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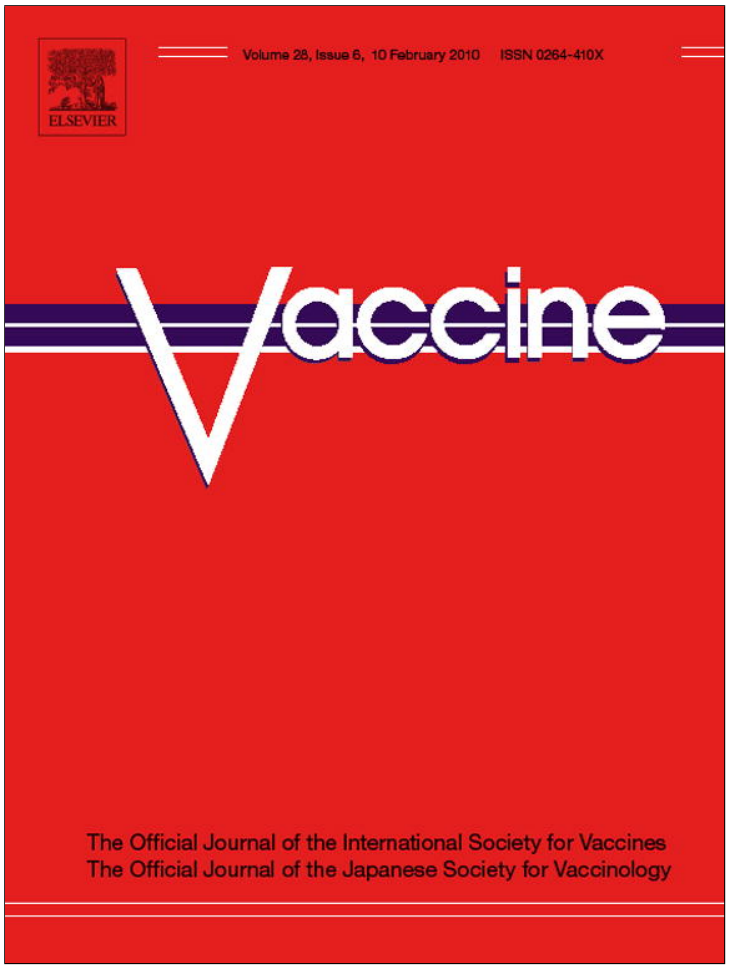

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Novel *Chlamydia pneumoniae* vaccine candidates confirmed by Th1-enhanced genetic immunization

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ABSTRACT

Identification of highly immunogenic antigens is critical for the construction of an efficacious subunit vaccine against *Chlamydia pneumoniae* infections. A previous project used a genome-wide screen to identify 12 protective *C. pneumoniae* candidate genes in an A/J mouse lung disease model (Li et al. [14]). Due to insufficient induction of Th1 immunity, these genes elicited only modest protection. Here, we used the *Escherichia coli* heat-labile enterotoxin as a Th1-enhancing genetic adjuvant, and re-tested these 12 genes, in parallel with six genes identified by other investigators. Vaccine candidate genes *cutE* and Cpn0420 conferred significant protection by all criteria evaluated (prevention of *C. pneumoniae*-induced death, reduction of lung disease, elimination of *C. pneumoniae*). Gene *oppA_2* was protective by disease reduction and *C. pneumoniae* elimination. Four other genes were protective by a single criterion. None of the six genes reported elsewhere protected by reduction of lung disease or elimination of *C. pneumoniae*, but three protected by increasing survival.

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1. Introduction

Chlamydia (*C.*) *pneumoniae* is an obligate intracellular bacterium that causes community-acquired respiratory infection and pneumonia in humans [1]. It has also been strongly associated with chronic inflammatory diseases such as atherosclerosis [2]. These public health concerns indicate a need for control of such infections.

Antibiotic therapies have only limited success against *C. pneumoniae* infections [3], especially after infection and pathology are established, in which case antibiotics may even enhance chlamydial dissemination [4,5]. For instance, in large scale field trials, antibiotic treatment did not reduce atherosclerosis, despite its association with increased *C. pneumoniae* antibody levels and detection of agent in lesions [6].

Genetic vaccines have been explored against chlamydial infections, due to inocula consistency and ease of manipulation, production, storage, and delivery [7]. A number of rationally selected *C. pneumoniae* genes, based on their known or presumed surface location, have been tested for protection in rodent models. In one study, heat-aggregated CopN (chlamydial outer protein N) protein, when intranasally administered in high dose together with *Escherichia coli* heat-labile toxin (LT), protected BALB/c mice against intranasal *C. pneumoniae* challenge [8]. In a different BALB/c mouse study, immunization with plasmids encoding the major outer membrane protein (MOMP) or an ADP/ATP translocase (Npt1) of *C. pneumoniae* resulted in a reduced bacterial load in the lung after challenge [9]. Finco et al. [10] showed that subcutaneous immunization with recombinant *C. pneumoniae* enolase (*Eno*) and several other proteins significantly decreased the amount of *C. pneumoniae* after an intraperitoneal challenge in hamsters. Svanholm et al. [11] showed that intranasal immunization with plasmid DNA encoding chlamydial heat shock protein 60 (HSP-60) reduced the *C. pneumoniae* lung loads by 5–20-fold in C57BL/6 mice, while also decreasing disease severity. Rodriguez et al. [12] showed that intranasal, but not intraperitoneal, genetic immunization with *C. pneumoniae* MOMP or HSP-60 conferred protection against *C. pneumoniae* infection, probably due to induction of cell mediated immune responses. Finally, Thorpe et al. [13] used recombinant LcrE, a potential component of the chlamydial type III secretion system to intraperitoneally immunize BALB/c mice. While a number of presumed surrogate parameters appeared to suggest protection, no statistically valid data indicated reduction of *C. pneumoniae* or any other form of actual protection of the mice. Overall, none of these antigens mediated protection that is close to the protection conferred by natural immunity after asymptomatic low-level *C. pneumoniae* infection, in which *C. pneumoniae* lung burdens are

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reduced at least 100-fold as compared to mock-vaccinated mice 10 days after inoculation. Thus, truly highly protective *C. pneumoniae* vaccine antigens still need to be identified as components of a vaccine with reasonable probability for successful human application.

