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Protective antigens against glanders identified by expression library immunization

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Burkholderia are highly evolved Gram-negative bacteria that primarily infect solipeds but are transmitted to humans by ingestion and cutaneous or aerosol exposures. Heightened concern over human infections of *Burkholderia mallei* and the very closely related species *B. pseudomallei* is due to the pathogens' proven effectiveness as bioweapons, and to the increased potential for natural opportunistic infections in the growing diabetic and immunocompromised populations. These *Burkholderia* species are nearly impervious to antibiotic treatments and no vaccine exists. In this study, the genome of the highly virulent *B. mallei* ATCC23344 strain was examined by expression library immunization for gene-encoded protective antigens. This protocol for genomic-scale functional screening was customized to accommodate the unusually large complexity of *Burkholderia*, and yielded 12 new putative vaccine candidates. Five of the candidates were individually tested as protein immunogens and three were found to confer significant partial protection against a lethal pulmonary infection in a murine model of disease. Determinations of peripheral blood cytokine and chemokine profiles following individual protein immunizations show that interleukin-2 (IL-2) and IL-4 are elicited by the three confirmed candidates, but unexpectedly interferon-γ and tumor necrosis factor-α are not. We suggest that these pathogen components, discovered using genetic immunization and confirmed in a conventional protein format, will be useful toward the development of a safe and effective glanders vaccine.

Keywords: *Burkholderia, B. mallei*, glanders vaccine, expression library immunization, functional genomics, genetic immunization, DNA vaccine, subunit vaccine

INTRODUCTION

Burkholderia mallei remains an endemic pathogen in many parts of the world including Africa, Asia, the Middle East, and South America (Elschner et al., 2011). This Gram-negative, facultative aerobic bacillus primarily infects solipeds, with horses considered the natural reservoir; however, human infections occur zoonotically. The course of disease depends on the route of infection. Direct contact with the skin leads to a localized cutaneous infection called farcy. Contact with nasal or oral mucosal surfaces or inhalation lead to a highly infectious, painful, and incapacitating disease called glanders (Nierman et al., 2004). Acute symptoms include coughing, fever, and the release of infectious nasal discharge. Nodular lesions form in the lungs and ulcers develop in the mucous membranes of the upper respiratory tract. Without aggressive antibiotic treatment disease progresses to septicemia and is nearly 100% fatal (Waag et al., 2005). Survivors can suffer chronic infections of the muscle liver and spleen for up to 20 years, and remain carriers (Whitlock et al., 2008). Even if quickly diagnosed, antibiotic treatments have shown low efficacy and require long regimens with multiple drugs. Moreover, no vaccine candidate has even progressed to a clinical trial (Kenny et al., 1999; Leelarasamee, 2004; Bondi and Goldberg, 2008). These therapeutic hurdles arise from a number of pathogen characteristics including its ability to reside in the vacuoles of eukaryotic cells. Other immune evading tactics, including high genomic variability (Nierman et al., 2004), have rendered the performance of even live

vaccines disappointing (Ulrich et al., 2005). In addition to being a naturally occurring disease, glanders has been used as a bioweapon against cavalry in the American Civil War, World War I, and World War II (Nierman et al., 2004). The severe nature of the disease and the lack of treatment options, combined with the potential for microbe aerosolization, have made *B. mallei* a member of the Center for Disease Control and Prevention category B Priority Agent list.

Immune correlates of protection for *B. mallei* are not well developed. Its capacity to replicate inside host cells suggests that cell-mediated responses are likely to be linked to protection. However, vaccine studies with heat-killed (HK) *B. mallei* strains, either encapsulated or not, elicited mixed T helper cell type 1/type 2 responses in mice that were only weakly protective (Amemiya et al., 2002; Whitlock et al., 2008). A live capsule-deficient *B. mallei* mutant was not protective; however, aerosol immunization with a live-attenuated auxotrophic mutant conferred 50% survival against aerosol challenge, although the mice were unable to clear the infection. This strain was shown to elicit a Th1-skewed antibody response (IgG2a > to IgG1; Ulrich et al., 2005). In a recent study, the type III secretion system protein BopA was shown to improve survival rate, but also failed to provide sterilizing immunity. It generated a strong Th1-skewed antibody response and T cells that produced γ-interferon (IFNγ; Whitlock et al., 2010).

Although not optimal, the protection conferred by the live *B. mallei* strain and BopA indicate that developing a *Burkholderia* vaccine is possible. The inherent advantage of a live-attenuated vaccine is that no antigen identification or development is required, and broad antigenic diversity is possible because all components are delivered. Molecular component vaccines are an alternative to the use of live or killed preparations. A component design enables immense control relative to whole-pathogen compositions for improving manufacture, product safety and consistency, and vaccine efficacy; however, antigen discovery is required. Discovery is a considerable challenge for *Burkholderia* species because of the unusual fluidity and large size of their genomes, the functional and antigenic diversity of isolates, and the exceptional complexities of the immune responses that infection can elicit (Holden et al., 2004; Nierman et al., 2004; U'Ren et al., 2007). These factors suggest that if a component design is pursued, multiple components will need to be discovered to confer robust protection. Toward this purpose, over 1500 complete bacterial genome sequences are currently published (Relman, 2011). However, the characteristics of a pathogen component that make it protective are poorly understood. Furthermore, a substantial portion of the open reading frames (ORFs) in microbial databases remain without known function (Cui et al., 2005), thereby restricting sequence-based analyses as a method of candidate discovery.

In addition to *in silico* analyses, the large microbial sequence databases can be used for the design and construction of genes and proteins for empirical testing as antigens. New molecular technologies have begun to provide solutions for making production and testing possible on genomic scales. For example, through genetic immunization (Tang et al., 1992) libraries of constructed genes expressing the full coding capacity of a pathogen genome can be delivered into an animal to elicit immune responses to the pathogen proteome in a process termed expression library immunization (ELI; Barry et al., 1995). These libraries are introduced into animals as organized sub-library pools from which individual ORFs are identified for further study based on their ability to stimulate immunity against pathogen challenge. To avoid thousands of time-consuming and bias-ridden cloning steps, we developed linear expression elements (LEEs) that can be rendered genetically active by simply attaching desired expression sequences such as a promoter and terminator (Sykes and Johnston, 1999b). This study demonstrates the utility of a modified ELI method for the discovery of *B. mallei* component vaccine candidates. BALB/c mice were immunized with LEE pools expressing the *B. mallei* ATCC23344 ORF-eome. To address the individual dose diluting effects of the highly mixed ORF pools, a two-dimensional pooling strategy with divergent complexities was applied. In addition, LEEs were gene gun administered using a modified DNA/gold-particle formulation that prevented DNA loss during bombardment. Challenge–protection assays identified 12 ORFs as potential vaccine candidates. Testing of 5 of these 12 candidates individually in a protein subunit format confirmed that three confer significant partial protection against pulmonary challenge and showed that they elicit a CD4-like T cell cytokine response.

MATERIALS AND METHODS

B. MALLEI CULTURING AND GENOMIC DNA ISOLATION

Burkholderia mallei strain ATCC 23344 (China 7) was cultured on Luria–Bertani (LB) agar plates supplemented with 4% glycerol agar plates for 48 h at 37°C. Isolated colonies were sub-cultured to LB broth with 4% glycerol, and incubated at 37°C until exponential growth phase was reached. To obtain HK inoculums, bacterial suspensions were incubated at 85°C for 3 h and stored at 4°C until use. The absence of live *B. mallei* organisms in the HK preparations was confirmed by plating 10% of the total inoculums (v/v) and incubating these at 37°C for 48 h. All procedures were performed under biosafety level-3 containment.

