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A DNA vaccine targeting the receptor-binding domain of *Clostridium difficile* toxin A

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Abstract

Clostridium difficile is a pathogen with increasing severity for which host antibody responses provide protection from disease. DNA vaccination has several advantages compared to traditional vaccine methods, however no study has examined this platform against *C. difficile* toxins. A synthetic gene was created encoding the receptor-binding domain (RBD) of *C. difficile* toxin A, optimized for expression in human cells. Gene expression was examined *in vitro*. Mice were inoculated then challenged with parenteral toxin A. Vaccination provided high titer antibodies and protected mice from death. This represents the first report of DNA vaccine inducing neutralizing antibodies to *Clostridium difficile* toxin A.

Keywords

Vaccine; *Clostridium difficile*; DNA

1.0 Introduction

Clostridium difficile is a gram-positive, anaerobic, spore-forming bacterium recognized as a major cause of hospital-acquired diarrhea [1–6]. While as many as 3% of healthy outpatient adults may be colonized with the organism, this rate increases dramatically following hospitalization [7,8]. Disease results from the production of one of two major cytotoxins, toxins A and B, whose clinical manifestations range from asymptomatic carriage to diarrhea, toxic megacolon, and death [3,9–12]. Since 2000, extensive publications describe changes in the rates and severity of *C. difficile* disease generating renewed interest in novel approaches to disease treatment and prevention, including toxin-specific vaccines [4,13–20].

It has been observed that toxin-specific, host antibodies influence the outcome of *C. difficile* colonization and infection [21]. Patients with anti-toxin A antibodies at the time of colonization with *C. difficile* spores are at lower risk of progression to active and severe disease [22]. Once

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infected, individuals who develop strong anti-toxin antibody responses clear their disease following antimicrobial treatment and remain disease free [23]. Such studies provide scientific rationale for development of a vaccine against *C. difficile* toxins. While numerous studies have presented candidate *C. difficile* vaccines [21,24–28], to date, none has examined the DNA vaccine platform. DNA vaccination has several advantages versus other modalities including established safety, ease of manufacturing, and the potential to include immunogenic coding sequences.

As proof of principle, we created a synthetic gene encoding the RBD of *C. difficile* toxin A, optimized for expression in human cells. The following data demonstrate that this gene is well expressed *in vitro*, is immunogenic in mice, and protects mice from a lethal toxin challenge. To our knowledge, this is the first report of a DNA vaccine targeting *C. difficile* toxins capable of inducing protective immune responses.

2.0 Materials and Methods

2.1 Plasmid design

The amino acid sequence corresponding to the receptor-binding domain of *C. difficile* toxin A (strain VPI 10463, Genbank Accession number CAA63564.1, residue positions 1839–2710) was identified. [3] The amino-acid sequence was back-translated *in silico* to provide a gene composed of those codons most commonly employed by human cells (<http://www.entelechon.com/>). Restriction sequences, a kozak sequence, and a methionine start site were incorporated as shown in Supplemental Figure 1 [29,30]. Following commercial synthesis (BlueHeron Biotechnology, Seattle, WA) the gene was inserted into the commercial vector, pVAX (Invitrogen, Carlsbad, CA) with or without a tissue plasminogen activator (tPA) sequence as previously described [31]. TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA) were transformed and positive clones confirmed by restriction digestion and DNA sequencing (GeneWiz, North Brunswick, NJ.). The resulting two plasmids differ only in the presence or absence of a tPA leader sequence following the ATG start codon and are referred to as 1. **TxA-RBD** and 2. **tPA-TxA-RBD** (Figure 1).

2.2 Protein Expression

293T cells were split and plated in a 12-well dish at a concentration of $3\text{--}4 \times 10^5$ cells per well in DMEM with 10% inactivated FBS (v/v) (Invitrogen, Inc. Carlsbad, CA) and 1% penicillin-streptomycin (v/v) (Invitrogen, Carlsbad, CA). Twenty-four hours post-plating, cells were transfected with 2 μ g of TxA-RBD, tPA-TxA-RBD, or pVAX expressing green fluorescent protein as a negative control using lipofectamine 2000 (Invitrogen, Carlsbad, CA) per the manufacturers instructions. Forty-eight hours post-transfection, supernatant and cell lysates were collected and stored at -20°C . Supernatant was clarified by centrifugation at $22,000 \times g$ for 30 minutes prior to immunoblot.