In previous experiments, we used expression library immunization to identify from the *C. pneumoniae* genome a total of 12 vaccine candidate genes that are capable of conferring high level protection to mice, as indicated by lower lung weights and better chlamydial elimination as compared to the mock-vaccinated controls [14]. In a subsequent re-test, however, these antigens did not confer complete protection, either by gene gun or a combined intramuscular–intradermal genetic immunization. We speculated that the poor vaccine efficacy was due to Th2-biased immunity elicited by gene gun vaccination [14]. However, early and robust induction of a Th1 response is critical for protective immunity against chlamydial infections. This has prompted us to use a vaccine adjuvant that particularly promotes Th1 immune responses.

Arrington et al. [15] have used both the A and B subunits of cholera toxin (CT) or the *E. coli* heat-labile enterotoxin (LT) as genetic adjuvants for particle-mediated genetic vaccines. Co-immunization with either of these vectors significantly elevated Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) levels. While both Th1 and Th2 cytokine production were enhanced in this experiment, the LT vectors have elicited more Th1-like biased responses in other systems. For example, HBcAg-specific IgG2a/IgG1 ratios were elevated and the IFN-γ (but not IL-4) responses were augmented [15]. Therefore, we used the LT subunit A and B plasmid vectors as a genetic adjuvant for re-evaluation of the *C. pneumoniae* vaccine candidates. In this investigation, we have re-tested the genes ranked highest for protection against *C. pneumoniae* in our previous genome-wide screen [14] delivered in the Th1-modulated vaccination regimen. We have identified gene vaccine candidates that confer protection levels comparable to a live *C. pneumoniae* vaccine.

2. Materials and methods

2.1. C. pneumoniae

C. pneumoniae strain CDC/CWL-029 (ATCC VR-1310) was grown in Buffalo Green Monkey Kidney monolayer cell cultures, purified by differential centrifugation, and quantified as previously published [16]. Purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer, stored in aliquots at −80 °C, and their infectivity was confirmed in female A/J mice.

2.2. Animals

Inbred A/J female mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and maintained in ventilated cages of 5 mice each with ad libitum access to water and a 19% protein/1.33% L-arginine standard rodent maintenance diet. Two weeks prior to the challenge infection the mice were started on a custom diet containing 24% protein/1.8% L-arginine (Harlan Teklad, Madison, WI). All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

2.3. Confirmation of the Th1 immunostimulatory effect of the LT adjuvant

The *Chlamydia abortus* protective vaccine candidate gene *dnaX2* was cloned into genetic immunization vector, pCMVi-UB as described earlier [17]. Plasmid-coated gold particles for gene gun immunization were prepared in a standard protocol (Bio-Rad Laboratories, Hercules, CA) using endotoxin free plasmid DNA preparations. Each vaccine dose contained a total of 1 μg of a plasmid DNA mix. The mix contained 0.9 μg of the DnaX2-encoding plasmid and 0.1 μg of the LT genetic adjuvant. This adjuvant was a 1:4 mixture of two plasmids encoding the B and A subunits of *E. coli* heat-labile toxin (LT A + B), which has been shown to induce a strong and Th1-biased immune response [15]. The coding sequence for subunit A was modified to change the R at position 192 to G to detoxify the gene [18]. DNA was delivered by gene gun (Bio-Rad Laboratories, Hercules, CA) into each ear lobe of each mouse (5 mice/group). An accelerated vaccination schedule was used to immunize mice on days 0, 21, and 42. Sera from mice were obtained by saphenous vein bleeding 4 weeks after the last vaccination.

For large-scale protein production of recombinant *C. abortus* DnaX2 antigen, sequence-confirmed DnaX2 was subcloned into pEXP5-NT (Invitrogen, Carlsbad, CA). The expression construct was used to transform the host strain BL21(λ)DE3. Cells were grown to mid-logarithmic phase and induced with 0.5 mM IPTG according to recommended protocols. Cells were harvested 3–4 h after induction by centrifugation and the resulting cell pellet lysed by resuspension in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitors (Roche Applied Sciences, Indianapolis, IN). Cell walls were permeabilized with 10 mg of lysozyme and subjected to 3 freeze/thaw cycles between −80 °C and room temperature. The viscous lysate was cleared in a 1 h incubation at 4 °C with 10 g/mL of DNase I and 20 mM $MgCl_2$. The lysate was centrifuged at 27,000 × *g* for 10 min at 4 °C, and the supernatant containing the soluble material was transferred to a fresh tube. The insoluble material, remaining in the pellet of the cleared lysate, was washed 4 times in PBS containing 1% Triton X-100 and 0.5 M guanidine followed by 3 washes with PBS. Cells were collected between washes by centrifugation at 3000 × *g* for 5 min at room temperature. After the final PBS wash, the inclusion bodies were resuspended in PBS, flash-frozen in liquid nitrogen, and stored at −80 °C until ready for use. To solubilize the inclusion bodies, the pellets were resuspended in PBS containing 8 M urea and 10% glycerol. Insoluble material was removed by centrifugation at 14,000 × *g* for 5 min at room temperature, and the soluble protein was collected in the supernatant and dialyzed against PBS.

Total IgG, IgG1, and IgG2a antibody concentrations against *C. abortus* DnaX2 were determined by ELISA of 1:2000 diluted sera. Briefly, 0.1 μg of recombinant *C. abortus* DnaX2 protein was coated per well by dilution in 0.05 M $NaHCO_3$, pH 9.6. After incubation of diluted sera, bound antibodies were detected by use of horseradish-peroxidase conjugated goat antibodies against mouse IgG, IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL) followed by TMB substrate (Thermo Scientific Pierce, Rockford, IL). The substrate reaction was stopped with sulfuric acid, and antibody concentrations were determined as absorbance at 450 nm. The background signal of antisera in a well without DnaX2 antigen was subtracted from the data.

