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Vaccine 24 (2006) 2917–2927
Vaccine
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Mouse model of respiratory *Chlamydia pneumoniae* infection for a genomic screen of subunit vaccine candidates

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Received 28 July 2005; received in revised form 11 December 2005; accepted 14 December 2005
Available online 4 January 2006

Abstract

An inbred A/J mouse respiratory challenge model was validated for vaccine testing against *Chlamydia* (*C.*) *pneumoniae* and used to screen the *C. pneumoniae* genome for vaccine candidates by expression library immunization (ELI). Biolistic delivery of genetic vaccine constructs elicited Th2-like immunity that was associated with inefficient elimination of *C. pneumoniae*. Delivery by injection elicited protective Th1-like responses. Since biolistic delivery of pools of ORFs was used in first round screening, the screen presumably selected against potent immunogens. Nevertheless, it was sufficiently accurate to identify three weakly protective antigens among all putative *C. pneumoniae* ORFs. The results suggest ELI discovery of highly protective *C. pneumoniae* vaccine candidates requires tight control of the Th1 immunity elicited by the genetically delivered library of test antigens.
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Keywords: *Chlamydia pneumoniae*; Expression library immunization; Vaccine candidates; Murine respiratory disease model

1. Introduction

Chlamydia (*C.*) *pneumoniae* is a major agent of community-acquired respiratory infection and pneumonia. In addition, *C. pneumoniae* is strongly associated with atherosclerotic coronary heart disease in developed countries, and is thought to be involved in the pathogenesis of asthma [1]. These public health concerns indicate a requirement for control of such infections.

Antibiotics can be successfully used for the treatment of acute pulmonary infection caused by *C. pneumoniae* [2]. However, once infection and pathology are established, antibiotic treatment has little effect on the outcome of chlamydial diseases [3]. For instance, in large-scale field trials, antibiotic treatment did not influence atherosclerosis that had been associated with increased antibody levels against *C. pneumoniae* and the presence of the agent in lesions [4].

Initial attempts to develop effective vaccines against *C. trachomatis* infections and diseases were made in the 1950s and 1960s [5]. Such inactivated vaccines were only partially protective, and some even increased disease in a subpopulation of vaccinees [6]. These results discouraged further vaccination attempts in humans, and prompted searches for vaccines in animal models [7,8]. While heat-inactivated whole *Chlamydia* organisms induced expression of co-stimulatory molecules, they failed to induce full differentiation of monocytes to antigen presenting cells, and thus failed to induce full immunity [9]. Attenuated live vaccines have been developed for animals, but were also inefficient in inducing protection [10]. Vaccination by transfer of dendritic cells pulsed with

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doi:10.1016/j.vaccine.2005.12.035

inactivated *C. trachomatis* induced sustained IL-12 production, and directed a protective Th1 cell-dominated response [11]. However, this approach is presently not practical for clinical use, and dendritic cells pulsed ex vivo with chlamydial major outer membrane protein (MOMP, a major vaccine candidate antigen, induced strong, but non-protective Th2 immunity [12]. Thus, attempts to date have not yielded a safe, efficacious, and clinically usable chlamydial vaccine, and there is a need to develop such a vaccine for immunization against *C. pneumoniae*.

As an alternative to these whole pathogen vaccines, recent trends in vaccine development have turned to component or subunit vaccine compositions. These are far safer and more consistently manufactured but have often shown reduced efficacy relative to live or inactivated pathogen vaccines. This has been attributed to reduced complexity and inefficient adjuvants; however, another consideration is that the best antigens are rarely if ever established for a vaccine. A solution to this antigen discovery problem is expression library immunization. ELI is a recombinant DNA pooling strategy that enables to assay the full repertoire of genome-encoded components of a pathogen for protective antigens using genetic immunization (GI).

Since the original demonstration of ELI by intramuscular injection of genetic vaccine constructs for protection against *Mycoplasma pulmonis* pneumonia in mice [13,14], a number of methods have been used to deliver genes into a host to raise immune responses against the encoded product. The most commonly used ones have been injection into intramuscular (IM) or intradermal (ID) sites and DNA-coated particle delivery into skin epidermis with a gene gun [13]. In an ELI screen, the whole genome of a pathogen is reconstructed as gene fragments [14]. This library of fragments is manipulated into mammalian expression constructs, partitioned into sublibrary pools, and then used as inocula for test animals. Following pathogen exposure, vaccine utility is evaluated by the single criterion of disease protection [15]. Another technology has been developed to speed construction and improve the quality of expression libraries. Linear expression elements (LEEs) are recombinant-DNA constructs that are built wholly in vitro. Namely, there is no amplification or propagation step that uses a live system such as bacterial cloning. LEEs are built by generating an open reading frame (ORF) by PCR, gene assembly, or some other in vitro DNA construction method, and then covalently or non-covalently attaching gene control elements such as a promoter and terminator [16,17]. The desired recombinant expression vector is constructed completely in vitro and ready to deliver directly in vivo.

The complete 1230 kb genome sequence of the CDC/CWL-029 strain of *C. pneumoniae* has been published by Kalman et al. [18] Using bioinformatics approaches, this knowledge allows identification of all putative ORFs for production of LEE vaccine constructs. Thus, all ORFs can be screened for protective candidate antigens for use in a vaccine against *C. pneumoniae*. The present report describes the validation of a mouse model for the screening of *C. pneumoniae* vaccine candidates, and its use to test a library of LEEs by ELI for subunit vaccine candidates. We have established that the animal model and screening approach can be effectively used to identify immunogens. However, discovery of highly protective immunogens will require tight control of immune reactivities stimulated by LEE immunization.

2. Materials and methods

2.1. Chlamydia pneumoniae

C. pneumoniae strain CDC/CWL-029 (ATCC VR-1310) was grown, purified and quantified as described [19]. Briefly, Buffalo Green Monkey Kidney cells (Diagnostic Hybrids, Inc. Athens, OH) were used as host cells for propagation of chlamydiae. For purification, elementary bodies (EB) in supernatant culture medium were concentrated by sedimentation, followed by low-speed centrifugation for removal of host cell nuclei, and by step-gradient centrifugation of the supernatant in a 30% RenoCal-76 - 50% sucrose step-gradient. Sediments of purified infectious EBs were suspended in sucrose–phosphate–glutamate (SPG) buffer and stored at −80 °C.

