cells in mice at an up to 3-fold higher frequency than that following vaccination with Rep^{Wt} *M. smegmatis* strains harbouring the same plasmid. The frequencies of gp120-specific CD8+ T-cell responses observed after immunization with the recombinant *M. smegmatis* were lower than those observed after intramuscular DNA immunization with a eukaryotic expression vector ($pgp120^{E}_{h}$), however, the immunization with the *M. smegmatis* recombinant generated more durable memory T-cells as compared to intramuscular DNA vaccination. These observations encourage the further development of mycobacteria as efficient DNA vaccine delivery vectors.

Hepatitis B virus proteins

Yue et al. [38] constructed eukaryotic and prokaryotic expression vectors and recombinant *M. smegmatis* which carried the fused genes of the CS1 antigen,

a fusion protein that comprises a truncated core protein (amino acids 1–155) and the preS1 peptide (amino acids 1–55) of the protein responsible for the attachment of the hepatitis B virus (HBV).

The antigen-specific cellular and humoral immune responses were characterized after inoculation with the recombinant *M. smegmatis* or a recombinant DNA based vaccine, which expressed the fused proteins *in vitro*. CS1-specific DNA immunization induced only a minor humoral response and low levels of T cell immunity. Vaccination with live recombinant *M. smegmatis* induced a stronger cellular immune response and a longer period of humoral immune response, which are crucial for virus-specific vaccines. Thus, this recombinant *M. smegmatis* has a potential for the prevention of HBV infection.

Outer membrane protein 26 kDa antigen (Omp26) of Helicobacter pylori

H. pylori induces chronic inflammation of the stomach mucosa, causing chronic gastritis and peptic ulcer. The bacteria infect approximately 50% of the world's population. Studies in mice have shown that prophylactic or therapeutic oral immunization with *H. pylori* lysate results in significant, but rarely sterilizing, immunity against *H. pylori*. Lü et al. [39] developed a recombinant *M. smegmatis* expressing the Omp26 antigen, then evaluated the capacity of the recombinant to reduce *H. pylori* colonization in the stomach and induce immune responses after therapeutic immunization in mice. The oral therapeutic immunization with recombinant *M. smegmatis* induced a significant reduction in the number of *H. pylori* bacteria in the stomachs, compared to the control infected mice. The serum anti-*H. pylori* specific antibody level was increased with a Th1 and Th2 profiles and also a local overproduction of cytokines in the stomach and spleen was observed. These results suggested that Omp26 is a promising vaccine candidate antigen for use in a therapeutic vaccine against *H. pylori,* and *M. smegmatis* provide new tools to analyse the immune mechanisms mediating protection and the identification of proteins which are important in protection.

Conclusion

Several recombinant *M. smegmatis* have been produced and evaluated in animal models as new vaccine candidates. It is hoped that the utilization of one or more of these new vaccines, combined with the development of modern produc-

tion technologies, will contribute substantially to the control of these important infectious diseases.

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