2.4. Confirmatory C. pneumoniae vaccine candidate screen

Previously, the genome sequence of *C. pneumoniae* isolate CDC/CWL-029 (ATCC strain VR-1310) was extracted from Genbank (AE001363, 1,230,230 bp) and all ORFs were tested in two rounds of screening through expression library immunization (ELI) [14]. In Round 1, the 1263 ORFs of 1.5 kb or less were PCR amplified and constructed as linear expression elements (LEEs) by linking to a CMV promoter and a human growth hormone terminator sequence. The LEE library was arranged in three different sets of 30 random pools, each with ∼42 ORFs, and used as inocula for 3 gene gun immunizations in groups of 5 mice. Each test inoculum contained 200 ng of a mixture of ∼42 ORFs and 800 ng of pUC118 carrier DNA. All mice were challenged by intranasal inoculation of 1×10^8 *C. pneumoniae*

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elementary bodies and sacrificed 10 days later, and *C. pneumoniae* lung loads were determined by FRET-qPCR [14]. Protection scores for each group were determined by calculating the geometric mean bacterial genome count (log value) measured in each mouse. The corresponding inoculating pools were subjected to two analyses to derive a ranking for individual ORFs. First, a matrix analysis was applied by taking advantage of the overlapping pooling strategy used to create the inocula. By mapping the positively scored planes onto the pooling matrix, ORFs at the intersections were identified and inferred to be potentially responsible for the observed group protection. In a second analysis, the pools with each set of 30 were ranked relative to their protection score and then a rank value was assigned to each gene by summing the ranks of its three resident pools. We have found that the two analytical methods identify predominantly the same ORFs, but each can advantageously pinpoint additional ORFs. For example, the matrix intersection method treats all positive pools identically, such that significant or non-significant differences among positive groups do not influence the output. The ranking method accommodates for the possibility that a protective gene may reside in a pool carrying an unfavorable gene. If the other two resident pools score well, the useful ORF can be captured despite one poor score. Using the combined approach, 46 *C. pneumoniae* ORFs were selected for further testing in the individual vaccine candidate screens in Rounds 2 and 3 (Table 1).

In this investigation, the highest ranked 12 candidates of those 46 candidates were cloned as full (10 candidates) or partial genes (2 candidates) and tested individually in Round 3, in a high-dose *C. pneumoniae* challenge using a day-10 LD_{50} inoculum. This experiment was designed as a rigorous challenge of the protective efficacy of the final candidate genes. The readouts were evaluation of protection from disease by survival of mice and determination of lung weight increase, as well as elimination of *C. pneumoniae* organisms by determination of total chlamydial lung loads.

Genetic immunization was performed by biolistic delivery of recombinant mammalian expression vectors carrying individual bacterial genes under control of a eukaryotic promoter. This genetic immunization vector, pCMVi-UB, which has been previously described [19], carries the eukaryotic cytomegalovirus immediate-early promoter enhanced by a chimeric intron (CMVi). The expression cassette contains a mouse ubiquitin gene (UB) and a human growth hormone terminator (hGH-term). Chlamydia sequences were cloned into unique BglII and HindIII restriction sites in frame with ubiquitin. ORFs were PCR amplified from *C. pneumoniae* genomic DNA with sets of gene-specific primers, then re-amplified with adapter primers and cloned into pCMVi-UB. The assembled expression cassettes and the correct integration was confirmed by DNA sequencing.

Genetic immunization with these plasmids was performed as described for the LT genetic adjuvant testing in Section 2.3. Mice were challenged with 5×10^8 *C. pneumoniae* elementary bodies 4 weeks after the last immunization. Also cloned and used in this round were the genes encoding six *C. pneumoniae* proteins, CopN, Npt1, Enolase, Momp, GatA and HSP-60, that had been reported as protective chlamydial antigens in the literature. These vaccine candidates were tested by immunizing groups of 10 female A/J mice.

2.5. Negative and positive controls

In this study, mice immunized with 5×10^6 genomes of viable *C. pneumoniae* 1 month prior to the vaccine challenge served as positive protection controls (live vaccination), and mice treated with SPG buffer (mock-vaccinated), and then challenged served as negative protection controls. Groups were scored for protection by calculating the percent lung weight increase over that of age-matched unchallenged female A/J mice (138.4 mg), and by calculating the mean logarithm of total *C. pneumoniae* per lung. These values were then converted to a relative protection score by normalizing them to the lung weight increase or logarithm of total *C. pneumoniae* lung load that was calibrated by control groups. A CMVi-UB LEE construct encoding the luciferase gene served as a control for LEE immunizations, and a plasmid construct pCMVi-UB carrying the same luciferase insert was used as the control for plasmid immunizations.

Table 1
Genetic vaccine fragments of *C. pneumoniae* genes selected in Round 1 for further testing in Round 2, and selected in Round 2 for final testing in Round 3.