2.2. Animals

Inbred A/J and C57BL/6 female mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) at 5 weeks of age. Udel "shoebox" type cages with spun fiber filter top were maintained in static air or ventilated cage racks. Five animals were housed per cage in a temperature-controlled room with a 12-h light/dark cycle, with ad libitum access to water and one of two diets. Mice were fed a 19% protein/1.33% L-arginine standard rodent maintenance diet. Beginning 2 weeks before challenge infection and during challenge infection, mice were fed a custom 24% protein/1.8% L-arginine diet (Harlan Teklad, Madison, WI). All components except protein/L-arginine were similar to the standard rodent maintenance diet. The custom diet was used because it was associated in preliminary experiments with enhanced immune responses and lower variance than the standard diet composed of non-chemically defined nutrient components. All animal protocols followed NIH guidelines and were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

3. Genetic immunization

3.1. ELI screen of the complete C. pneumoniae genome

The genome sequence of *C. pneumoniae* isolate CDC/CWL-029 (ATCC strain VR-1310) was extracted from

Genbank (AE001363, 1,230,230 bp). The 1052 annotated genes of *C. pneumoniae* were imported into a gene-splitting and primer prediction program; primer pairs to amplify 1263 ORFs of 1.5 kb or less were exported. A 1.5 kb maximum ORF length was chosen to ensure sufficient PCR quality and yields, and this generated a few additional fragments. The sequence-specific primers each carried at the 5′-end a common 15 base stretch in which deoxy-uracil bases were interspersed every third position. This design rendered the 5′-ends of all PCR products susceptible to uracil–DNA–glycosylase (UDG) cleavage. Genomic DNA was isolated from purified *C. pneumoniae* stock as described [17] and used as a template. All products were PCR-amplified from *C. pneumoniae* genomic DNA.

First-pass PCR conditions were: 20 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min followed by 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and lastly 72 °C for 7 min. This amplified all but 364 ORFs. Failed PCR reactions were repeated at different annealing temperatures, and all but 38 were amplified. New primers were synthesized, but not re-designed, and all but 16 ORFs were amplified. These ORFs were amplified with new, re-designed primers. ORF PCR products were purified by gel-filtration with Sephadex G-50. The purified PCR products were vacuum-concentrated in a Speedvac centrifuge as needed to keep the volume below 200 μl. The pooled PCR products were phenol:chlororform extracted, chloroform extracted and ethanol precipitated.

The products were arrayed into microtiter wells for pooling. The ORFs were combined into 90 pools of ∼42 ORFs. Each ORF was a member of three unique pools, and the complete genomic set of ORFs is represented in three different sets of 30 pools. This pooling strategy can be conceptualized as a 3-dimensional grid. The purpose is to enable multiplex analyses of the subsequent ELI results and thereby facilitate the selection power of the screen.

To enable non-covalent linkage of expression elements, the ORF pools were exposed to UDG. These samples were combined with three expression elements also produced by PCR: the CMV promoter linked to a ubiquitin sequence, the CMV promoter linked to a secretory leader sequence, and a terminator sequence. These were also designed with UDG sensitive ends and prepared for ORF linkage by enzymatically exposing 3′ single stranded ends complementary to the ORFs, as described [15,18].

A/J mice were used in all vaccine screening experiments. For Helios gene gun immunization (Bio-Rad Laboratories, Hercules, CA), mice received an isoflurane inhalation anesthesia, and were immunized on the outside of each ear. Three gene gun immunizations were performed in 1-month intervals with five mice per vaccine pool. The individual vaccine dose per ORF LEE was ∼50 ng DNA/mouse (1/42 dose), resulting in a total DNA dose of ∼2 μg DNA/mouse per pool, split into two immunization doses per mouse.

3.2. *C. pneumoniae* vaccine candidate screens

3.2.1. Experiment 1

After the total *C. pneumoniae* genome screen, the 46 highest scoring inferred candidates were tested individually. Subsequent steps were identical to those described above. The inocula per gene gun-dose were comprised of 200 ng of the candidate ORF and 800 ng of pUC118 filler DNA. Each mouse received two doses and each group had 10 mice. All other gene gun vaccination parameters were identical to the previous experiment.

3.2.2. Experiment 2

After the initial vaccine candidate screen in Experiment 1, seven ORFs with a protection score above 50% were further tested. Each ORF was synthetically built as a codon-optimized version from overlapping 60-mer oligonucleotides [20]. Large ORFs were synthesized as fragments of less than 500 bp, that overlapped one another by 50% of their length. These ORFs were constructed for expression as LEEs and as plasmids (pCMVi-UB) [13]. The plasmid inserts of full length mammalianized ORFs were verified by DNA sequencing. Each LEE construct was delivered at 200 ng ORF and 800 ng filler DNA per dose. Each plasmid construct was delivered at 5 ng ORF and 1 μg filler DNA per shot. Mice received two gene doses per immunization, with 10 mice per group.

3.2.3. Experiment 3

Another test of the seven ORFs from Experiment 2 was performed using the pCMVi-UB constructs in a combined intramuscular and intradermal injection protocol [21,22]. Ten mice per ORF plasmid were anesthesized by intraperitoneal injection of ketamine/xylazine. Each quadriceps and tibialis anterior muscle received an injection of 12.5 μg plasmid DNA in 50 μl PBS. Ten micrograms plasmid DNA in 20 μl PBS was intradermally injected in four doses in the abdominal skin. This vaccination was repeated once after 2 weeks.

3.3. Negative and positive controls

In all experiments, unvaccinated (naïve) but challenged animals served as negative protection controls, and mice immunized with 5×10^6 genomes of viable *C. pneumoniae* 1 month prior to the vaccine challenge served as positive protection controls (immune). Groups were scored for protection 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 logarithm of total lung *C. pneumoniae* load that was calibrated by control immune (100% protection) and naïve (0% protection) groups. A CMVi-UB LEE construct encoding the luciferase gene (LUC) served as a control for LEE-based immunizations, and plasmid construct pCMVi-UB carrying the same LUC insert was used as the control for plasmid-based immunizations.