To isolate genomic DNA, bacilli were cultured at 37°C in LB broth for 18 h and then pelleted at 3000–5000 g for 5–10 min at room temperature. The cells were resuspended in 1 ml of buffer B1 (with RNase A; Qiagen, Valencia, CA, USA) to obtain efficient lysis of the bacteria, then 20 μl of lysozyme stock solution and 45 μl of Proteinase K solution were added and the samples were incubated at 37°C for at least 30 min. Buffer 2 (0.35 ml) was added and tubes were mixed and incubated at 50°C for 30 min in a heatblock. Lysates were added to a Qiagen Genomic-tip 500/G purification column and genomic DNA was extracted following manufacturer's instructions.

ORF AMPLIFICATION, LEE ASSEMBLY, AND GENETIC ADJUVANT PREPARATION

The genomic sequence of the two chromosomes of *B. mallei* ATCC23344 were downloaded from GenBank (accession no. CP000010; CP000011). The genome contains a predicted total of 5535 genes (Nierman et al., 2004). In-house software (GeneSplitter) was used to confirm coding sequence annotations and assign a project number to each gene. To design the project's ORF-eome, all genes of 1 kb or less were generated as full length gene ORFs; genes longer than 1 kb were generated as a set of ∼800 bp long sub-genes overlapping by approximately 100 bp, thereby increasing the number of total ORFs in the library. If no primers could be designed to specifically amplify individual

members of a gene family, then the whole family was permitted to be amplified with a single oligo pair and this was recorded. The resulting mixed PCR product was designated with a single ORF number, thereby decreasing the total number of numerically defined ORFs relative to the annotation. Using these rules, a total of 6630 unique *B. mallei* ORFs were electronically defined; a corresponding number of ORF specific primer pairs were predicted and obtained containing a 5′ common forward or reverse region in addition to the ORF specific sequences. In a first step PCR, ORFs were amplified by 20 cycles of denaturation at 98°C, 40 s of annealing at 66°C, and 2 min of extension at 72°C from ∼100 to 150 ng of *B. mallei* ATCC23344 genomic DNA. The MasterTaq Kit (5′) was used with 1× Taq buffer with $MgCl_2$, 1× Taq Master PCR Enhancer, 0.5 units of Taq, 200 μM dNTPs, and 200 nM of ORF specific primers with a common adapter region (forward primers: GGTATAGGCGGAAGCGGATTG; reverse primers: GTGGGAGGGAGGTTAGGT) in a total volume of 10 μl. Universal primers were designed for use in a second step PCR with the common adapter region preceded by a region containing deoxyuracil (dU) phosphoramidite bases at intervals of three within a short 5′ stretch of the ORFs (forward primer: *AGUAGUA GUAGUAGU*GGTATAGGCGGAAGCGGATTG; reverse primer: *AUGAUGAUGAUGAUGAU*GTGGGAGGGAGGTTAGGT). This reaction was set up as a 90-μl master mix containing 1× Taq buffer with $MgCl_2$, 1× Taq Master PCR Enhancer, five units of Taq (Invitrogen), 200 μM dNTPs, and 250 nM of the dU-universal primers, which was then added to the 10-μl first step PCR reaction product. Amplification was conducted with 5 cycles of 30 s denaturation at 98°C, 40 s of annealing at 50°C, and 2 min of extension at 72°C followed by 2 min of extension at 72°C, followed by 15 cycles of denaturation at 98°C, 40 s of annealing at 66°C, and 2 min of extension at 72°C. This dU extension of the ORFs rendered them sensitive to uracil-DNA glycosylase (UDG). This two-step amplification protocol was developed after many optimization tests in which a large number of protocols, kits, and reagents for amplifying ORFs from high-GC genomes were evaluated. Using this protocol 5760 of the 6630 *B. mallei* ORFs were amplified (87%) as high quality products (specific, correct molecular weight) of sufficient yield for LEE construction. ORF yields were quantified fluorimetrically using PicoGreen (Invitrogen). At least 1 pmol of each ORF was generated for use in the library screen.

The cytomegalovirus (CMV) promoter and murine ubiquitin (UB) gene sequences (1.3 kb) and the human growth hormone (hGH) gene terminator sequence (0.6 kb) from pCMVi–UB (Sykes and Johnston, 1999a) were amplified by PCR as two separate fragments, in bulk, with primers carrying dU stretches complementary to those present within the ORF end sequences. These sequence-compatible PCR fragments would serve as upstream and downstream elements for attachment to the ORFs. In preparation, the fragments were gel-fractionated and then purified from preparative agarose gels using QIAquick Gel extraction Kit (Qiagen), and stored at −20°C until LEEs were ready to be non-covalently assembled.

In addition to this library of ORFs, two non *B. mallei* ORFs were similarly prepared in the two-step protocol for use in control group immunizations. The firefly luciferase (LUC) and human α1-antitrypsin gene (AAT) genes were amplified in the first step from pCMViLUC+ and pCMViAAT (Sykes and Johnston, 1999b). The same universal primers described for the library were used in the second step amplification. The LUC and AAT-expressing LEEs would serve as irrelevant antigens for the challenge–protection assays; however, the LUC and AAT LEEs would also be positive controls for gene and genetic immunization activity, respectively. An extra group of mice were bombarded with the LUC-expressing LEEs prepared from the same batch of gene gun cartridges (bullets) as used for the immunizations. Mice were culled 24 h later and ears were harvested to assay LUC activity (Promega, Inc.; Svarovsky et al., 2008). The AAT-expressing LEE served as a positive control for genetic immunization efficiency, as immune sera was later tested for anti-AAT reactivity by ELISA (Sykes and Johnston, 1999b).

The genetic adjuvants heat-labile toxin subunit A (LTA) and subunit B (LTB) of *E. coli* were generated by cloning the respective subunit-encoding genes into the *Bgl*II and *Hin*dIII sites in the multi-cloning site of pCMVi (Sykes and Johnston, 1999a). LTA- and LTB-expressing covalently prepared LEEs containing the promoter, the LTA or LTB ORF, and terminator sequences were generated by digesting the pCMVi–LTA and pCMVi–LTB plasmids with *Dra*I and *Pvu*II. These enzymes serve to linearize the plasmid and cleave most of the plasmid backbone from the gene expression sequences. The LT-expressing LEE fragments were purified from the pCMVi backbone by fractionation using a size-exclusion (Sepharose) column. Fractions were analyzed on a 0.8% agarose gel to identify the LEE fragments, and these fractions were pooled. These plasmid sub-fragments were used for this large scale production of elements for the library of LEEs because large scale production of DNA by PCR is less efficient than that by standard plasmid production.

ORF POOLING, LEE ASSEMBLY, AND BIOLISTIC PARTICLE PREPARATION

The 5760 *B. mallei* ORFs were concentration normalized and then pooled as ORFs using a two-dimensional 96 × 48 pool grid design, such that each dimension held all ORFs of the library. Therefore each ORF was present once per dimension and twice within the full grid. The ORFs within the X-dimension were partitioned into pools by a different sorting method than the ORFs in the Y-dimension such that each ORF resided in a pool of different co-resident ORFs within one dimension relative to the other and these pools were of different ORF complexities in one dimension relative to the other. Since the total DNA dose per gene gun bombardment (shot) was constant, the effect of altering the pool complexity was to alter the dose per ORF. The X-dimension pools delivered 10.5 fmol of each ORF per shot and the Y-dimension pools delivered 5.25 fmole of each ORF per shot. This was accomplished as follows: The ORFs were arrayed into the B–G rows of 80 separate 96-well plates. The 96 X-dimension pools were comprised of 60 ORF components that were pooled by taking all of the ORFs from a specific column from each of 10 plates (for example column 1 of plates 1–10; 1 column/plate × 6 ORFs/column × 10 plates/pool = 60 ORFs/pool). The 48 Y-dimension pools consisted of 120 ORFs that were pooled by taking all of the ORFs from a specific row from each of 10 plates (for example the B row of plates 1–10; 1 row/plate × 12 ORFs/row × 10 plates/pool = 120 ORFs/pool). Upstream and downstream expression elements were added to each pool to equimolar ratio to total 40.3 pmol ORFs per pool. The LTA and LTB adjuvant expressing ORFs were added to

each pool in 1:9 ratio. The combined DNA mixtures representing each pool were ethanol precipitated, dissolved in water and 200 μl aliquots (3 + 1 tubes) were stores at −20°C until genetic immunization was scheduled. UDG (NEB) treatment of DNA in each pool was performed immediately prior to the preparation of gene gun cartridges. For each mixture, 1.25 U of UDG enzyme was added per picomole of dU ends in pool (200 U for 200 μl aliquot). Incubation of UDG reaction was carried out for 45 min at 37°C then 10 min at 60°C and cooling down to room temperature. The reaction mixtures were ethanol precipitated and dissolved in 100 μl water.