Gene	Round 1 total lung *C. pneumoniae* protection score	Round 1 rank	Round 2 total lung *C. pneumoniae* protection score[a]	Round 2 rank[b]
*mutL*_a	0.729	1	0.102	21
ldh	0.680	2	−0.074	36
atoC	0.663	3	0.349	11•
CPn0249_b	0.651	4	−0.295	45
gapA	0.622	5	−0.273	44
*ide*_b	0.621	6	0.857	3•
CPn0884	0.614	7	0.042	30
CPn0913	0.554	8	0.039	31
fabD	0.544	9	0.484	9•
*cutE*_a	0.542	10	1.287***	1•
CPn0420	0.541	11	1.102**	2•
CPn0755	0.539	12	0.071	24
ppa	0.537	13	0.230	15
yigN	0.521	14	−0.163	40
*efp*_2	0.519	15	−0.098	38
*glgX*_b	0.514	16	0.559	6•
CPn0330	0.512	17	0.125	18
CPn0095_a	0.508	18	0.276	13•
CPn0020_b	0.502	19	0.524	7•
CPn0174	0.502	20	−0.017	34
*ychM*_a	0.496	21	−0.109	39
CPn1072	0.495	22	0.086	22
CPn0044	0.495	23	0.133	17
CPn0155	0.495	24	0.228	16
CPn0523	0.492	25	−0.214	42
*oppA*_2_a	0.489	26	0.762	4•
CPn0554	0.488	27	0.043	28
yacE	0.484	28	−0.298	46
CPn0830	0.479	29	−0.086	37
fliI	0.476	30	0.118	19
rl1	0.472	31	0.401	10•
CPn0509	0.460	33	0.524	8•
CPn0981_b	0.458	34	0.025	33
CPn1020_b	0.438	37	−0.197	41
pyk	0.433	40	0.047	27
*ftsH*_a	0.416	43	0.053	26
CPn1061	0.414	44	0.070	25
CPn0927	0.405	47	0.316	12
CPn1070	0.405	48	0.028	32
*gidA*_b	0.403	49	0.112	20
CPn0553	0.396	50	0.076	23
rs5	0.392	53	0.043	29
CPn0602	0.345	70	−0.218	43
ssb	0.308	94	0.582	5•
CPn0369	0.299	100	−0.038	35
*pbp2*_b	0.290	109	0.244	14

[a] Asterisks in this column indicate significant differences (**$P < 0.01$; ***$P < 0.001$) from mock-vaccinated controls in a post hoc Dunnett's test for determination of the significant differences between a single control group mean and the remaining treatment group means in ANOVA.
[b] Dots in this column indicate genes selected for further testing in Round 3.

2.6. C. pneumoniae lung challenge infection

Mouse intranasal inoculation was performed as described [20], and optimal doses for live immunization and challenge inocula were determined in preliminary experiments. For intranasal inoculation, mice received a light isoflurane inhalation anesthesia.

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Vaccine protection control mice were inoculated with a low dose of 5×10^6 *C. pneumoniae* elementary bodies in 20 μl SPG buffer. In Rounds 1 and 2, higher dose challenge infection was performed 4 weeks after the last gene gun genetic vaccination or low dose inoculation of live *C. pneumoniae*, by intranasal inoculation of 1×10^8 *C. pneumoniae* elementary bodies in 20 μl SPG buffer. In Round 3, mice were challenged by an LD_{50} dose of 5×10^8 *C. pneumoniae* elementary bodies in 20 μl SPG buffer. Mice were sacrificed by CO_2 inhalation 10 days after inoculation, and lungs were weighed, snap frozen in liquid nitrogen, and stored at −80 °C until further processing. The lung weight values were converted to protection scores by normalizing to the lung weight increase of control immune (protection score 1 = 100% protection) and mock-vaccinated (protection score 0 = 0% protection) groups.

2.7. Mouse lung nucleic acid extraction

Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent in disposable tissue grinders (Fisher Scientific, Atlanta, GA) to create a 10% (w/v) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) [21].

2.8. Analysis of C. pneumoniae lung loads by real-time PCR

The primers and probes used in the PCR assay were custom synthesized by Operon, Alameda, CA. The copy number of *C. pneumoniae* genomes per lung was determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) qPCR [21]. The $\log_{10}$ *C. pneumoniae* lung loads were also converted to protection scores by normalizing to the positive (protection score 1 = 100% protection) and negative protection control groups (protection score 0 = 0% protection).

2.9. Statistical analysis

All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Data of *C. pneumoniae* genome copies were logarithmically transformed. Normal distribution of data was confirmed by the Shapiro–Wilk's *W* test, and homogeneity of variances by Levene's test. Data were evaluated by mean plots ± 95% confidence intervals, and analyzed by analysis of variance (ANOVA). Post hoc comparisons of means were performed under the assumption of no *a priori* hypothesis by the Tukey honest significant difference (HSD) test, or by Dunnett's test for determination of the significant differences between a single control group mean and the remaining treatment group means. Survival data were analyzed by one-sided Fisher Exact test.

3. Results

3.1. The E. coli heat-labile enterotoxin stimulates the Th1 immune response in mice

To evaluate the Th1 immunostimulatory effect of *E. coli* LT used as genetic adjuvant, mice were immunized with the *C. abortus dnaX2* gene, which we previously identified in a genomic screen as being protective [17], with or without LT. To determine the overall immune response, and of Th1 vs. Th2 immunity, sera obtained 4 weeks after the last immunization were analyzed for total IgG, IgG1, and IgG2a antibodies against *C. abortus* DnaX2 antigen. For total IgG levels, 2000-fold diluted sera from mice immunized with *C. abortus dnaX2* in combination with the LT subunit genes had an average optical density of 1.095, whereas sera from mice immunized with only *C. abortus dnaX2* had 0.863 ($n = 5$, $P = 0.00013$, Student's *t*-test).

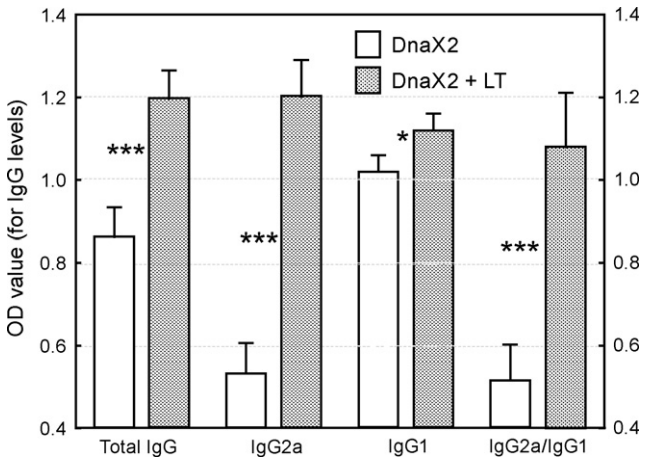

Fig. 1. Use of *E. coli* heat-labile enterotoxin (LT) as genetic adjuvant boosts total and Th1 immune response. Groups of 5 mice were immunized with *C. abortus dnaX2* in the presence (white bars) or absence (shaded bars) of LT adjuvant. Mice ($n = 5$/group) were vaccinated 3 times in 3-week intervals, and antibody levels at 4 weeks after the last vaccination were evaluated by ELISA using recombinant DnaX2 protein as antigen, goat-anti-mouse horseradish-peroxidase conjugates, and TMB substrate. Data are shown as means of absorbance of 1:2000 diluted sera or the ratio of the IgG2a/IgG1 OD values ± 95% confidence intervals. Asterisks indicate significant differences between adjacent groups (*$P < 0.05$; ***$P < 0.001$; Student's *t*-test).