Immunoblots were performed using the Invitrogen SureLock system according to the manufacturer's recommendations. Briefly, 32.5 μ l of sample was added to 12 μ l NuPAGE LDS loading buffer and 5 μ l of reducing agent and heated to 70°C for 10 minutes. Samples were subjected to electrophoresis in a 10% BisTris gel (Invitrogen, Carlsbad, CA) at a constant voltage of 200V. Samples were transferred to PVDF membranes and blocked for two hours in blocking buffer (5% dry milk, 0.5% bovine albumin in PBS (Invitrogen, Carlsbad, CA)). Membranes were incubated with primary goat polyclonal anti-toxin A (List Biological Laboratories, Inc.) 1:2000 in blocking buffer overnight at 4°C . Membranes were then washed with wash buffer (PBS with 0.05% Tween, Sigma, Inc. St.Louis, MO) and HRPconjugated anti-goat secondary antibody (Sigma Inc., St.Louis, MO) 1:8000 in blocking buffer was added for 1 hour at room temperature. Membranes were washed as above and developed using the

Amersham ECL development system (GE Healthcare, Piscataway, NJ). The procedure was repeated as described and samples analyzed using anti- β actin primary antibody (murine host) (Sigma Inc., St. Louis, MO) and anti-mouse IgG secondary antibody (Amersham Biosciences, Inc. Piscataway, NJ) 1:25,000 in blocking buffer to evaluate variations in loading volumes.

2.3 Animal Inoculations

6–8 week old BALB/c mice (6 mice per group) and CD-1 Swiss-Webster mice (5 mice per group) were obtained (Charles River Laboratories, Wilmington, MA) and housed in at the Laboratory Animal Research Center of The Rockefeller University. All procedures were carried out as described under protocols approved by the Institutional Animal Care and Use Committee of The Rockefeller University. Each experiment was repeated in two independent experiments.

Endotoxin free (<100IU) plasmid DNA for vaccination was obtained (Aldevron Inc., Fargo, ND). Five groups of BALB/c (6 per group) or CD-1 (5 per group) mice were vaccinated at weeks 0 and 2 with 50 μ g of plasmid DNA divided into two doses delivered by either standard syringe injection (IM) or *in vivo* electroporation (IM) into each of the rear limbs. Groups were inoculated with 1. pVAX alone; 2. tPA-TxA-RBD (IM); 3. TxA-RBD (IM); 4. tPA-TxA-RBD (EP); and 5. TxA-RBD (EP) as shown in Figure 3. Serum was obtained at week 6 post injection for immunologic evaluation prior to toxin challenge. Animals were sedated with ketamine and xylazine prior to the procedure. *In vivo* electroporation was performed using the TriGridTM electroporation system for mice as recommended by the manufacturer, Ichor Medical Systems, Inc. (www.ICHORMS.com).

2.4 ELISA

96-well high protein-binding polystyrene EIA plates (Costar 9018, Corning Inc., Corning, NY.) were coated with 50 ng of purified, whole toxin A from *C. difficile* (List Biological Laboratory) in PBS overnight at 4°C. The following day, plates were blocked for 1.5 hours with blocking buffer (PBS-T, 5% dry milk w/v, 0.5% BSA w/v). Serum samples were added in duplicate following serial ten-fold dilution in blocking buffer and incubated for two hours at 37°C. Plates were washed five times with wash buffer (PBS-0.05% Tween 20) and incubated for 45 minutes with AKP-conjugated goat anti-mouse secondary antibody (1:10,000 in blocking buffer, BD Pharmingen, San Diego, CA). Plates were developed using the AMPAK ELISA development kit according to manufacturer's specifications (DAKO Corporation, Carpinteria, CA). Optical density of plate wells was determined at 490 nm (Dynex Technologies, Chantilly, Va). The end-point antibody titers represent the reciprocal dilution of the last dilution providing an O.D. 2-fold higher than the O.D. of sera controls at the lowest performed dilution.