For biolistic immunization, LEE pools were loaded onto positively charged (polyethyleneimine, PEI)-gold micro/nano particles as previously described (Svarovsky et al., 2008, 2009) and prepared onto gene gun cartridges for gene gun discharge.

GENETIC IMMUNIZATION AND *B. MALLEI* CHALLENGE

Female, 6- to 8-week-old, BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were acquired and housed under specific-pathogen-free-conditions with food and water *ad libitum* on a 12-h dark/light schedule for 1 week before use. The mice were organized into groups of eight mice genetically vaccinated in the ear skin pinnae with a Helios Gene Gun (Bio-Rad, Hercules, CA, USA) loaded with DNA coated gold particles (Svarovsky et al., 2009) and delivery at a helium pressure of 350 p.s.i. Two shots were delivered to each mouse, one in each ear. Genetic boosts with identical genetic inocula were administered at week 6 post-prime. Negative control mice were either unimmunized or immunized with a LEE expressing the irrelevant (non *B. mallei*) AAT antigen. Vaccine control mice were vaccinated with 0.5 μg of HK *B. mallei* [with Interleukin-12 (IL-12)] by intraparenteral (i.p.) injection using a 25-gage syringe. Mice were challenged with 5×10^5 CFU/50 μl of live *B. mallei* (∼2 LD_{50}) by intranasal (i.n.) delivery 12–13 weeks following the boost. Aliquots from the inocula were plated to confirm the infecting dose. The challenge experiment was carried out in four cascaded sub-experiments due to animal housing limits; however, the same naïve, irrelevant LEE, and positive vaccine controls were included in each sub-experiment. All procedures and animal protocols used in this study were approved by the Biosafety and IACUC committees at UTMB and conducted in either BSL-3 or ABSL-3 laboratories. Survival of mice was monitored at 12 h intervals over time. Survival readouts were analyzed as detailed in the Section "Results." In the sub-experiment testing X1 through X40, no mice died from challenge including the unimmunized controls, indicating a sublethal dose had been administered. Since survival could not be scored as a valid readout for these groups, mice were sacrificed and lungs were weighed as a measure of the severity of inflammatory disease. Challenge results are provided in **Table A1** in Appendix. Lung weight increase has been associated with increased disease (Huppert et al., 1976). However since these readouts could not be combined statistically with the survival data, these groups were excluded from the protection score grid analyses.

RECOMBINANT PROTEIN PRODUCTION OF A SUBSET OF *B. MALLEI* CANDIDATES

Cloning and protein expression protocols were followed as described previously (Whitlock et al., 2010). Briefly, target sequences were subjected to bioinformatics analysis using SignalP v.3.0 (Bendtsen et al., 2004), TMHMM v.2.0 (Krogh et al., 2001), and PHYRE v.0.2 (Kelley et al., 2009) to identify putative N-terminal amino acid (AA) secretion sequences, transmembrane domains, and homologies to published crystal structures, respectively. Protein subcellular localization was predicted using PSORTb v.3.0.2 (Yu et al., 2010) and CELLO v.2.5 (Yu et al., 2004). Informatically predicted signal peptides and transmembrane sequences were identified so as to avoid them if possible in the design of the ORFs for cloning into bacterial expression constructs. This was intended to facilitate recombinant production. The redesigned ORFs were PCR-amplified from genomic *B. mallei* DNA and cloned into pET28a(+) (Novagen), or pcDNA3.1 (Invitrogen) expression vector, in frame with either an N- or C-terminal 6× His affinity tag, or both. Oligo primers introducing specific restriction enzyme sites were purchased from Integrated DNA Technologies. In addition to the new targets, the GroEL gene (BMA_2001) was cloned to be produced and purified for use as a previously characterized immunogen (Amemiya et al., 2007).

For target protein production, *E. coli* host Rosetta (λDE3) was transformed with the expression constructs. Expression was induced by growth in Overnight Express instant TB medium (Novagen) for 18–20 h. Bacterial pellets were lysed using 10× CelLytic B (Sigma), and 6× His-tagged proteins purified by Ni^{2+} affinity chromatography. Purified proteins were dialyzed against two changes of 10 mM HEPES/150 mM NaCl, pH 7.4, aliquoted and stored at −80°C. Protein concentrations were determined using the BCA kit (Pierce) using bovine serum albumin (BSA) as a standard. Five of the 12 candidates and the previously identified antigen GroEL (hsp60) protein were sufficiently produced, with either an N- or C-terminal tag, or both, for vaccine testing. BMA_2001/GroEL (residues 1–550) and BMA_2821 (residues 418–753) were produced with both N- and C-terminal His tags. BMA_2804 (residues 17–370), BMA_A0768 (residues 20–489), and BMA_0816 were produced with C-terminal tags. BMA_0816 was produced in three sub-fragments (residues 43–334, 35–668, and 669–930) with C-terminal tags.

PROTEIN IMMUNIZATION AND VACCINE TESTING OF INDIVIDUAL CANDIDATES

Female, 6- to 8-week-old, BALB/c mice (groups of 12) were immunized by intramuscular (i.m.) injection with 10 μg of one of the purified candidate or control proteins, each formulated with 12.5 μg immune stimulating complexes (ISCOMs) AbISCO 100 (Isconova AB, Sweden) and 12.5 μg murine class C CpG motif oligos (ODN 2395; Coley Pharmaceuticals, Wellesley, MA, USA) in HEPES buffer (pH 7.4). A negative control group was administered the adjuvants only. Mice were boosted at week 4 post-prime with identical inocula and then challenged at week 7 by i.n. route with 2 LD_{50} (1×10^5) *B. mallei* ATCC23344. Deaths were recorded twice daily. Survival results were analyzed by a log-rank statistic test; Mantel–Haenszel survival curves were generated and plotted (PRISM, Graphpad, Inc.). The results from the groups of mice immunized with the same antigen but with an N versus a C-terminal tag were similar; therefore, these data were combined for analysis creating groups of 24 mice. The results from the N-terminal and the central sub-proteins of BMA_816 were similarly protective (the C-terminal sub-protein conferred no protection);

therefore, the results from these two groups were combined for analysis creating a group of 24 mice.

BIOPLEX ANALYSES OF CYTOKINE AND CHEMOKINE EXPRESSION

Peripheral blood sera levels of 23 cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α) were determined using CD4 Pro Mouse Cytokine 23-plex Assay (Bio-Rad #M60-009RDPD). Blood samples from mice were collected 1 week prior to initial immunization (week −1) and 1 week after the second immunization (week 5 post-prime), and sera were analyzed as per manufacturer's instructions. Replicates of three animals per group per time point were used to determine cytokine and chemokine levels in the blood sera. An unpaired Student's t-test was used to evaluate significance ($p < 0.05$).

RESULTS

DESIGN AND VALIDATION OF LINEAR EXPRESSION ELEMENTS FOR MURINE GENETIC IMMUNIZATIONS

The *B. mallei* ATCC23344 genomic sequence was used to construct a library of 5760 unique ORFs for construction into LEEs. The upstream expression element carries both a CMV promoter and the short coding sequence of a mouse ubiquitin (UB) subunit for proteosome targeting and efficient antigen processing (Sykes and Johnston, 1999a). The downstream element carries a stop codon in all three frames and the efficient transcriptional terminator from hGH gene. The tripartite molecules are annealed to form functional LEEs (**Figure 1** inset).