The sera from dnaX2- and LT-administered mice showed higher levels of both IgG1 and IgG2a than sera from mice immunized with dnaX2 alone; however, the difference between these two sera samples is much more significant for IgG2a (1.203 vs. 0.533, $P < 0.0001$) than for IgG1 (1.120 vs. 1.078, $P = 0.01$). The IgG2a/IgG1 ratio is also significantly higher in sera from LT co-immunized mice than receiving dnaX2 alone (1.080 vs. 0.514, $P < 0.0001$), indicating a strong bias of the T helper cell response towards Th1 (Fig. 1).

In a parallel experiment, mice were vaccinated with the human alpha-1 antitrypsin (*aat*) gene, with and without the LT subunit genes. Again, overall antibody responses increased with the LT adjuvant, but in particular the level of IgG2a subtype specific anti-AAT antibodies (1.592 vs. 0.521, $P < 0.0001$) and the IgG2a/IgG1 ratio of OD values (1.276 vs. 0.389, $P < 0.0001$) indicate a Th1-like biased response. These two separate experiments validate the Th1-enhancing effect of the *E. coli* heat-labile enterotoxin when used as a genetic adjuvant [15].

3.2. Efficacy confirmation of protective antigens identified previously by expression library immunization

All 1052 annotated genes of *C. pneumoniae* have been previously screened for vaccine candidates as 1263 full or partial gene ORFs by independently partitioning the ORFs into 30 pools of ORFs, 3 times, and then using these 90 pools of linear expression constructs (LEEs) [22] to genetically immunize mice [14]. All ORFs were evaluated by calculation of a protection score, and 46 *C. pneumoniae* ORFs were selected for further individual vaccine candidate screening in that investigation (Table 1). The first 31 of the candidates were selected strictly on the basis of protection score and the intersections of ORFs common among the protective pools. The remaining 15 candidates were selected on the basis of protection score as well as low variance of the scores. These 46 partial or full-length ORFs were individually evaluated as LEE gene vaccines in Round 2. Total lung *C. pneumoniae* protection scores and the ranking of the genes based on these scores are shown in the rightmost columns of Table 1. The results of Round 2 identified the following *C. pneumoniae* genes, in this ranking, as candidates for final testing and confirmation in Round 3: *cutE*, Cpn0420, *ide*, *oppA_2*, *ssb*, *glgX*, Cpn0020, Cpn0509, *fabD*, *rl1*, *atoC*, and Cpn0095 [14].

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Table 2
Survival of *C. pneumoniae* high-dose-challenged mice in Round 3 vaccinated with plasmid-cloned *C. pneumoniae* genes selected in Round 2.

Vaccine	Day-10 survival (%)[a]
Mock-vaccinated	8/14 (57)
Live vaccine	15/15 (100)**
Control vaccine	9/10 (90)
cutE	10/10 (100)*
Cpn0420	10/10 (100)*
*ide*_ab	9/10 (90)
*oppA*_2	9/10 (90)
ssb	9/10 (90)
Cpn0509	9/10 (90)
fabD	3/10 (30)
glgX	7/10 (70)
Cpn0020	10/10 (100)*
atoC	8/10 (80)
rl1	8/10 (80)

[a] Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) from mock-vaccinated controls in the Fisher Exact test.

These 12 highest ranking ORFs were now cloned as full-length genes into genetic immunization plasmid CMVi-UB. Candidates *ide* and Cpn0095 were represented as subgene ORFs *ide*_ab and Cpn0095_a in the screen, and were cloned as these same fragments. The remaining genes were represented as full-length clones. Mice were genetically vaccinated with the candidate expressing constructs together with a genetic adjuvant comprised of plasmids expressing mutant, non-toxic *E. coli* enterotoxin A and B subunits [15]. Animals were challenged with an inoculum of 5×10^8 *C. pneumoniae* elementary bodies that was 5-fold higher than that used in the Round 2 screen. This elicited severe disease in intranasally inoculated mock-vaccinated female A/J mice and approximately 50% lethality within 10 days (LD_{50}). Cpn0095_a was not used in this challenge since it was tested in an independent experiment. This Round 3 high-dose challenge was used to evaluate protective efficacy of the vaccine candidates for (i) prevention of *C. pneumoniae*-induced death, (ii) lung disease, and (iii) bacterial elimination.

The survival data in Table 2 show that in addition to the live vaccine positive control, immunization with genes *cutE*, Cpn0420, and Cpn0020 prevented death, whereas 43% of the mock-vaccinated mice died ($P<0.05$, Fisher Exact test). In the remaining test groups, at least one animal died, and the survival in these groups against this severe challenge was not significantly different from mock-vaccinated mice. Thus, genes *cutE*, Cpn0420, and Cpn0020 were confirmed as mediating significant protection from death following high-dose challenge with *C. pneumoniae*.

Next, the efficacy of the vaccine constructs in reducing *C. pneumoniae*-induced lung disease (interstitial bronchopneumonia) was evaluated by analyzing lung weight increases of surviving challenged mice when they were sacrificed on day 10 after inoculation. Lung weight increase relative to unchallenged age-matched animals is proportional to lung infiltration with inflammatory cells, and therefore reflects disease intensity [20]. The 64.5% average lung weight increase of the mock-vaccinated mice was set as 0% protection; the 32% lung weight increase of live vaccine administered mice was set as 100% protection. Data shown in Fig. 2A indicate that genes *cutE*, Cpn0420, *oppA*_2, and *ssb* significantly reduced the increase in lung weight of infected as compared to mock-vaccinated mice and thus mediated significant protection from lung disease ($P<0.05$, Dunnett's test).