3.4. C. pneumoniae lung challenge infection

Mouse intranasal inoculation was performed as described [23], and optimal doses for live-immunization and challenge inocula were determined in preliminary experiments (data not shown). For intranasal inoculation, mice received a light isoflurane inhalation anesthesia. Vaccine protection control mice were inoculated with a low dose of 5×10^6 *C. pneumoniae* elementary bodies in 30 μl SPG buffer. High-dose challenge infection was performed 4 weeks after the last gene gun genetic vaccination or low dose inoculation of live *C. pneumoniae*, and 6 weeks after the last intramuscular-intradermal genetic vaccination, by intranasal inoculation of 1×10^8 *C. pneumoniae* elementary bodies in 30 μl SPG buffer. Mice were sacrificed by CO_2 inhalation 2 h, 3 days, 10 days, or 15 days after inoculation, and lungs and spleen were weighed, snap frozen in liquid nitrogen, and stored at −80 °C until further processing. In all screening experiments, mice were sacrificed 10 days after inoculation. From selected animals, terminal blood was collected in heparinized microcentrifuge tubes by axillary incision under isoflurane anesthesia. Plasma was obtained by centrifugation at $5000 \times g$ for 20 min in a microcentrifuge. Percent lung weight increase was based on naïve lung weights of 138.4 mg for adult A/J mice and 133 mg for adult C57BL/6 mice.

3.5. 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% (wt/vol) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) [24,25] and for mRNA extraction using oligo $(dT)_{20}$ silica beads.

For mRNA extraction, a suspension of oligo $(dT)_{20}$-coated silica beads (25 mg/ml in dH_2O; 1 μm particle size, Kisker GbR, Steinfurt, Germany) was used. First, 100 μl of 10% lung suspension was mixed with 10 μl oligo $(dT)_{20}$ silica bead suspension diluted in 230 μl dilution buffer (0.1 M Tris–HCl, pH 7.5, 0.2 M LiCl, 20 mM EDTA). For mRNA binding, samples were incubated at 72 °C for 3 min followed by room temperature for 10 min. The silica beads were sedimented by centrifugation at $13{,}000 \times g$ for 2 min, supernatants removed by decanting, the beads resuspended in 100 μl DNase buffer (20 mM Tris–HCl, pH 7.0, 1 M NaCl, 10 mM $MnCl_2$) containing 100 U of RNase-free bovine pancreatic DNase I (Roche Applied Science, Indianapolis, IN) and incubated for 15 min at room temperature. Subsequently, beads were washed three times with wash buffer (10 mM Tris–HCl, pH 7.5, 0.2 M LiCl, 1 mM EDTA) by vigorous vortexing for 2 min followed by sedimentation at $13{,}000 \times g$, and mRNA was eluted by resuspension of the beads in 200 μl DEPC-treated ddH_2O followed by incubation at 72 °C for 7 min, centrifugal sedimentation, and removal of the supernatant mRNA. The purified nucleic acids samples were stored at −80 °C until used for real-time PCR assays.

3.6. Analysis of lung nucleic acids by real-time PCR

The primers and probes used in all PCR assays were custom synthesized by Operon, Alameda, CA, and the sequences are listed in Table 1. The copy number of *C. pneumoniae* genomes per lung was determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy

Table 1
Oligonucleotide primers and probes used in real-time PCRs

Gene name length exon	Primers probes[a]	Sequence (5′ → 3′)[a]
Chlamydia	UP	GGGGTTGTAGGGTYGAGRAIAWRRGATC
23S	DN	GAGAGTGGTCTCCCCAGATTCARACTA
rRNA	BOD	BOD-CCTGAGTAGRRCTAGACACGTGAAAC-P
168 bp	FLU	ACGAAARAACAARAGACKCTAWTCGAT-6-FAM
Tim3	UP	CTGAAATTAGACATCAAAGCAG CCAAGGT
200 bp	DN	GTTCTGATCGTTTCTCCAGAGTCCTTAATTTC
exon	BOD	BOD-GGAACAAAAATTTCCACATGGGCTGA-P
2–5	FLU	ACAGACACTGGTGACCCTCCATAATAACAA-6-FAM
GATA3	UP	AAGCCCAAGCGAAGGCTGTCG
171 bp	DN	TTTCTTCATAGTCAGGGGTCTGTTAATATTATGAAG
exon	BOD	BOD-GCAATGCCTGTGGGCTGTACTACAAG-P
3–5	FLU	GAACGCTAATGGGGACCCGGTC-6-FAM
CD45RO	UP	CACAGAAGTCTTTGTCACAGGGCAAACA
157 bp	DN	GTTCCCAAACATGGCAGCACATGTT
exon	BOD	BOD-GGCTGGCACCATCACTGGGTGTA-P
2–9	FLU	GCCCAGAGTGGATGGTGTAAGAGTTGTG-6 -FAM

I: deoxy inosine; K = G/T; R = A/G; W = A/T; Y = C/T.

[a] BOD, amine-reactive Bodipy 630/650 fluorophore attached to 5′ terminus; 6-FAM, 6-carboxyfluorescein attached to 3′-O-ribose; P, phosphate group attached to the 3′ terminus

transfer) qPCR [24]. One-step duplex RT-qPCR for analysis of lung transcript concentrations was performed in a Lightcycler as described [25]. RT reaction and PCR amplification for the analyte transcripts (Table 1) and an internal reference housekeeping gene transcript (porphobilinogen deaminase, PBGD) were performed in the same tube. All analyte transcript concentrations are expressed as copies per 1000 PBGD reference transcripts. Tim 3 is a CD4 Th1 cell-specific surface protein [26] (GenBank #AF450241), GATA-3 is a CD4 Th2 cell-specific GATA sequence transcription factor [27–29] (GenBank #X55123), CD45RO is a memory T cell surface protein [30,31] (GenBank #NM_0112100).

3.7. ELISA determination of anti-C. pneumoniae IgG1 and IgG2a antibodies

Plasma samples collected on day-10 pi were tested as 1:100 dilutions by enzyme-linked immunoabsorbent assay (ELISA) for IgG1 and IgG2a antibodies against an antigen of lysed *C. pneumoniae* elementary bodies, using a modification of a previously reported chemiluminescent assay [32] with 1:6000 diluted biotinylated secondary goat antibody against mouse IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL). The secondary antibody was detected by incubation with avidin–biotin complexed alkaline phosphatase (Vector Laboratories, Burlingame, CA) followed by chemiluminescent alkaline phosphatase substrate (Roche Applied Science, Indianapolis, IN). Emitted light was measured in a SpectraFluor Plus reader with luminescence setting, maximum dynamic-range 500 ms integration and optimal gain (Tecan, Durham, NC). Results were reported as relative light units per second (rlu/s). The background signal of a positive control plasma in a well without *C. pneumoniae* antigen (80–350 rlu/s in all assays) was subtracted from the data, and results were normalized between assay plates by use of a calibration positive control plasma.