Before large scale library production, pilot constructs were prepared as described above in order to validate gene vaccine activity in mice. The genes encoding five *B. mallei* proteins predicted to be secreted via the type III secretion system (Whitlock et al., 2007) were used; one of these was recently tested as a protective antigen (Whitlock et al., 2010). BopA (BMA_A1521), BopE (BMA_A1523), BipD (BMA_A1528), BipC (BMA_A1530), and BipB (BMA_A1531) were constructed and prepared as LEEs for mammalian expression. They were individually delivered into groups of five mice by gene gun together with LEEs encoding both subunits A and B of *E. coli* lethal enterotoxin (LTA/B) as genetic adjuvant (Peppoloni et al., 2003). One control group was immunized with LEEs expressing the non-glanders (irrelevant) luciferase (LUC) antigen with LTA/B; another control group was not immunized. Mice were genetically immunized at weeks 0, 3, and 6 followed by blood draws for evaluation of sera reactivity to recombinantly produced antigens. At week 9, i.p. challenges with 6×10^7 *B. mallei* were conducted. Survival results indicated several of the antigens conferred extended time to death; BopA and BipD provided 20% endpoint survival from the i.p. challenge (**Figure 1**). These results confirm that the LEE genetic immunization constructs are immunologically functional *in vivo*.

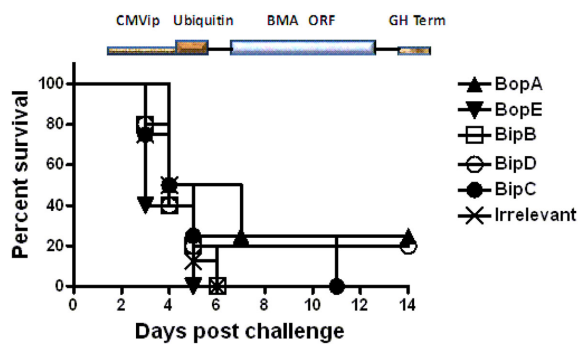

FIGURE 1 | Validation of ORF construction and expression *in vivo*. A set of five genes encoding *B. mallei* proteins predicted to be secreted were generated and constructed as linear expression elements (LEEs). These LEEs were constructed by the same protocols as intended for use in preparing and testing the *B. mallei* ORF library for vaccine candidates. Inset cartoon shows LEE design. These five LEEs were co-delivered with LEEs encoding the *E. coli* heat-labile enterotoxin subunits A and B (LTA/B) as adjuvant by gene gun at weeks 0, 3, and 6 into groups of five mice. At week 9, mice were evaluated immunologically in challenge–protection assays. Survival curves of immunized animals are plotted following i.n. delivery of 1×10^5 bacteria (2 LD_{50} *B. mallei* strain ATCC23344). The irrelevant group was immunized similarly but with a LEE expressing the luciferase (LUC) as a negative control for vaccine activity. This construct also served as a positive control for LEE gene expression activity (data not shown).

B. MALLEI EXPRESSION LIBRARY CONSTRUCTION AND STRATEGY FOR PROTECTIVE ANTIGEN SCREENING

Bioinformatic analyses of the genome identified a number of challenges for gene production relative to standard PCR amplification, including the high-GC content (70%) of the genome's coding sequences. In addition, several gene sequences displayed high homology; specific PCR amplification of these ORFs required detailed analyses of gene flanking sequences and individualized reaction conditions. Further design hurdles included the large number of transposable elements, bacterial introns, and overlapping transcripts that are found throughout the genome. Using the optimized reagents and protocol conditions, 5760 of the 6630 enumerated ORFs were amplified and constructed into LEEs, representing 87% of the coding capacity of *B. mallei*.

The ELI screen was designed to assay the vaccine potential of each *B. mallei* library component twice. This was done by distributing the complete library of 5760 ORFs into two immunization pools, by independent strategies that were designated X and Y. The feasibility of performing this multiplexed protection experiment design for antigen discovery was previously demonstrated (Stemke-Hale et al., 2005; Borovkov et al., 2009). For this project, each of the X-pools held 60 ORFs, creating 96 X's; each of the Y-pools held 120 ORFs, creating 48 Y's. Every ORF resided once in the X library and once in the Y library, but at doses that differed by twofold. Unlike earlier strategies, this enabled sensitivity to be optimized in the X-dimension and screening efficiency to be optimized in the Y-dimension. Two-dimensional matrix analyses of these organized distributions of ORFs would enable a single animal experiment of 144 groups to be used to infer which individual ORFs were responsible for disease protection.

GLANDERS ELI SCREEN

The inocula comprising the X and Y LEE pools were co-delivered with the LTA/B genetic adjuvant into groups of eight BALB/c mice by gene gun on charged-gold micro/nanoparticles (Svarovsky et al., 2009). Genetic boosts were delivered at week 6 post-prime using the same inocula. At week 18 post-prime, a lethal i.n. challenge of *B. mallei* (2 LD_{50}) was administered; mice were monitored

and deaths were recorded twice daily. As negative controls, naïve mice were not immunized and an irrelevant antigen group was administered an AAT-expressing LEE. As a positive control for protection, a group of mice was i.p. immunized with a HK *B. mallei* preparation with IL-12p70 as adjuvant.

Protection scores for all of the test and control groups were determined by recording the hours survived by each mouse, then deriving an average survival time for each group (**Table A1**). Confidence intervals (95%) and ANOVA scores were calculated to generate corresponding *p*-values for each test group relative to controls. In the X library of ORF-expressing LEE pools, X57, X59, and X89 conferred significantly extended times to death; within the Y library pools Y15 and Y19 were significantly protective ($p < 0.05$). These results are detailed in **Table 1**. In addition to the protection score analysis, log-rank Mantel–Haenszel survival curves were generated, with respective *p*-values calculated relative to controls (Prism 4.0, Graphpad Software, San Diego, CA, USA). This analysis indicated that mice immunized with pool Y24 also displayed significant extended time to death. Endpoint group survival rates are shown alongside each group's survival curve statistic in **Table 1**. Animals immunized with pool X89 showed 87.5% endpoint survival following lethal i.n. infection. Immunization with pools X57 and X59 provided animals 75% endpoint survival following the i.n. challenge. The mouse groups administered the positive vaccine control (HK *B. mallei* with IL-12p) displayed 100 and 62.5% survival rates in the X and Y challenge assays, respectively; the irrelevant antigen groups showed 25 and 0% survival in X and Y experiments, respectively. These survival curves are displayed in **Figure 2**; the top panel displays significantly different test group and also control group survival curves from the X challenge assay and the bottom panel similarly shows the Y challenge assay survival curves.

Together, the protection score and survival curve analyses indicated that three X-pools and three Y-pools were significantly protective. However, the pooling strategy of the two-dimensional screen designated Y-pools with twice the ORF complexity of the X-pools (120 versus 60 ORFs). Since a matrix analysis would be most informative if a similar number of ORFs were crossed from each dimension (library), the X-pools ranked 4, 5, and 6 (X75, X76, and X95 with *p*-values of 0.17, 0.20, and 0.018, respectively) were taken into the cross-hair analyses. Their protection scores, survival rates, and statistics are provided in **Table 1**.

Table 1 | Analyses of ELI challenge–protection screen.