Finally, efficacy of the top 12 vaccine candidates in facilitating elimination of *C. pneumoniae* as compared to mock-vaccinated mice was evaluated. To maximize sample size, protection scores based on the logarithm of total *C. pneumoniae* lung loads on day

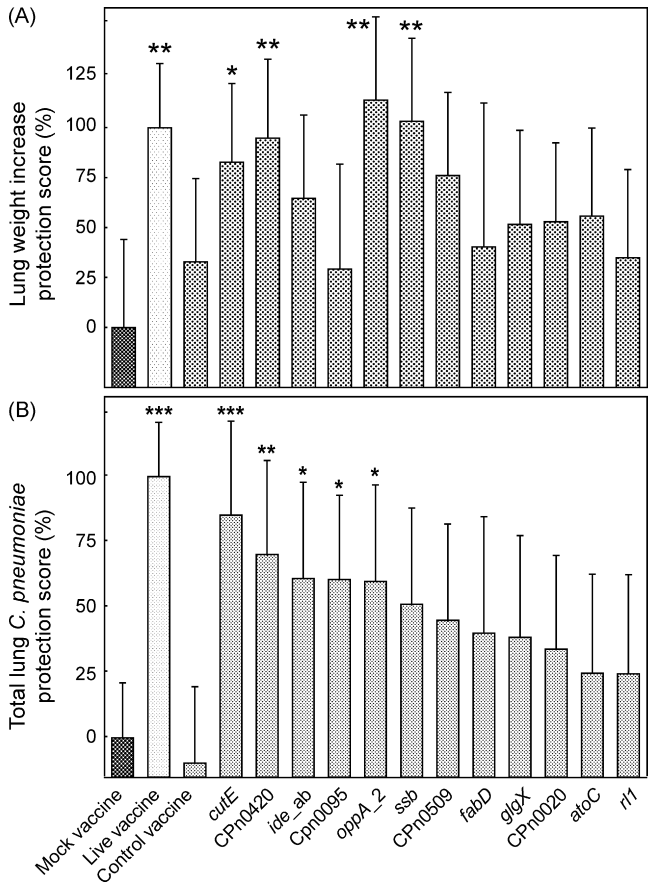

Fig. 2. Disease protection efficacy of final vaccine candidates. After testing of 46 individual candidates in Round 2, 12 of these genes (Table 1) were cloned as full-length genes (except *ide*_ab and Cpn0095_a) into genetic immunization plasmid CMVi-UB and used for vaccination together with genetic vaccine adjuvant LT A + B in Round 3. Cpn0095_a was not included in the Round 3 high-dose challenge. Vaccinated mice ($n=10$/group) were intranasally challenged with an LD_{50} of 5×10^8 *C. pneumoniae* elementary bodies. Surviving mice (Table 2) were sacrificed on day 10 p.i., lungs were weighed, and the lung weight increase over the average lung weight of unchallenged age-matched female A/J mice was calculated. The lung weight increase is a reliable measure of disease intensity, and high increases reflect severe disease. (A) Lung weight increase data were linearly transformed into protection scores by setting the increase for unprotected mock-vaccinated mice at 0 and for optimally protected live-vaccinated mice at 1. (B) For vaccination Rounds 2 and 3 of the final vaccine candidate genes, protection scores were calculated based on the logarithm of the total *C. pneumoniae* lung load on day 10. Protection scores from Round 2 with the use of LEE constructs and from Round 3 with plasmid-cloned genes (full-length except for partial genes *ide*_ab and Cpn0095_a) were pooled and analyzed by one-way ANOVA. Data are shown as means ± 95% confidence intervals (mock-vaccinated, live vaccine groups $n=60$; genetic vaccine groups $n=13$–20). Asterisks indicate protected groups that are significantly different from mock-vaccinated mice (*$P<0.05$; **$P<0.01$; ***$P<0.001$; Dunnett's test).

10 from Rounds 2 and 3 were combined. Due to minor differences in mouse age, feeding, and challenge inoculum, responses of the mock-vaccinated and live vaccine mice were quantitatively different between different experiments. Therefore, protection scores were calculated so as to relate the efficacy of individual vaccine candidates to the mock-vaccinated and live-vaccine calibration groups within the same experiment. The adoption of normalized protection scores enables comparison of the three experiments, thereby making analyses of the combined dataset statistically possible. Efficacy of Round 2 LEE-based vaccination with gene fragments (*cutE*_a, *ide*_b, Cpn0095_a, *oppA*_2_a, *glgX*_b, Cpn0020_b) or full-length genes (Cpn0420, *ssb*, Cpn0509, *fabD*, *atoC*, *rl1*) with the plasmid-based vaccination with gene fragments (*ide*_ab, Cpn0095_a) or full-length genes (*cutE*, Cpn0420, *oppA*_2, *ssb*, Cpn0509, *fabD*, *glgX*_b, Cpn0020, *atoC*, *rl1*) was also combined. Cpn0095_a had been used in sepa-

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Table 3
Survival of *C. pneumoniae* high-dose-challenged mice in Round 3 vaccinated with plasmid-cloned *C. pneumoniae* genes reported as protective.

Vaccine	Day-10 survival (%)[a]
Mock-vaccinated	8/14 (57)
Live vaccine	15/15 (100)**
Control vaccine	9/10 (90)
copN	9/10 (90)
npt1	9/9 (100)*
Enolase	9/10 (90)
Momp	10/10 (100)*
gatA	10/10 (100)*
hsp60	8/10 (80)

[a] Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$) from mock-vaccinated controls in the Fisher Exact test.

rate Round 2 experiments both as LEE and as plasmid. Data shown in Fig. 2B indicate that genes *cutE*, Cpn0420, *ide*, Cpn0095, and *oppA_2* mediated significantly enhanced elimination of *C. pneumoniae* ($P<0.05$, Dunnett's test) as compared to the $10^{6.670}$ *C. pneumoniae* lung load of mock-vaccinated mice (0% protection) and $10^{4.044}$ of live-vaccinated mice (100% protection).