2.5 Toxin Challenge

Eight weeks post-vaccination, animals were challenged with 300ng of freshly reconstituted toxin A from *C. difficile* in 100 μ l of sterile saline delivered via intraperitoneal syringe injection (IP) [32]. Animals were examined twice daily for mortality up to 14 days post challenge.

2.6 Statistical Analysis

Comparison of group-specific ELISA titers was performed as follows. Groups were compared using Kruskal-Wallis non-parametric analysis for over-all significance. If positive ($p < 0.05$), groups were compared pair-wise using one-way ANOVA with a Bonferroni correction for multiple comparisons. Significance was determined at $p < 0.05$. Differences between median survival times post challenge were examined with pair-wise log-rank analysis. The Kaplan-Meier survival curves and overall statistic were calculated and plotted using MedCalcTM.

Kruskal-Wallis, one way ANOVA, and Log-Rank pair wise comparisons were calculated using SPSS version 14.

3.0 Results

3.1 Gene Expression

Cell lysates and supernatants were harvested forty-eight hours post transfection and examined for protein expression via immunoblot (Figure 2). Levels of protein present in cell lysates were significantly higher in the TxA-RBD sample as compared to tPA-TxA-RBD or the GFP plasmid control. Detection of β -actin provides a loading control and indicates an equivalent volume of sample in each well. Protein levels detectable in the clarified supernatant are comparable between the two vaccine vectors. The electrophoretic sizes of the observed proteins in the supernatant differ between the two vaccine vectors with tPA-TxA-RBD yielding a larger product than TxA-RBD.

3.2 Immunogenicity

Antibody responses to the vaccine plasmids were first examined in BALB/c mice (Charles River), an H2^d background strain commonly employed in vaccine studies [33–36]. Six to 8 week old female BALB/c mice were obtained and inoculated as described in Methods and in Figure 3. Serum was harvested 6 weeks post-injection and anti-toxin antibody responses evaluated by ELISA. The procedure was performed twice in independent experiments (6 animals per group in each experiment) and the results are presented in Figure 4.

Inoculation with vector only (pVAX) yielded negligible background reactivity. Vaccination with plasmid DNA including a tPA leader sequence did not result in significant antibody responses and was not significantly improved by electroporation (Figure 4). In contrast, the vaccine vector lacking the tPA-signal peptide provided significant immunogenicity. Among animals receiving TxA-RBD via syringe injection, 11/12 mounted detectable anti-toxin antibody titers, a result not statistically different from those groups receiving tPA-TxA-RBD ($p>0.05$). Finally, all animals receiving TxA-RBD by electroporation mounted strong antitoxin antibody responses with most animals reaching a 1:10,000 titer, a result significantly different from all other groups ($p<0.05$).

To confirm these results, the protocol was repeated in a second mouse strain. Outbred CD-1 mice (5 animals per group per experiment) were vaccinated using a schedule identical to that employed in the BALB/c mice. The procedure was performed twice in independent experiments and the results are presented in Figure 5. Consistent with the BALB/c strain, antibody responses in animals receiving vector control were negligible. In contrast, both vaccine plasmids yielded antibody responses in nearly all animals regardless of the mode of delivery. Antibody titers in animals receiving TxA-RBD via electroporation were significantly higher than those of all other groups except those of animals who received tPA-TxA-RBD via electroporation. A single animal which received the TxA-RBD plasmid via electroporation did not have detectable anti-toxin antibodies. In contrast to results in BALB/c mice, electroporation significantly improved the mean antibody titers in animals receiving the tPA-TxA-RBD plasmid ($p<0.05$) as compared to titers in animals who received standard IM injection.