	Protection score (*p*-value)	Protection rank	Endpoint survival (%) (*p*-value)
X-POOLS			
X89	423 (0.003)	1	87.5 (0.006)
X57	372 (0.03)	2	75 (0.029)
X59	365 (0.04)	3	75 (0.05)
X75	321 (0.17)	4	62.5 (0.137)
X95	318 (0.18)	5	62.5 (0.158)
X76	317 (0.20)	6	62.5 (0.156)
Irrelevant	126 (0.59)		25
HK + IL-12	456 (0.0005)		100
Y-POOLS			
Y19	270 (0.02)	1	50 (0.027)
Y15	269 (0.02)	2	50 (0.08)
Y24	233 (0.10)	3	37.5 (0.05)
Irrelevant	146 (0.89)		0
HK + IL-12	336 (0.0003)		62.5

IDENTIFICATION AND TESTING OF SCREEN-INFERRED PROTECTIVE ANTIGENS

A matrix analysis was performed to pinpoint any antigens that conferred protection as a constituent within both its X-pool and Y-pool inocula. This was done by comparing the list of ORFs comprising the subset of X-pools selected for further evaluation

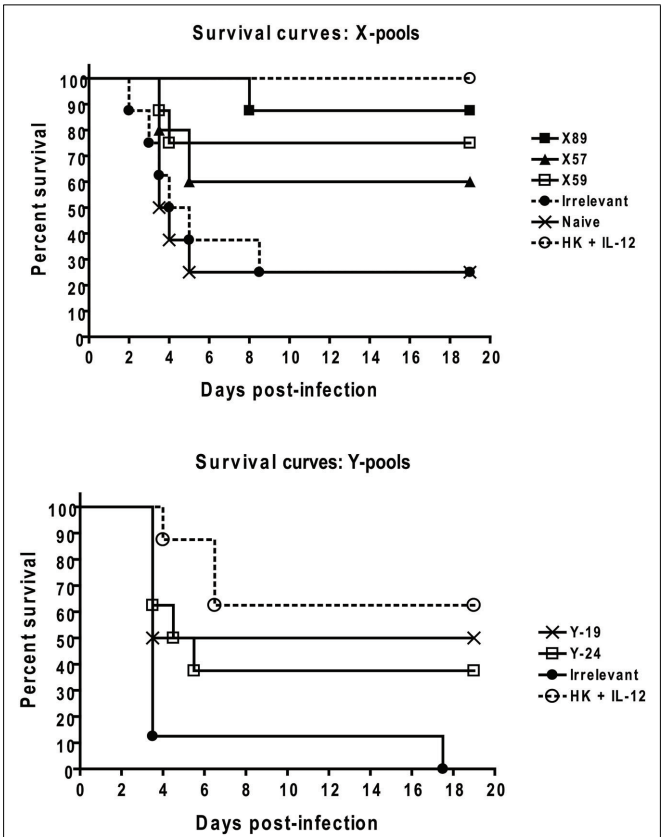

FIGURE 2 | Screening of the *B. mallei* ORF-ome for vaccine candidates by ORF-pool immunization and challenge–protection assay. Groups of eight mice were immunized once in each ear pinna with 1 μg dose by gene gun. Each discharge carried a pool of *B. mallei* ORFs as LEEs (0.9 μg) and the genetic adjuvant LTA/B (0.1 μg). The LTA and LTB-expressing LEEs were mixed 1:5 (0.08 and 0.02 μg, respectively). Two libraries of 5750 ORFs, labeled X and Y, were partitioned into 96 pools of 60 ORFs (X-pools) or 48 pools of 120 ORFs (Y-pools). Controls were heat-killed (HK) *B. mallei*, the non *B. mallei* antigen α1-antitrypsin (AAT), and naïve mice. Boosts were similarly administered at week 6. A 2 LD_{50} (1×10^5) dose of live *B. mallei* ATCC23344 was administered i.n. at week 10 and death was monitored twice daily. Survival curves were generated for all groups; however, only the curves corresponding to statistically protective groups ($p < 0.05$) and controls are plotted for clarity. Top graph displays results from animals immunized with protective pools from the X library. Bottom graph displays the survival courses of mouse groups administered the protective pools from the Y library.

to the list of ORFs comprising the Y-pools selected for evaluation. Furthermore, each ORF was considered to represent its full length target gene. This was done because a protective antigen often carries multiple protective epitopes (Crowe et al., 2010), and these are likely to rank differently in potency from one species to another as a result of divergent antigen presentation molecules and processes. Therefore, identification of any portion of a gene is likely to indicate a useful target antigen, as opposed to merely a target epitope. Twelve *B. mallei* genes were identified by this overlapping pool matrix analysis. These candidates are enumerated in **Table 2** along with their resident pools within the screen and any predicted protein functions or homologies found in existing databases.

To evaluate how genetic immunization leads might translate into more conventional protein subunit vaccine formulations, the 12 new gene vaccine candidates were subjected to structural analysis in preparation for testing in a new round of challenge–protection assays as individually administered protein inocula. Many of the target proteins carried multiple predicted transmembrane helices that would make recombinant protein production difficult. These were excluded from the expression constructs in favor of regions predicted to be immunogenic (see Materials and Methods). The BMA_0816 gene was cloned and expressed as three sub-fragments because of its large size. Five candidates (BMA_A0712, BMA_A0768, BMA_0816, BMA_2804, BMA_2821) and the previously described immunogen GroEL (BMA_2001; Amemiya et al., 2007) were bacterially produced with N- and/or C-terminal His tags and purified by standard nickel-affinity chromatography. Each of the six proteins was delivered i.m. with ISCOMs and murine class C motif CpG oligos as adjuvant into groups of 12 BALB/c mice. Boosts were administered at week 4 and then the mice were challenged with live *B. mallei* (2 LD_{50}) at week 7. Survival was monitored and recorded twice daily.

Protection was assessed by generating Mantel–Haenszel survival curves and deriving a log-rank statistic as described above. The results plotted in **Figure 3** show that three of the five tested candidates and GroEL were confirmed as conferring partial protection. Immunization with the protein encoded by BMA_A0768 provided 25% (3/12) endpoint survival while GroEL immunization provided 12.5% (3/24) endpoint survival and both displayed significantly extended times to death ($p = 0.025$ and $p < 0.001$, respectively). The proteins encoded by BMA_2821 and BMA_0816 also conferred significantly extended times to death ($p = 0.0023$ and $p < 0.0001$, respectively) relative to adjuvant only immunized controls. The three significantly protective candidates (BMA_A0768, BMA_2821, and BMA_0816) are highlighted in red in **Table 2**.

Table 2 | Individual *B. mallei* vaccine candidate genes identified from matrix analysis of the ELI challenge–protection assays.

ELI-derived vaccine candidate	Predicted function/homology	Resident ELI pools
BMA_A1219	Tetratricopeptide repeat protein	X95; Y24
BMA_A1678	Selenocysteine-specific translation elongation factor	X76; X95; Y24
BMA_A1678	Putative CpaE protein (pilus assembly)	X76; Y24
BMA_A1325	Major facilitator family sugar transporter	X95; Y24
BMA_A0712[1]	Major facilitator superfamily protein	X75; Y24
BMA_A0768[2]	Mannitol dehydrogenase family protein	X57; Y19
BMA_A0950	Major facilitator family transporter; MerR family transcriptional regulator	X89; Y24
BMA_2821[2]	Toxin secretion ABC transporter, ATP-binding protein (colicinV processing peptidase)	X75; Y15
BMA_2804[1]	Molecular chaperone, Hsp70	X75; Y19
BMA_2468	Glyceraldehyde-3-phosphate dehydrogenase, type I	X57; Y24
BMA_0092	Peptide ABC transporter, ATP-binding protein	X59; Y24
BMA_0816[2]	Maltooligosyl trehalose synthase (putative glycosyl hydrolase)	X89; Y24

[1] *Candidates tested individually are shown in boldface.*

[2] *Vaccine candidates found to be protective are indicated in red type.*

ELI VACCINE CANDIDATES ELICIT SERUM CYTOKINE AND CHEMOKINE RESPONSES

Blood was drawn from the animals described above, immunized with the five *B. mallei* protein antigen candidates or the adjuvant control, 1 week prior to immunization (week −1 relative to prime) as a baseline for each animal and then 1 week after the booster (= week 5 post-prime) as a measure of vaccine elicited responses. Measuring levels at these two time points was designed to display a profile of the hosts' responses to vaccination (**Figure 4**). The influence of the adjuvant on host responses was measured by comparing the pre-immunization profiles to that of the control sera, which was not drawn from naïve mice but from mice immunized with adjuvant only. Prior to immunization, GM-CSF (BMA_0768) and MIP-1β (BMA_0768) showed elevated levels relative to the adjuvant-immunized control sera; whereas IL-12p70 (BMA_A0712) was significantly lower in one of the pre-immune