Mice vaccinated with the constructs encoding the luciferase gene had a lung weight increase protection score of 33%, which is statistically not different from the mock-vaccinated control ($P=0.65$). The total *C. pneumoniae* lung load protection score was −10%, also not different from the mock-vaccinated control ($P=0.89$). This indicates that the protective effect is mediated by specific antigens rather than by genetic immunization alone.

3.3. Comparative analysis of 6 individual C. pneumoniae vaccine candidates reported in the literature

The strongest *C. pneumoniae* protective antigens reported in the literature are *copN*, *npt1*, enolase, Momp, *gatA*, and *hsp60*. The genes encoding these candidates were cloned, manipulated into pCMiUB and used to immunize mice as described above. To comparatively test their efficacy to those genes identified in our *C. pneumoniae* genomic screen, a challenge experiment was conducted as described above. Survival data shown in Table 3 indicate that mice immunized with genes *Npt1*, Momp and *gatA* had a survival rate of 100%, significantly higher than the mock-vaccinated mice ($P<0.05$, Fisher Exact test). In groups immunized with the remaining constructs, one or more animals died, and the survival in these groups was not significantly different from mock-vaccinated mice. Thus, genes *npt1*, Momp and *gatA* mediated significant protection from *C. pneumoniae*-induced death.

The efficacy of these 6 candidates in reducing *C. pneumoniae*-induced lung disease was also evaluated by analyzing day-10 lung weight increases of surviving challenged mice. Data shown in Fig. 3A indicate that none of the previously identified genes mediated significant protection from lung disease. These data also conform to the ranking results of the Round 1 screen of all *C. pneumoniae* genes. These ranks were: *copN* = 77, *npt1* = 1100, *ompA* (Momp) = 633, *gatA* = 953, enolase = (Cpn0800) = 1230, *hsp60* (Cpn0134) = 572 and 784 (two fragments). With the exception of *copN*, all genes are ranked low in the list.

Finally, efficacy of the final vaccine candidates in enhancing elimination of *C. pneumoniae* as compared to mock-vaccinated mice was evaluated. Data shown in Fig. 3B indicate that none of the previously identified genes mediated significantly enhanced elimination of *C. pneumoniae*.

In summary, vaccine candidates *cutE* and Cpn0420, identified in the *C. pneumoniae* genome-wide vaccine screen, were individually

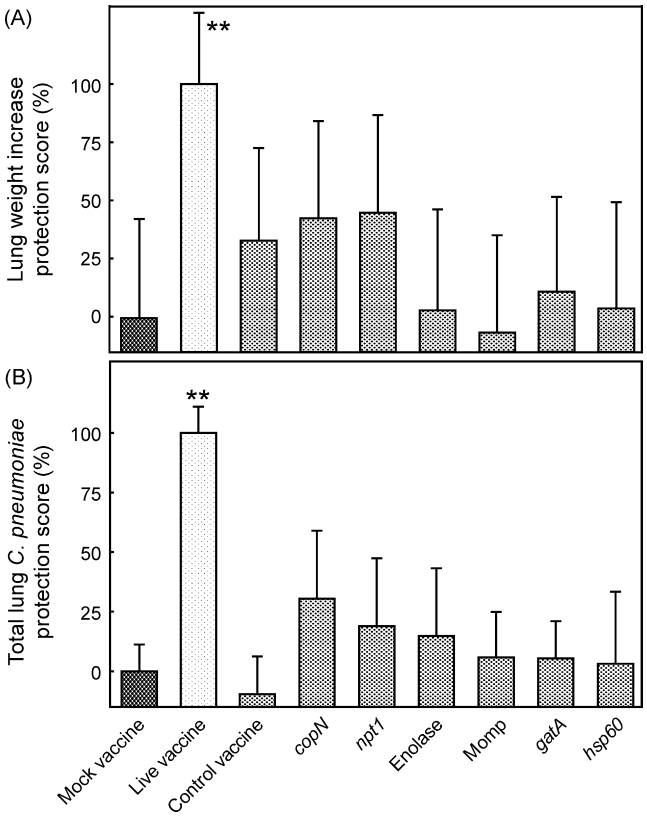

Fig. 3. Vaccine candidates reported as protective do not confer protection against *C. pneumoniae* disease. Genes previously reported as protective [8–12,17] against *C. pneumoniae* or *C. abortus* challenge were tested for direct comparison to the vaccine candidates found in this study. (A) Lung weight increase data were converted into protection scores as described and analyzed by one-way ANOVA. (B) Reported protective genes were compared to the vaccine candidates found in this study. The logarithm of the total *C. pneumoniae* lung load was converted into protection scores as described. One-way ANOVA results are shown as means ± 95% confidence intervals (**$P<0.01$; Dunnett's test).

protective by all criteria (survival, disease reduction, and *C. pneumoniae* elimination). Gene *oppA_2* was protective by dual criteria (disease reduction and *C. pneumoniae* elimination). Genes protective by a single readout were (i) *ssb* by disease reduction, (ii) *ide* and Cpn0095 by *C. pneumoniae* elimination, and (iii) Cpn0020 and previously reported *npt1*, Momp and *gatA* by survival. Given the overall robust protection mediated by genes *cutE*, Cpn0420, and *oppA_2*, their combined use in a recombinant vaccine may mediate broad based protection similar to a natural infection or live vaccine.