3.3 Toxin A Challenge

Mice are sensitive to purified *C. difficile* toxin A with LD₅₀ values of approximately 50ng following intraperitoneal (IP) challenge [37]. Thus, eight weeks post-vaccination, mice were challenged with 300ng ($6 \times \text{LD}_{50}$) of purified *C. difficile* toxin A via IP injection and examined every 12 hours for two weeks. Survival between groups was compared using pair-wise, log-rank analysis. Results are presented in Figure 6. Consistent with the sensitivity of mice to toxin

A, 100% of control animals died within 60 hours of toxin challenge, with most succumbing within 24 hours. By contrast, animals which had received TxA-RBD via electroporation demonstrated 100% survival. Mice in other groups manifested intermediate survival phenotypes. Animals inoculated with tPA-TxA-RBD suffered significant mortality regardless of delivery modality that was not significantly different from controls (2/12). Animals which received TxA-RBD by IM injection showed intermediate survival (4/12), a result significantly different from animals which received the tPA-TxA-RBD plasmids. Detailed results of the pair-wise comparison of mean survival times are provided in Supplemental Table 1.

The procedure and analysis was repeated as above, now using CD-1 mice. Similar to the BALB/c mice, 100% of control animals were dead at 24 hours post-challenge. In contrast to BALB/c mice and consistent with the uniformly high antibody titers, most animals in all other groups survived. Ten of 10 animals inoculated with TxA-RBD IM and tPA-TxA-RBD-EP survived while 9/10 animals receiving TxA-RBD-EP survived (NS, $p>0.05$) and 8/10 animals inoculated with tPA-TxA-RBD-EP survived (NS, $p>0.05$). Of note, the single animal in the TxA-RBD-EP group non-responsive to the vaccine by ELISA accounted for the sole mortality in that group, while the two animals in the tPA-TxA-RBD-IM group which succumbed to challenge had titers of 1:1000. Detailed results of the pair-wise comparison of mean survival times are provided in Supplemental Table 2.

4.0 Discussion

This report presents the first description of a DNA vaccine targeting *C. difficile* toxin A. Over the past decade, a variety of vaccines and immunotherapeutics intended to treat or prevent *C. difficile* disease have been tested in murine and hamster animal models [21,25–28,38–42]. The inactivated toxoid and monoclonal antibody platforms are the furthest in clinical development [43,44]. In fact, recently released phase II results of a monoclonal antibody product has provided the first evidence of the efficacy of an immunotherapeutic product in preventing recurrent diarrhea in affected human subjects [45]. While conceptually straight-forward, inactivated toxin strategies have certain disadvantages. Large proteins rely on the exogenous pathway of antigen presentation by antigen-presenting cells (APCs) and produce TH2 biased immune responses, particularly when administered with alum [46]. The lack of approved adjuvants (to date, only alum and MF-59) for co-formulation limits the ability to significantly alter this immunology [46]. Further, toxoids are based upon inactivation of whole, enzymatically active protein and carry the risk of toxicity due to inadequate inactivation procedures or difficulties in purification [47,48]. Finally, the inactivation process itself may alter structural domains important for antibody recognition [49].

DNA vaccines on the other hand, have several advantages as a vaccine platform. First, DNA vaccination involves the injection of recombinant genes encoding a protein of interest and relies upon endogenous synthesis of antigen by the inoculated host to induce effector immune responses [50]. As such, the DNA platform is often compared to viral infection in its ability to provide both humoral and cell-mediated immunity [51]. This technique permits rational inactivation or removal of potentially toxic enzymatic domains as well as inclusion of species-specific molecular adjuvants in a single product [52]. Indeed, the synthesis of DNA plasmids by bacterial cells during manufacturing process ensures the presence of potentially immunogenic CpG motifs [53]. However, despite examples of successful DNA vaccination against bacterial and toxin-mediated diseases in animal models [54–58], a DNA vaccine against *C. difficile* toxin A presented several challenges.