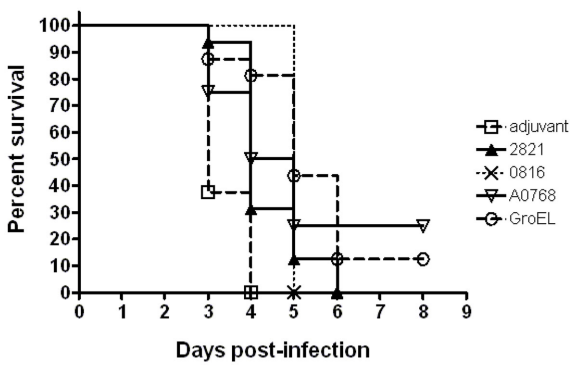

FIGURE 3 | Evaluation of a subset of the vaccine candidates identified by ELI, tested individually. Groups of 12 BALB/c mice were i.p. injected with the five candidates and GroEL (BMA 2001) delivered as recombinant bacterially produced proteins. These were formulated with ISCOMs and CpG's as adjuvants (adjuvant). Boosts were administered at week 4 post-prime and challenged by i.n. route with 2 LD_{50} (1×10^5) *B. mallei* ATCC23344 at week 7. Survival was monitored, recorded and analyzed as described in the Section "Materials and Methods." The Mantel–Haenszel survival curves for the control groups and for the three test groups that displayed statistically significant protection ($p < 0.05$) are plotted. GenBank BMA gene numbers are shown.

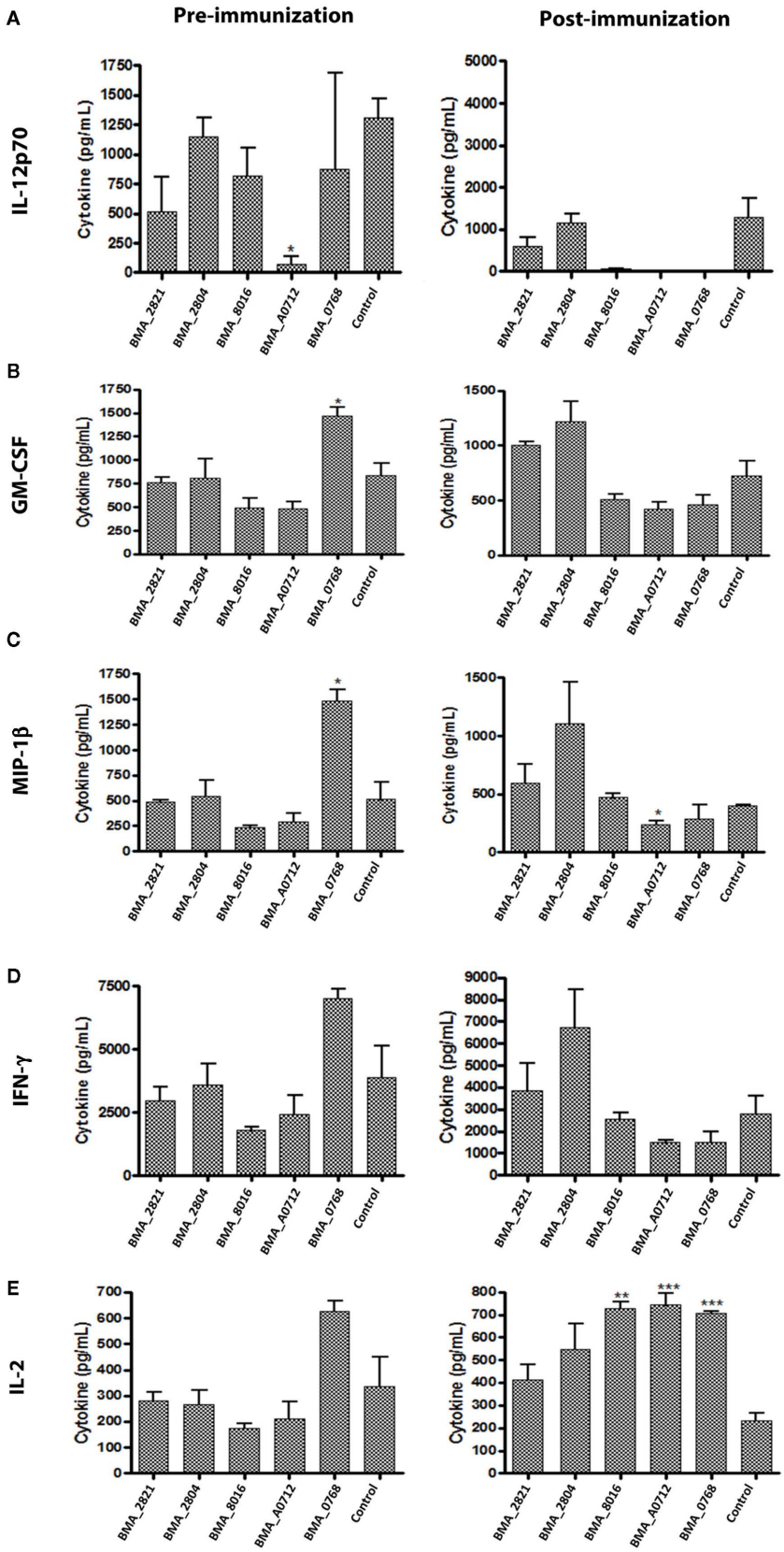

FIGURE 4 | Continued

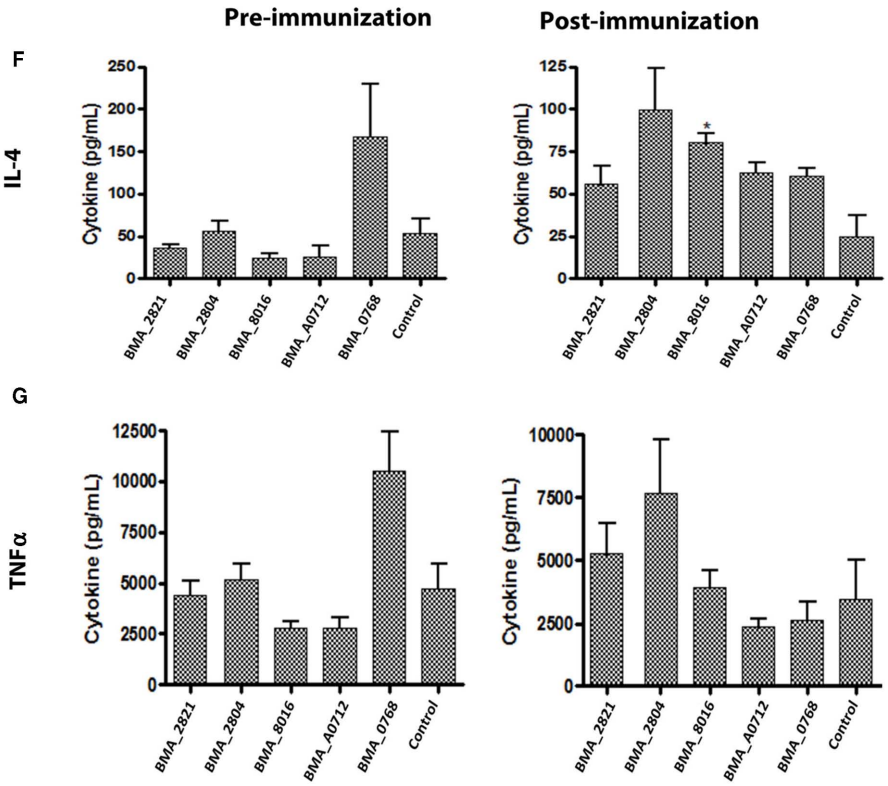

FIGURE 4 | Bioplex analyses of proinflammatory cytokines elicited in peripheral blood of immunized versus unimmunized mice. Blood was drawn (i) 1 week prior to immunization and (ii) 1 week post booster immunization (=5 weeks post-prime) of the mice described in **Figure 3**. Results from these time points are graphed on the left (pre-immunization) and right (post-immunization) panels. Control sera were drawn from adjuvant-immunized mice. Replicates of three animals per group per time point were used to determine the level of cytokine or chemokine indicated above each pair of graphs **(A)** through **(G)**. Sera were collected and analyzed per manufacturer's instructions. Unpaired Student's *t*-test was utilized to determine significance ($p < 0.05$). Error bars are SD with group sera.