4. Discussion

This study is an extension of a previous study in which we were unable to confirm the protective effect of *C. pneumoniae* vaccine candidate genes identified by expression library immunization. This result was presumably due to a Th2 immune shift caused by biolistic genetic immunization of individual instead of complex mixtures of genes [14]. In this project, we first evaluated the promotion of Th1-biased immunity by use of a detoxified version of the *E. coli* heat-labile enterotoxin as genetic immunization adjuvant. The results showed a strong Th1-promoting effect of the LT genetic adjuvant, and we subsequently proceeded to re-test the best *C. pneumoniae* vaccine candidate genes. We had assayed all 1263 putative ORFs of the *C. pneumoniae* genome by ELI for vaccine candidates before [14], and have now confirmed several ORFs that effectively protect mice against *C. pneumoniae* lung infection. Most significantly, vaccination with *C. pneumoniae* genes *cutE* and

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Cpn0420 significantly protected mice against *C. pneumoniae* mediated death, reduced lung disease, and increased elimination of the *C. pneumoniae* organisms in the mouse lungs. Four other genes, *ide*, *oppA*, *ssb*, and Cpn0095, also mediated considerable protection against one or more readouts of disease but not all three. While not top candidates, these genes may be more protective in other host backgrounds, or be useful in combination with other antigens.

Of the six candidate genes selected from the literature that have been reported to confer protection against chlamydial infections, only *copN*, *Momp* and *gatA* protected mice from *C. pneumoniae*-induced death; none ameliorated lung disease, or facilitated clearance of the pathogens.

Reactivity to some genes tested in this study, such as *fabD*, *glgX*, *atoC*, *rl1*, and *hsp60*, resulted in deaths of several mice between 4 and 9 days after the challenge infection. This is typically the result of shock precipitated by an uncontrolled release of cytokines ("cytokine storm") during a strongly polarized Th1 immune response [23]. This immune response elicited by these genes in surviving mice, however, did not result in later protection from disease or efficient elimination of *C. pneumoniae*. Thus, use of these genes in a vaccine is not advisable.

Conversely, vaccination with genes Cpn0020, *npt1*, *copN*, Momp, and *gatA* provided complete protection from death; however, they did not reduce subsequent disease or *C. pneumoniae* lung loads. This suggests that these genes did elicit a limited Th2 response that was protective early in infection, but this response was not sufficient to clear the chlamydiae or the disease that resulted from the continuous chlamydial presence. Thus, these genes are also not preferable as vaccine candidates.

The protective antigens, used individually or preferably in combination, must be further evaluated in vaccine formulations that are appropriate for administration to humans. Two main issues apply: (i) do the *C. pneumoniae* vaccine candidates identified in the mouse have functionally the same role during human infection, and thus can they serve similarly in humans as highly visible target to an effective immune response and (ii) are the vaccine candidates similarly presented by different MHC-II molecules? While no data are available for humans, we have demonstrated that protective *C. abortus* genes identified by ELI in a mouse model are also protective in cows [17]. This suggests a high probability that the *C. pneumoniae* vaccine candidates identified here will function similarly in humans. Presentation of the vaccine candidate proteins by different MHC-II molecules can be tested in inbred mouse lines. Our unpublished data show that the *C. abortus* vaccine candidates identified in BALB/c mice (haplotype d) are equally protective in A/J (haplotype a) and C57BL/6J (haplotype b) mice. Furthermore, the protection mediated in outbred cattle also suggests that they function equally on different MHC-II backgrounds. Nevertheless, it may be a good strategy to include several full-length vaccine candidates in a human vaccine to maximize the probability that any of their peptides will be presented on any possible MHC-II molecule of an outbred vaccinee population, as suggested by Igietseme et al. [24]. For example, Ifere et al. [25] have shown that a vaccine composed of MOMP and PorB (porin B) induced a higher Th1 response than single subunit vaccines. It is likely that a combination of the candidate genes identified in this study may provide pronounced protection that is close or equal to the level of protection mediated by prior natural infection.

It is very important to identify suitable adjuvants, since they can selectively induce appropriate immune responses and improve protective efficacy by facilitating specific presentation of the antigens to macrophages or dendritic cells, or facilitate consistent release of the antigens [26]. Aluminum salts have been shown to be effective, but are not preferred for this vaccine because they induce a Th2-biased humoral immune response. Liposomes and MF59, a squalene-based sub-micron emulsion, have also been tested [27]. Arrington et al. [15] used cholera toxin (CT) and the *E. coli* heat-labile enterotoxin (LT) as genetic adjuvants, and both elicited a strong Th1-biased immune response, typically effective against intracellular pathogens. Our previous tests also have confirmed that the LT adjuvant boosts Th1 immunity as evidenced by elevated mouse IgG2a/IgG1 ratios, but also increases overall levels of antibodies (data not shown).

In design of efficacious vaccines against intracellular *Chlamydia* pathogens, an efficient delivery system is critical for mediation of a long-term protective immunity [28]. Some researchers have used bacteria or bacterial antigens as delivery vehicles and achieved considerable success. As an example, He et al. [29] used a live attenuated recombinant influenza A/PR8/34 virus as a vaccine vector for intranasal delivery of a subunit vaccine (a chlamydial epitope) against *Chlamydia trachomatis* infection, and a strong Th1 response against chlamydial EBs was detected. Additionally, *C. trachomatis* shedding was decreased and long-term protective immunity correlated with the preservation of specific Th1 cells and elevated IgG2a in genital secretions.

Another potential avenue for delivery of a *C. pneumoniae* vaccine is protein and gene vaccine formulations. These subunit vaccines are safer alternatives to killed or live attenuated whole organism vaccines, and genetic formulations are more stable and amenable to multi-component inocula. Unfortunately, they have been less effective in humans and livestock than in mouse models [26]. However, in the genetic vaccines, more recent use of electroporation and gene gun delivery protocols have yielded much better results than needle injections. Nevertheless, work will continue to identify optimal strategies for protein and gene vaccine delivery, adjuvantation, and antigen presentation.

In conclusion, this study has identified the best suited subunit vaccine candidate antigens among all annotated *C. pneumoniae* proteins. These antigens will be the basis for further formulation of an experimental vaccine in a commercially viable format. This vaccine will then be tested in animal models of lung disease as well as *C. pneumoniae*-enhanced insulin resistance [5], and ultimately may enter human trials.

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