3.8. Data analysis

All analyses were performed with the Statistica 7.0 software package (StatSoft, Tulsa, OK). Data of *C. pneumoniae* genome copies, RT-PCR gene transcripts, and anti-*Chlamydophila* IgG1 and IgG2a antibody relative light unit values 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). Comparisons of means were performed under the assumption of no a priori hypothesis by the Tukey honest significant difference (HSD) test.

3.8.1. ELI screen of the C. pneumoniae genome

The numbers of *C. pneumoniae* genomes per lung were logarithmically transformed, and the means of all immunization pools determined. The protective capacity of each pool of ∼42 ORF immunization constructs was determined as protection score in a linear equation in which the Log_{10} of the lung *C. pneumoniae* genomes of the low-dose immunized positive protection controls equaled 100% protection, and that of the naïve controls 0% protection. Groups that had higher *C. pneumoniae* lung loads than the naïve controls had negative protection scores. The protective potential of the *C. pneumoniae* ORFs was matrix analyzed in two ways: (1) by ranking in descending order the sum of the protection scores of the X, Y, and Z pools in which any one ORF was a member, and (2) by residency of an ORF in 3 protective groups (one each from the x, y, and z sets), which represents an intersection of planes X, Y, and Z. Using both analyses of inferred protection, 46 candidates were identified.

3.8.2. C. pneumoniae vaccine candidate experiments

In Experiment 1, 46 screen-inferred candidate ORFs were tested individually. They were re-amplified from genomic DNA, prepared as before in LEE constructs and then used to immunize mice at 200 ng LEE and 800 ng pUC118 DNA per shot. The challenge-protection experiment identified seven protective ORFs, displaying significantly higher protection scores than the control vaccine based on *C. pneumoniae* lung loads ($p < 0.05$, Tukey HSD). In Experiments 2 and 3, linear *C. pneumoniae* lung loads of the candidate immunized groups were used for comparison with the positive and negative vaccine control groups.

4. Results

4.1. A/J mice optimally identify immune protection on day 10 after challenge

Prior to conducting the vaccine screen, we evaluated several parameters of the murine model that we deemed critical for an optimal challenge-protection assay. Two mouse strains, A/J and C56BL/6, were evaluated. These were chosen because of their known differences in inflammatory responses and putatively divergent susceptibility to *C. pneumoniae* disease. To calibrate the range of achievable protection, groups of naïve and immunized mice were prepared by intranasal inoculation with 5×10^6 live *C. pneumoniae* EBs or were mock inoculated 4 weeks before the high dose challenge with 10^8 organisms [33]. Total lung load of *C. pneumoniae* and lung weight increase were used as readouts for protection (Fig. 1).

A 15-day time course of infection was analyzed in both mouse strains, either naïve or immune to *C. pneumoniae*. A/J mice had lower disease than C57BL/6 mice, expressed as percent increase over the average lung weight of unchallenged mice. Disease in immune mice peaked on day-3 post inoculation (pi), in naïve mice between days 10 and 15 pi (Fig. 1A and B). *C. pneumoniae* lung loads in naïve mice, determined by real-time PCR as genome copies per lung, tended to be lower in C57BL/6 mice than in A/J mice (Fig. 1C and D),

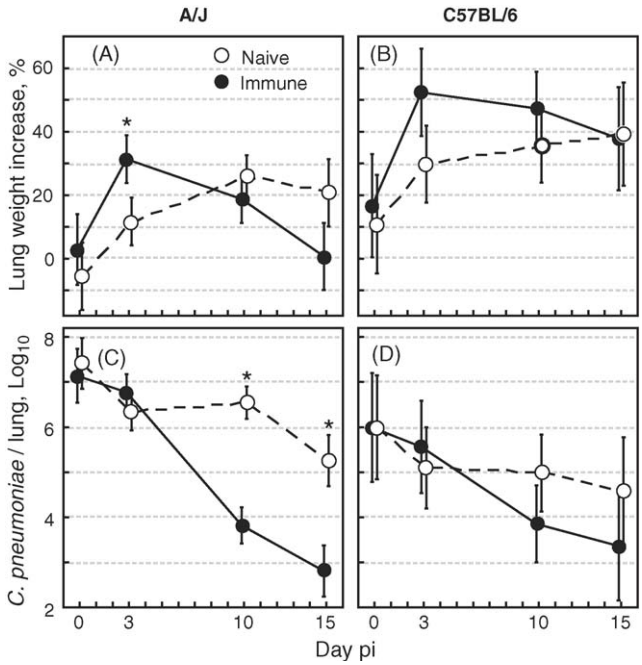

Fig. 1. Fifteen-day time course of *C. pneumoniae* lung infection in A/J and C57BL/6 mice. Six-week-old female mice received a pre-challenge mock inoculum (naïve) or a low-dose *C. pneumoniae* inoculum (5×10^6 EB; immune), were intranasally challenged 4 weeks later with 1×10^8 *C. pneumoniae*, and sacrificed 2 h (day 0), 3 days, 10 days, or 15 days after inoculation. Lung total nucleic acids were extracted, and *C. pneumoniae* genomes were determined by *C. pneumoniae* 23S rRNA FRET real-time PCR. Data are means ($n = 10$) ±95% confidence intervals. Asterisks indicate significant differences between groups ($p < 0.05$, Tukey HSD test). (A) Time course of lung weight increase in naïve and *C. pneumoniae*-immune A/J mice, and (B) in naïve and *C. pneumoniae*-immune C57BL/6 mice. (C) Time course of *C. pneumoniae* lung burdens in A/J mice and (D) in C57BL/6 mice. Lung weight increases of immune C57BL/6 mice on days 10 and 15 pi are significantly higher than of A/J mice ($p < 0.01$). Immune A/J mice on day 3 post inoculation (pi) have a higher lung weight increase than naïve A/J mice ($p = 0.008$). On days 10 and 15 pi, immune A/J mice, but not C57BL/6 mice, have highly significantly lower lung *C. pneumoniae* loads than naïve mice ($p < 0.001$).

but significantly lower only on day 10 ($p = 0.038$). On days 0 and 3 pi, lung loads of immune mice were not different from naïve mice. On days 10 and 15 pi, lung *C. pneumoniae* of immune A/J were approximately 300-fold reduced (2.5 log reduction) as compared to naïve A/J mice ($p < 0.001$), while lungs loads of immune and naïve C57BL/6 mice did not differ significantly (Fig. 1C and D). The strong elimination of *C. pneumoniae* by immune A/J mice unambiguously identifies A/J mice, but not C57BL/6 mice, as suitable for identification of *C. pneumoniae* vaccine candidates. The kinetics of elimination of *C. pneumoniae* in A/J mice indicate that day 10 pi is the optimum time point for identification of vaccine candidates that promote immune elimination of *C. pneumoniae* organisms. At later time points, the immune response induced by the challenge inoculation potentially interferes with pre-existing immunity against vaccine candidates, preventing the identification of protective *C. pneumoniae* antigens.