Firstly, the toxin A gene is over 9 kilobases in length and encodes a protein product of over 300kD. Genes encoding protein products of such size are difficult to construct, may express poorly, and provide poor immunogenicity when delivered as DNA vaccines. Fortunately,

neutralizing antibodies map to the carboxy-terminal receptor binding domain [59–63] permitting a significant reduction in size. Secondly, the genome of *C. difficile* contains significant A–T content. While not universally true, several DNA vaccines encoded by AT rich sequences have proven poorly immunogenic as a result of low protein expression [58, 64] or a paucity of endogenous CpG motifs [53,58]. The DNA vaccine candidates employing AT-rich wild-type genes which have proven immunogenic often express well *in vitro*, despite their AT content [55,65,66]. For example, a study comparing codon-optimized versus non-optimized DNA vaccines against *C. tetani* toxin showed minimal expression of the non-optimized vaccine *in vitro* and no immunogenicity *in vivo* despite controlling for CpG content [58]. To avoid this issue, a synthetic *C. difficile* gene was created employing only the most common codons used in the human genome (<http://www.entelechon.com>).

The third hurdle in the design of this vaccine was whether to retain eukaryotic asparagine-linked glycan residues (N-linked glycans). Most published data describing DNA vaccines targeting other prokaryotic toxins reveals conservation of N-glycan signals. This bias may follow from their direct assembly from amplified genomic sequences rather than intentional design [54,55,57,65–67]. One study directly examined the effect of elimination of N-glycan signals in a recombinant DNA vaccine against anthrax PA- and LF-toxins [55]. In both cases elimination of N-glycans dramatically reduced *in vitro* expression and subsequent immune responses in vaccinated mice [55]. With this work in mind, all N-glycan signals were retained in the synthetic *C. difficile* gene.

Following confirmation of *in vitro* expression, studies were undertaken to describe vaccine immunogenicity in mice. While often effective in mice, DNA vaccines suffer from lackluster performance in larger animals, possibly due to dose limitations and limited plasmid uptake by host cells [68,69]. Methods which augment DNA uptake by host cells, whether natural (bacterial [70], viral [71]) or artificial (electroporation [72], gene-gun [73], biojector [74]) provide the means to improve DNA vaccine immunogenicity. This study employed *in vivo* electroporation (Ichor Medical Systems, Inc.), a method proven to logarithmically increase antigen expression and the resulting immune responses, in particular, humoral immune responses [75–77]. The results in the BALB/c strain of mice strongly support the utility of *in vivo* electroporation as a method to enhance immune responses to DNA vaccines. In this animal strain, electroporation significantly improved performance of the TxA-RBD vaccine such that all animals who received the vaccine via electroporation were protected from toxin challenge. While less obvious, antibody titers in CD-1 mice also segregated by delivery method with those animals receiving vaccine by electroporation showing significantly higher antibody titers.

Antibody titers observed in the BALB/c strain were higher in animals receiving the vector without a tPA leader sequence. The marked difference in protein production seen in the *in vitro* analysis is one likely explanation. Further, the obvious size difference in the protein products observed in the cell supernatant suggests that the tPA sequence was not cleaved from the corresponding protein. Thus, this tPA sequence may not function as expected in association with this protein sequence. Finally, potential differences in glycosylation between the protein products produced by the two vectors may have influenced subsequent immune responses.

The final question examined in this study was whether the vaccine-specific immune responses were able to protect animals from toxin challenge *in vivo*. Mice are not susceptible to gastric challenge with *C. difficile* spores but are very sensitive to parenteral delivery of active, purified *C. difficile* toxins [32,37,78]. While not recapitulating gastrointestinal disease, this method is a first step to examine the functionality of antibody responses to *C. difficile* toxins *in vivo*. In this study, survival corresponded best to groups with anti-toxin antibody titers $\geq 1:1000$, regardless of strain. In BALB/c mice, 100% of animals who received TxA-RBD via electroporation were protected from toxin A challenge while 100% of animals inoculated with

vector control succumbed to toxin. The other vaccine regimens produced intermediate survival phenotypes. Consistent with their high antibody titers, all CD-1 animal groups receiving vaccine experienced high survival rates following toxin challenge regardless of plasmid or delivery method. The survival rates demonstrated in the BALB/c mice receiving TxA-RBD via electroporation and in all CD-1 groups receiving vaccine compare favorably to published reports of the protection provided by inactivated toxoid and carrier conjugate vaccines [32, 78].