sera groups relative to the sera from adjuvant expose mice. This indicates that IL-12p70 may be elicited by the ISCOM and CpG formulation alone. Post-immunization, IL-2 levels were significantly elevated in sera from mice immunized with three of the five antigen candidates (BMA_0816, BMA_A0712, BMA_0768), two of which were protective (**Figure 3**). IL-4 (BMA_0816) and MIP-1β (BMA_A0712) were also significantly different ($p < 0.05$) from the adjuvant-immunized control sera levels, with IL-4 elevated and MIP-1β reduced. These levels represent antigen-specific activity elicited by the vaccine candidates.

DISCUSSION

A functional genomics vaccine candidate screen does not need to assume anything about the properties or characteristics of protective antigens. However the screen presented here does assume that a protective gene can be detected among many simultaneously expressed ones. Support for this assumption lies in its parallel with the antigenic complexity of a live or whole-pathogen vaccine, or a pathogen infection, situations in which the immune system is exposed to a mixture of many antigens. We used ELI to directly test the *B. mallei* ORF-eome for protective components by genetic immunization followed by lethal challenge. A two-dimensional, matrix analysis of a genomic-scale protection assay following vaccination with pools of ORFs indicated 12 gene candidates as being responsible for conferring survival rates as high as ∼90% against i.n. challenges. Five of these 12 vaccine candidates were produced as proteins and tested as individual antigens in the challenge–protection assays; three were found to provide significant, partial protection from a lethal pulmonary infection with *B. mallei*.

The protection level measured following immunization with any of the five tested protein candidates was lower than that achieved by the ORF pools in which the candidates were originally resident. The simplest explanation for this outcome is that the more highly protective candidates were among the seven untested of the 12 matrix-inferred candidates. Even for the five tested candidates, only the portions of the target proteins selected for recombinant expression by bioinformatics were administered; more strongly protective epitopes may have been unintentionally excluded. Alternatively the antigen delivery format, which differed between the two rounds of challenge–protection assays, may be critical. First, the genetic immunization constructs expressed the antigens endogenously within the skin cells of the host, and these constructs carried proteasomal targeting (UB) sequences intended to promote cellular immune responses against the UB-fused antigen. By contrast, the candidates were redesigned in the second

round without UB fusion or their natural secretory leader peptides for *ex vivo* protein production. We had elected to reformulate the candidates from genes into proteins for the secondary round of vaccine testing because of the generally higher level of acceptance of this more traditional subunit vaccine format. Namely, several protein-based vaccines have already been FDA approved (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM093833) while gene vaccines are at this time still in trials. Protein formats have been shown to promote T helper cell 2-like (Th2) responses and antibody production, relative to *in vivo* produced antigen (Chow et al., 1998; Iborra et al., 2007). These responses may not be as protective as Th1 responses against *B. mallei*. As a second difference in delivery format between the two rounds, the mixed versus individual context of the antigen may be relevant. The significance of this format distinction is consistent with several previous ELI screens in which all candidates were tested, and all tested as genes in both initial and secondary screens. In these studies, the mixed pool inocula were frequently more protective than the individually evaluated candidates (Stemke-Hale et al., 2005; Li et al., 2006; Borovkov et al., 2009), as found in this work.

We suggest that the complexity of the ORF pools is important. In this project, we intentionally designed a grid with differing complexities (either 60 or 120 in the X and Y-pools respectively) potentially capturing the undetermined effects of antigen dose, complexity, and competition on immunity. If there were neither negative nor positive interactions among the constituents of the mixed inocula, then we anticipated that maximizing dose would be most important in maximizing any response elicited. Since we delivered constant total genetic doses, this would mean that the lower pool complexity (higher individual ORF doses) of the X-pools would be advantaged over the Y-pools. However the average levels of protection conferred by the X versus the Y-pools, relative to HK *B mallei* and irrelevant antigens were similar. This indicates that dose was not a strong factor in the survival readout. Alternatively, if immunological interactions do occur among the pool constituents, then an ORF's individual performance level may be evaluated relative to that within a pool; however, the performance of an ORF in two different pools of two different complexities cannot be quantitatively interpreted. Antigenic competition has been well described among co-delivered protein antigens (Adorini et al., 1989); however, a range of results have been reported for immune interactions among co-delivered genetic antigens (Braun et al., 1998; Sedegah et al., 2004; Hirao et al., 2011). A combination of competitive and cooperative activities among the antigen pool components would have opposing effects that would blur minor differences in antigen dose. This is consistent with our observations. Nonetheless, the performance of both the X and Y antigen pools were reproducibly superior relative to that of the individually administered antigens. These results suggest that the overarching effect of gene vaccine complexity is cooperative. The three confirmed individual candidates may play a critical role as a specific antigen, while one or more of the remaining constituents of their highly protective resident pools may provide non-specific, or even additional specific, immunological help and modulation. This may be addressed in vaccine development in a number of ways. For example, a small set of cooperatively acting components may be identified in future studies that are highly effective. Protection levels may also be enhanced by immunomodulators that direct responses to those determined to be most effective. Mixed modality immunization regimens such as DNA-prime/protein- or live vectored-boosts may serve to widen the breadth of responses to facilitate greater protection levels. Notably, the ELI screen was done by genetic immunization and a long immunization regimen (18 weeks), whereas the individual components were delivered as proteins in an abbreviated regimen (7 weeks). Perhaps their combination in a mixed modality prime-boost regimen, and an extended immunization period, would be productive.

Bioplex determinations of a set of 23 cytokine and chemokines identified IL-2 as the principal cytokine elicited by the vaccine candidates. IL-2 was significantly elevated relative to controls in the sera from three of the five groups of mice immunized with individual protein antigens; two of these groups displayed significant protection from challenge. IL-4 was also significantly elevated in sera from mice immunized with one of the tested candidates that conferred protection. Notably, the cytokine profile elicited by these subunit vaccines is divergent from those stimulated following exposure to HK *B. mallei*, or the BopA subunit, which includes IFNγ and TNFα τ cell secretion (Whitlock et al., 2008, 2010). This suggests that improved protection might be achieved by either formulating these antigens with a different adjuvant or preparing the antigens in another format (such as genes or gene pools, or live vector) that would modulate the cytokine profile closer to that elicited by other vaccines. However, the strong CD4 T cell and antibody response indicated by expression of IL-2 and IL-4 is consistent with the importance found for B cells in the protective response to HK *B. mallei* (Whitlock et al., 2008). Notably, class C motif CpG oligos were selected as the adjuvant in this study because of the role demonstrated for antibodies in addition to cell-mediated responses in clearing *B. mallei* infections (Bondi and Goldberg, 2008). A more divergent explanation is that these three new antigens direct the host immune system to generate a unique protective response not engendered by previously tested subunits or by the pathogen itself.