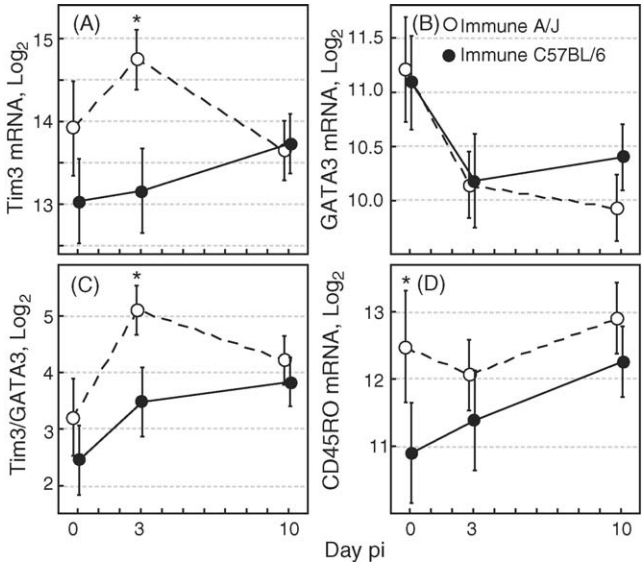

Fig. 2. Time course of lung transcripts of immune-associated genes in immune A/J and C57BL/6 mice after challenge inoculation. Mice immunized by a previous low-dose inoculation were challenged intranasally with 1×10^8 *C. pneumoniae*, and sacrificed 2 h (day 0), 3 days, or 10 days after inoculation ($n = 10$). Lung poly(A)$^+$ RNA was extracted, and transcripts were quantified by one-step duplex real-time RT-PCR. Data are expressed as numbers of the respective transcripts per 1000 transcripts of the PBGD reference gene (means ± 95% confidence intervals). Asterisks indicate significant differences between groups ($p < 0.05$, Tukey HSD test). (A) Time course of lung Tim3 transcripts (associated with Th1 immunity). (B) Lung GATA3 transcripts (associated with Th2 immunity). (C) The transcript ratio of Tim3:GATA3. (D) Lung CD45RO transcripts (memory T cell-associated). Immune A/J mice show higher early Tim3 transcripts and Th1 immune bias (Tim3/GATA3), and overall higher memory T cell CD45RO transcripts than C57BL/6 mice (combined CD45RO data; $p = 0.008$).

4.2. Immune A/J mice show enhanced Th1 immunity against C. pneumoniae

The levels of several key immune-related transcripts were evaluated as indicators of the type and intensity of the local lung tissue response to the *C. pneumoniae* challenge (Fig. 2). Tim3 transcripts, indicative of Th1 cells, peaked on day-3 pi in A/J mice and were significantly higher than in C57BL/6 mice ($p = 0.004$; Fig. 2A). Early GATA3 transcripts, indicative of Th2 cells [34,35], did not differ between the mouse strains (Fig. 2B). Hence, the ratio of Tim3/GATA3 on day 3 was significantly higher in A/J mice than in C57BL/6 mice ($p < 0.001$; Fig. 2C), which is consistent with a Th1-biased immune profile. CD45RO transcripts, indicating memory T cells, were higher in A/J mice ($p = 0.008$ for combined days 0, 3, and 10 data; Fig. 2D). These data show that pre-immunized A/J mice mount a stronger and more Th1 biased early immune response than C57BL/6 mice during challenge with *C. pneumoniae*. This confirms that A/J mice are appropriate for a respiratory challenge model for identification of *C. pneumoniae* vaccine candidates.

Day-10 pi plasma antibody responses against *C. pneumoniae* of naïve and immune A/J mice were determined by

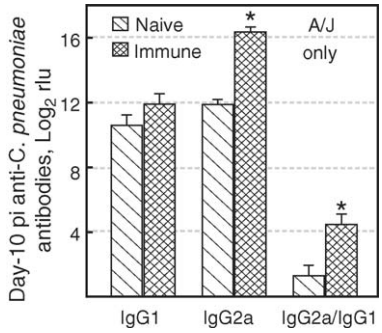

Fig. 3. Day-10 pi plasma levels of anti-*C. pneumoniae* antibody isotypes in A/J mice. Naïve A/J mice and A/J mice immunized by a previous low-dose inoculation were challenged intranasally with 1×10^8 *C. pneumoniae*, and plasma was obtained on day 10 pi. Mouse IgG1 and IgG2a antibodies binding to *C. pneumoniae* lysate antigen were determined by chemiluminescent ELISA. Data are expressed in relative light units (rlu) as means ($n = 20$) ±95% confidence intervals. Immune animals have highly significant higher IgG2a antibody levels and IgG2a:IgG1 ratio than naïve mice on day 10 after challenge ($p < 0.001$).

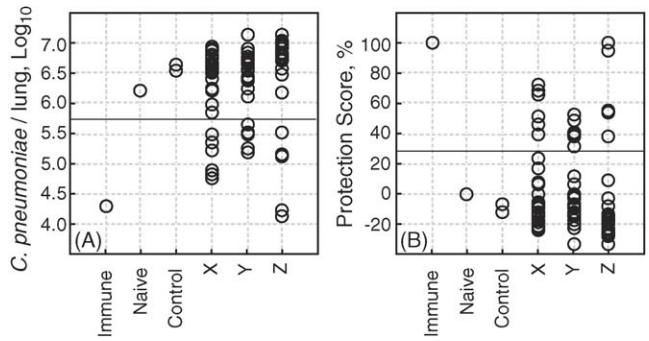

Fig. 4. ELI screen of the complete *C. pneumoniae* genome for ORFs with protective capacity in A/J mice. The LEE library of *C. pneumoniae* ORFs was arrayed into 90 pools (30 X-, 30 Y-, and 30 Z) of ~42 LEE constructs each that were used as inocula for 3 gene gun immunizations in 4-week intervals ($n = 5$ mice/pool). Each test inoculum contained 200ng of a mixture of ~42 ORFs and 800 ng of pUC118 carrier DNA. Four weeks after the last immunization, all mice were challenged by intranasal inoculation of 1×10^8 *C. pneumoniae* organisms and sacrificed 10 days later. Positive control, immune mice received a low-dose inoculum of *C. pneumoniae* 4 weeks prior to high dose challenge. Immune and naïve groups ($n = 20$) were used to calibrate the range of possible protection. Another set of negative control animals was immunized with a construct expressing an irrelevant (LUC) gene product ($n = 10$). (A) Group means of total *C. pneumoniae* lung loads (genomes) determined by real-time PCR. The area below the horizontal line corresponds to the area above the protection threshold line in panel B. (B) Protective capacity of all test groups. The protection scores are calibrated by a 100% protection score of the immune group and a 0% protection of the naïve group. The area above the horizontal line contains the vaccine pools that were used to select candidate protection ORFs. ORFs were ranked using the sum of protection scores of the ORF's respective XYZ pools three-way intersection approach of pools above the protection threshold. The combined approach selected 46 *C. pneumoniae* ORFs among which seven candidates were identified in vaccine candidate screen 1, but proved non-protective in screen 2.

ELISA (Fig. 3). Absolute levels and the ratio of IgG2a (Th1-associated) and IgG1 (Th2-associated) antibodies confirmed a highly significant Th1 shift of the immune response to *C. pneumoniae* in immune as compared to naïve A/J mice ($p < 0.001$).

4.3. *C. pneumoniae* genome screen for vaccine candidates

The full repertoire of 1263 ORFs was independently partitioned into 30 pools of approximately 42 *C. pneumoniae* ORFs each, three times as described in Section 3.1. Mice were immunized three times by gene gun at monthly intervals, and 1 month later challenged using the respiratory inoculation model as defined earlier. Protective ORFs were identified that were common constituents of a positively scored X, Y, and Z pool (Fig. 4). This represents the virtual equivalent of the intersections of all positively scored cubic planes. Each individual ORF was also assigned a genomic score by summing the relative protection scores corresponding to its three resident pools. These two analyses were used to select 46 *C. pneumoniae* ORFs for individual vaccine candidate screening.

4.4. Individual protection candidate ORF tests

The 46 library-derived ORFs selected as testing candidates were PCR-amplified and individually prepared as LEEs. A/J mice were immunized by gene gun delivery (200 ng/shot) and then subjected to *C. pneumoniae* challenge. ORFs below $10^{5.7}$ *C. pneumoniae* genomes per lung were significantly lower than the control vaccine ($p < 0.05$, Tukey HSD test; data not shown). This threshold specified 7 ORFs for further evaluation. The remaining 39 ORFs were designated as non-protective and not further tested.

The seven ORFs that had stimulated significant protection were prepared for further testing by redesigning them as codon-optimized sub-ORFs of 500–600 bp that overlap one another by 50% of their length. This approach was chosen because (i) codon-optimization of bacterial genes for mammalian expression increased product expression, and (ii) splitting genes into fragments enhanced or broadened antigenicity. These synthetically built sub-ORFs were prepared for expression as both LEE and plasmid constructs. They were linked to the same LEE elements used previously and cloned into plasmid vector pCMVi-UB [13]. The inocula were gene-gun delivered once into each mouse ear at doses of 200 ng LEE or 5 ng plasmid. After two boosts at monthly intervals, animals were challenged as described. The results indicated that the seven *C. pneumoniae* ORFs induced either no significant effect or reduced elimination of *C. pneumoniae* as compared to the negative control plasmid expressing an irrelevant LUC antigen gene (data not shown). Thus, the protective effect displayed by delivery of a set of natural sequence full length ORFs was not generated when the same ORFs were delivered as codon-optimized, overlapping sub-ORF fragments, either as LEEs or as plasmids. Instead increased chlamydial lung burdens were observed.

4.5. Gene gun genetic immunization with C. pneumoniae vaccine candidates reduces the early Th1 immune response against C. pneumoniae

Toward understanding why the seven ORF candidates, conferring protection as wild type ORFs, displayed non-protective responses as expression-optimized versions, we examined the Th1-Th2 balance of gene gun-vaccinated mice on day-3 and day-10 after challenge in comparison to positive (protected) and negative (naïve) control mice. We hypothesized that we may have increased immune responsiveness but in an unproductive direction. In particular, live *C. pneumoniae*-immunized, positive-control mice display a Th1-biased response. Therefore, we assumed that successful vaccination needed to pre-establish an antigen response that modifies the lung microenvironment so as to drive early Th1 immunity. Fig. 5A shows that gene gun immunization with the codon-optimized sub-ORFs displays reduced Th1 activity on day 3 (Th2 shift) as compared to the positive control (immune) mice ($p < 0.001$) and even the negative control mice (naïve and LUC-immunized) ($p \leq 0.034$). By a later time point (day 10), peak disease lungs in test and naïve groups showed Th1-driven inflammation as evidenced by the significantly increased ratio of Th1 associated cytokine expression relative to that of the immune controls ($p \leq 0.003$).