In the microbial world, the genomes of *Burkholderia* species rank as some of the most complex, carrying nearly as many base pairs as a eukaryotic yeast genome (Holden et al., 2004; Nierman et al., 2004). *Through the empirical discovery approach described here, we have identified 12 new vaccine candidates for further evaluation* (**Table 2**). A number of these pathogen components were unlikely to have been selected informatically from the genome since the pSORT B-predictions indicated cytoplasmic localizations (**Table 3**). The preliminary testing of a subset of only five of these candidates established that *three conferred significant protection against a pulmonary challenge with virulent B. mallei bacilli.* Of these three protective candidates, two are predicted to encode cytoplasmic enzymes. First, BMA_A0768 displays homology to a mannitol dehydrogenase (MTD) gene, encoding a member of an oxidoreductase family. Mannitol and MTD are known to play roles in host defense and pathogen counter-defense (Jennings et al., 1998). Second, BMA_0816 is homologous to a maltooligosyl trehalose synthase gene, encoding an enzyme that catalyzes transglycosylations (Nakada et al., 1995). In contrast to these genes, the third vaccine candidate, BMA_2821, encodes a membrane protein. It shows homology to a member of the ATP-binding cassette (ABC) transporter family that secretes the peptide

Table 3 | Characteristics of individually protective subunit vaccine candidates.

B. mallei vaccine candidate	Cellular location	Percentage identity to Bpm 96243	Survival curve significance, *p*-value
BMA_A0768	Cytoplasmic[a,b]	99.8	0.025
BMA_2821	Inner membrane[a,b]	99.7	0.002
BMA_0816	Cytoplasmic[a,b]/outer membrane[b]	99.5	<0.0001

[a] *Determined by PSORTb v.3.0.2.*

[b] *Determined by CELLO v.2.5.*

antibiotic colicinV (Zhong et al., 1996). Colicin is encoded on a virulence plasmid and the ABC efflux transporter secretes it (Waters and Crosa, 1991). By conventional criteria, this surface-located virulence-factor secreting protein might have been classified as a putative protective antigen. Notably, another ABC transporter was within the set of 12 ELI-derived candidates, in addition to a sugar transporter; however, neither of these ORFs (BMA_0092 and BMA_A1325, respectively) was individually tested in the vaccine assay.

Sequence alignments of the protective *B. mallei* ORFs to the *B. pseudomallei* K96243 (Bpm 96243) strain homologs show a very high percentage of identity (**Table 3**), suggesting that these antigens may be able to cross-protect against *B. pseudomallei* infection. Further characterization and optimizations of the protective immune responses induced by these new candidates, and exploration of the remaining candidates, may reveal novel pathways to immunity against *B. mallei*, and possibly *B. pseudomallei*. In summary our results provide a basis for the development of a safe and effective *Burkholderia* vaccine, comprised of a set of genome-encoded pathogen components, against pulmonary glanders disease.

AUTHOR CONTRIBUTIONS

Kathryn F. Sykes, Alfredo G. Torres, and D. Mark Estes designed the research project; Gregory C. Whitlock, Mark D. Robida, Barbara M. Judy, Arpaporn Deeraksa, Katherine Taylor, C. Shane Massey, Andrey Loskutov, Alex Y. Borovkov, Jose A. Cano, and Kevin Brown executed the experiments and contributed to the analyses of results; Omar Qazi and Katherine A. Brown designed and produced critical reagents; Kathryn F. Sykes drafted the manuscript, and all authors contributed to the manuscript review and final preparation.

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APPENDIX

Table A1 | Challenge protection results from ELI screen.

Group	Protection score (*p*-value)	Rank	Group	Protection score (*p*-value)	Rank
Y1	129 (0.63)	31	X41	222 (0.85)	53
Y2	131 (0.66)	26	X42	128 (0.09)	52
Y3	81 (0.15)	46	X43	126 (0.09)	55
Y4	177 (0.62)	14	X44	83 (0.02)	56
Y5	107 (0.35)	37	X45	129 (0.10)	51
Y6	179 (0.60)	9	X46	174 (0.35)	46
Y7	197 (0.38)	4	X47	228 (0.92)	28
Y8	177 (0.62)	12	X48	186 (0.45)	39
Y9	131 (0.66)	26	X49	177 (0.37)	45
Y10	134 (0.70)	24	X50	231 (0.96)	23
Y11	149 (0.93)	19	X51	269 (0.59)	21
Y12	110 (0.38)	36	X52	273 (0.54)	13
Y13	81 (0.15)	46	X53	276 (0.51)	10
Y14	131 (0.66)	26	X54	230 (0.94)	26
Y15	269 (0.02)	2	X55	273 (0.54)	13
Y16	81 (0.15)	46	X56	273 (0.54)	13
Y17	131 (0.66)	26	X57	372 (0.03)	2
Y18	92 (0.22)	41	X58	270 (0.57)	18
Y19	270 (0.02)	1	X59	365 (0.04)	3
Y20	177 (0.62)	14	X60	183 (0.42)	40
Y21	146 (0.89)	18	X61	227 (0.91)	30
Y22	95 (0.24)	39	X62	210 (0.71)	37
Y23	180 (0.58)	7	X63	231 (0.96)	24
Y24	233 (0.11)	3	X64	291 (0.37)	7
Y25	138 (0.77)	32	X65	183 (0.42)	54
Y26	135 (0.72)	22	X66	180 (0.40)	41
Y27	86 (0.17)	43	X67	159 (0.24)	49
Y28	93 (0.23)	41	X68	222 (0.85)	35
Y29	134 (0.70)	21	X69	180 (0.40)	41
Y30	125 (0.57)	33	X70	180 (0.40)	41
Y31	174 (0.67)	17	X71	134 (0.11)	50
Y32	84 (0.19)	44	X72	221 (0.83)	36
Y33	96 (0.25)	38	X73	237 (0.96)	22
Y34	176 (0.65)	16	X74	273 (0.54)	13
Y35	84 (0.16)	44	X75	321 (0.17)	4
Y36	179 (0.60)	9	X76	317 (0.20)	6
Y37	131 (0.66)	26	X77	180 (0.40)	41
Y38	191 (0.45)	5	X78	224 (0.87)	33
Y39	179 (0.60)	9	X79	270 (0.57)	17
Y40	138 (0.77)	20	X80	270 (0.57)	18
Y41	135 (0.72)	22	X81	189 (0.48)	38
Y42	182 (0.56)	6	X82	173 (0.33)	47
Y43	134 (0.70)	24	X83	176 (0.36)	48
Y44	180 (0.58)	8	X84	270 (0.57)	18
Y45	119 (0.49)	35	X85	230 (0.94)	25
Y46	177 (0.62)	12	X86	276 (0.51)	10
Y47	93 (0.23)	40	X87	276 (0.51)	8
Y48	126 (0.59)	34	X88	227 (0.91)	30
Naïve 1	146 (0.89)		X89	423 (0.003)	1
Irrelevant	126 (0.59)		X90	224 (0.87)	33
Vaccine 1	336 (0.0003)		X91	227 (0.91)	30
Vaccine 2	456 (<0.0001)		X92	276 (0.51)	8

X93	276 (0.51)	10
X94	228 (0.92)	29
X95	318 (0.19)	5
X96	230 (0.94)	26
Naïve 1	224 (0.87)	
Naïve 2	183 (0.42)	
Irrelevant	192 (0.51)	
Vaccine	456 (0.0005)	

Group	**Average spleen weight (g)**	**Rank**
X1	1.43	28
X2	1.25	18
X3	1.67	38
X4	1.53	33
X5	1.04	8
X6	1.13	11
X7	1.19	15
X8	1.46	29
X9	1.13	11
X10	1.02	6
X11	1.51	32
X12	1.29	20
X13	1.32	22
X14	1.61	34
X15	1.30	21
X16	1.62	35
X17	1.62	35
X18	1.50	31
X19	1.62	35
X20	1.35	25
X21	1.49	30
X22	1.71	39
X23	1.20	17
X24	1.90	40
X25	1.07	9
X26	1.18	14
X27	1.19	15
X28	1.33	24
X29	1.02	7
X30	1.01	5
X31	1.32	22
X32	0.91	1
X33	1.27	19
X34	1.15	13
X35	0.97	4
X36	0.95	3
X37	0.91	1
X38	1.36	26
X39	1.11	10
X40	1.36	26
Naïve uninfected	1.00	
Naïve infected	1.25	