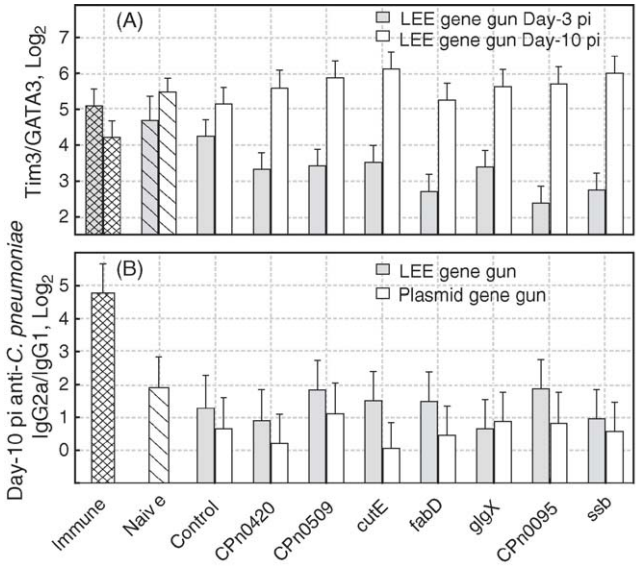

Fig. 5. Examination of the protection failure of *C. pneumoniae* vaccine candidates. LEE gene gun vaccinations with the seven individual *C. pneumoniae* vaccine candidate ORFs were performed with two groups of 10 A/J mice each, mice were sacrificed on day 3 or 10 after challenge, and Th1-(Tim3) and Th2-associated (GATA3) transcripts were determined in lung tissue. Anti-*C. pneumoniae* IgG2a (Th1-associated) and IgG1 antibodies (Th2-associated) in day-10 plasma from *C. pneumoniae* vaccine candidate ORF LEE- and plasmid-vaccinated mice were determined. (A) Tim3/GATA transcript ratios of days 3 and 10 are shown. Early infection (day-3) lungs of all *C. pneumoniae* ORF-vaccinated groups show a highly significant reduction in Th1/Th2 transcripts (Th2 shift) as compared to protected immune mice ($p < 0.001$). Conversely, peak disease (day-10) lungs of all vaccinated groups show a significant increase in the Th1/Th2 transcript ratio (Th1 shift) as compared to immune mice ($p \leq 0.003$). (B) Day-10 plasma of all gene gun vaccinated mice show a highly significant reduction in the IgG2a/IgG1 ratio (Th2 shift) as compared to the immune mice ($p < 0.001$).

The early Th1 downshift in the test groups is also evident in the plasma anti-*C. pneumoniae* IgG2a/IgG1 antibody isotype ratio. LEE- and plasmid-vaccinated mice showed a highly significantly reduced ratio as compared to live-immunized controls ($p < 0.001$; Fig. 5B). Thus, both transcript and antibody ratios indicate that gene gun vaccination with the *C. pneumoniae* vaccine candidate ORFs induced a Th2 dominance of the early immune response to *C. pneumoniae* respiratory challenge infection followed by a Th1 response.

4.6. Genetic immunization by intramuscular injection elicits protective Th1-biased immunity against C. pneumoniae

Previous analyses of genetic immunization have shown that gene gun delivery methods frequently elicit Th2 immunity, while IM or ID injections favor a Th1-biased response [36–38]. These methods also differ substantially in gene doses delivered. A gene gun shot delivers 1 μg of DNA or less whereas 25–200 μg of DNA is generally injected by needle. To determine whether any of the candidates was protective as high-dose injected inoculum, we vaccinated mice with the seven plasmids by a combined IM-ID immunization route. The results in Fig. 6 show that two of the genes delivered by this method generated significant levels of protection against infection. IM-ID vaccination with genes CPn0095, fabD, and the pool of all vaccine candidates, generated responses that resulted in moderately, but significantly reduced total *C. pneumoniae* lung loads as compared to control mice vaccinated with a plasmid expressing a non-*Chlamydia* ORF ($p \leq 0.019$).

To analyze a further aspect of protective immunity, reduction or prevention of systemic chlamydial dissemination, day-

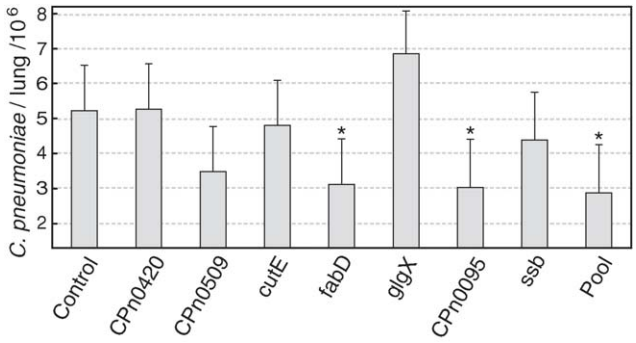

Fig. 6. Evaluation of seven *C. pneumoniae* vaccine candidate ORF plasmids by combined intramuscular-intradermal immunization. Mice received two intramuscular-intradermal (IM-ID) vaccinations in a 2-week interval, each with a total of 60 μg plasmid DNA of a *C. pneumoniae* vaccine candidate ORF (50 μg IM into 4 muscles + 10 μg ID into four abdominal skin depots), and were challenged 6 weeks later. Day-10 *C. pneumoniae* genomes per lung are means ($n = 10$) ±95% confidence intervals. Groups that differ significantly from the control vaccine are indicated by an asterisk ($p \leq 0.019$, Tukey HSD).

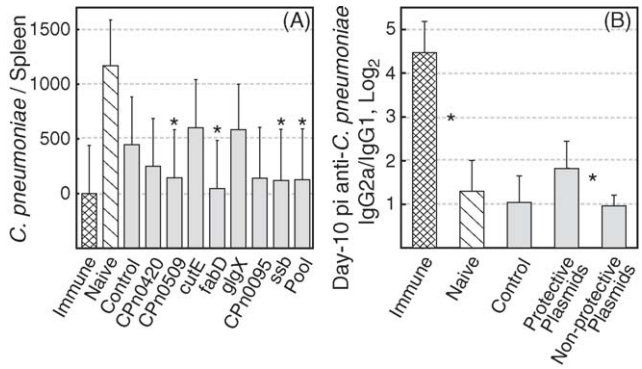

Fig. 7. Examination of protection by plasmid IM-ID genetic immunization with *C. pneumoniae* vaccine candidate ORFs. For further evaluation of protective effects, *C. pneumoniae* burdens in spleens and plasma anti- *C. pneumoniae* antibody levels of IM-ID plasmid-vaccinated and *C. pneumoniae*-challenged mice were examined. Data are means ($n = 10$) ±95% confidence intervals. Asterisks indicate significant differences in panel A between vaccine groups and the naïve control, and in panel B between groups on either side of the asterisk. (A) Vaccination with three *C. pneumoniae* ORFs and with the pool of vaccine candidates significantly reduces the *C. pneumoniae* spleen burden in challenged mice ($p \leq 0.048$, Tukey HSD). (B) Day-10 anti-*C. pneumoniae* IgG2a/IgG1 ratios are significantly different between immune and naïve mice ($p = 0.004$) and between the mice vaccinated with lung- and spleen-protective candidate ORFs (Cpn0095, fabD, Cpn0509, ssb) and non-protective ORFs ($p = 0.012$).

10 *C. pneumoniae* spleen loads were determined (Fig. 7A). Mice vaccinated with candidate genes ssb, fabD, CPn0509, and the pool of all seven candidates, as well as live-immunized controls, showed significant reduction of spleen chlamydial loads as compared to naïve, non-protected control mice ($p \leq 0.048$). Candidate gene CPn0095, which significantly reduced lung chlamydial loads, was borderline spleen-protective ($p = 0.054$). Thus, spleen chlamydial load data indicate that IM-ID vaccination-mediated protection reduces secondary organ infection after intranasal *C. pneumoniae* inoculation.

To examine if protection was associated with Th1 immunity, we analyzed IgG2a and IgG1 anti-*C. pneumoniae* antibody ratios in day-10 pi plasmas. As shown in Fig. 7B, the Ig2a/IgG1 isotype ratio of the combined groups vaccinated with lung- and/or spleen-protective candidates (Cpn0095, fabD, Cpn0509, ssb, pool) was significantly higher ($p = 0.012$) than that of the mice vaccinated with non-protective candidate ORFs (Cpn0420, cutE, glgX). Thus, analogous to the Th1 shift in protected live-immunized mice as compared to naïve mice ($p = 0.004$; Fig. 7B), *C. pneumoniae* immunity of mice injection-vaccinated with protective candidate ORFs showed a significant Th1 shift. Collectively, lung and spleen *C. pneumoniae* load data and anti-*C. pneumoniae* isotype ratios after IM-ID genetic immunization show that gene gun immunization with low doses of the ORFs elicits an early Th2-biased immune response that is not protective. Needle injection of mice with high doses of the same ORFs identified a set of partially protection antigens.

5. Discussion

In the present study, we first examined the critical parameters of host strain and time after *C. pneumoniae* inoculation. Conditions were identified that maximized the amplitude between chlamydial lung burden of naïve mice and of immune mice protected by a low-dose live *C. pneumoniae* inoculation. These preliminary studies demonstrated that the optimum protection readout time point was 10 days after challenge infection, and that A/J, but not C57BL/6 mice, were the host inbred mouse strain in the respiratory challenge model suitable for identification of protective *C. pneumoniae* protein antigens. The corollary of this finding is that an appropriate host genetic background will be essential for protective efficacy of any *C. pneumoniae* vaccine, presumably not only in inbred mouse strains, but also in an outbred human vaccinee population. Vaccine candidates identified using this animal model of disease will be chlamydial antigens that are presented to and recognized by the immune system in a manner that stimulates a productive host response. However, successful use of vaccine antigens in individuals that are genetically refractory to immune protection against *C. pneumoniae*, as are C57BL/6 mice, will require an understanding of the factors that normally prevent immune protection. This will enable immunity to be manipulated more productively.

We identified a total of four *C. pneumoniae* genes that mediate protection against infection as determined by the reduction of *C. pneumoniae* organisms in the lung and/or the spleen. Since all four mediate modest levels of protection, these genes are unlikely to merit further development as vaccine candidates. However, we anticipate that analysis of the reasons why this screen did not identify highly protective candidates will facilitate the design of future vaccine screens. In particular, our work confirms the importance of an early, robust Th1 response for protective immunity against chlamydial infection. This study also demonstrates the impact of vaccine delivery routes on the type of specific immunity raised by the host, and consequently on the outcome of chlamydial challenge infection.

Our immunization regiment elicited early stage Th2-favored immune responses, even in the pre-selected A/J mouse strain. We assume that this biased our screen against strongly immunogenic antigens. Nevertheless, the ELI protocol was sensitive enough to distinguish modestly protective antigens in a large population of non-protective ones. In the ELI screen, the full set of library components (ORFs) was delivered by gene gun in complex mixtures in which each ORF was represented at 4 ng per pool. When the natural sequence ORFs derived from the screen were retested individually at 200 ng per dose, 7 ORFs conferred significant protection. However, when these ORFs were manipulated to increase immune reactivity, protection was not generated. When the same candidates were delivered via an alternative route at much higher dosage by needle injection, several of the candidates conferred partial protection.

A consequence of the Th2 bias in the present screen is that any highly immunogenic gene would have caused even stronger Th1 down-regulation and even lower elimination of chlamydiae than the genes ranked for highest protective capacity in round 1. Thus, the best vaccine candidate antigens most likely were associated with the highest *C. pneumoniae* loads. In fact, a reverse evaluation of the round 1 results for pools with significantly increased *C. pneumoniae* loads revealed nine tightly clustered genes that were shared at the XYZ intersections of such high-load pools. Experiments are planned that will test the protective efficacy of these genes after IM-ID genetic immunization.

Directed ELI screening for identification of protective antigens of pathogens takes advantage of the knowledge of pathogen genomes. It involves production of thousands of ORFs and the assembly of these products into expression constructs. The breadth of the project makes methods that require minimal amounts of genetic material very attractive. Therefore, we used the most efficient genetic immunization method: gene gun delivery. The results of this study suggest that releasing the immune constraints imposed by the gene gun delivery might lead to the identification of highly protective components of chlamydiae. Thus, the next step will be to determine the feasibility of eliciting biolistically Th1 rather than Th2 biased responses biolistically. Sudowe et al. [39] have shown that transcriptional targeting of dendritic cells (DC) by gene gun immunzation leads to Th1 biased specific immunity against the genetic vaccine antigen. They used the DC-specific Fascin promoter instead of the ubiquitously active CMV promoter, and achieved selective expression of vaccine antigens in mature antigen presenting cells, eliciting potent IFN-γ production. Another approach to generate Th1 immunity by gene gun vaccination might be the co-administration of plasmids directing the expression of co-stimulatory or cytokine molecules that mediate development of an antigen-specific Th1 immune response [40,41], or by fusing the antigen to sequences that will drive a strong Th1 response. Such methods will need to be explored prior to future attempts at identification of chlamydial vaccine candidates by ELI.

Acknowledgments

The authors thank Dannialle Clayton and Yihang Li for excellent help and contributions to this project. This investigation was supported by NIH Public Health Service R01 Grant AI47202 to B.K. and K.S. and by an Auburn University Graduate Research Fellowship to T.K.

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