One unexpected observation made in the study was the mouse strain dependent difference in vaccine immunogenicity. Several similar reports specific to BALB/c and CD-1 mice have been published previously. First, Pirzadeh and colleagues published an examination of the immunogenicity of a DNA vaccine encoding the glycoprotein 5 (GP5) of porcine reproductive and respiratory syndrome virus (PRRSV) in BALB/c and CD1 mice [79]. Animals were inoculated with 50µg of a DNA vaccine encoding PRRSV-GP5 protein fused to glutathione *S*-transferase or control plasmid. By day 65 post-inoculation, CD-1 mice mounted binding antibody titers of >12800 (reciprocal of the highest dilution by ELISA) while titers in BALB/c mice reached only 2560. Interestingly, serum from CD-1 mice was not capable of neutralizing virus in an *in vitro* neutralization assay while serum from BALB/c mice demonstrated a viral neutralization titer of 102. Authors hypothesized strain dependent differences in the recognition of linear B-cell epitopes as a possible explanation. A study published by Toapanta and Ross examined the immune responses to a DNA-based HIV vaccine expressing gp120 fused to multiple copies of the complement component C3D in three inbred (BALB/c, C57BL/6, and C3H/He) and one outbred mouse strain (CD-1) [80]. Overall antibody titers at the end of study were similar, however, differences were observed between inbred and outbred strains in antibody isotype, antibody avidity, and in the profile of cytokines released by splenocytes following gp120 stimulation. Antibody responses in the inbred strains were of a predominantly IgG₁ isotype and manifested low to moderate avidity. Splenocyte cytokine release from vaccinated inbred mice was exclusively IL-4. In contrast, CD-1 mice manifested a mixed IgG isotype response (IgG₁ and IgG_{2a}) with high avidity. Splenocyte cytokine release from vaccinated CD-1 mice demonstrated both IFN-γ and IL-4. Finally, data published in 2004 by Ito and colleagues specifically examined the differences in antibody responses to DNA and protein vaccination between outbred (ICR, ddY), H-2 congenic strains (B10.A, B10.BR, B10.D2), and inbred strains (C57BL/6, A/J, C3H/He, BALB/c, C57BL/10) of mice [81]. Using vectors encoding green fluorescent protein (GFP) or β-galactosidase (β-gal), authors found differences that varied by encoded protein and strain. In one example, no inbred or H-2 congenic strain of mouse responded to immunization with plasmid expressing GFP while both outbred strains responded with high antibody titers. No differences were found in RNA transcript levels within transfected muscle and the difference was overcome by GFP peptide inoculation indicating possible differences in protein expression beyond the level of transcription. Unfortunately, tissue was not examined for *in vivo* GFP or β-gal expression. In contrast, immunization with DNA encoding β-gal induced antibody responses in all strains. However, antibody titers among the inbred and congenic strains segregated by H-2 background. Authors concluded that antibody response to DNA vaccination in mice was influenced by both *in vivo* antigen expression as well as the haplotype background of the animals.

In conclusion, we have developed a DNA vaccine encoding the receptor binding domain of *C. difficile* toxin A. This vaccine is well expressed *in vitro*, is immunogenic *in vivo*, and protects mice from lethal toxin challenge. A similar vaccine approach specific for the toxin B of *C. difficile* is underway. We believe such a combination vaccine may warrant further testing in other animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A schematic description of *C. difficile* toxin A and the vaccine vectors

A Linear depiction of the three major domains identified within *C. difficile* toxin A. (Modified from Voth and Ballard. *Clin Micro Reviews*, April 2005; p 247–263.) The region containing the vaccine sequence is indicated by the underline. **B.** Schematic depiction of the vaccine gene sequence as inserted into the eukaryotic expression vector, pVAX. These plasmids differ only in the presence or absence of a tissue plasminogen activator (tPA) signal peptide sequence.

Figure 2. Protein expression from vaccine vectors or control following transient transfection *in vitro*

Immunoblot of 293T cell lysates and supernatants following transient transfection with pVAX encoding green-fluorescent protein (Mock), TxA-RBD in pVAX, or tPA-TxA-RBD in pVAX for detection of expressed protein products. Supernatant was clarified at $20,000 \times g$ for 30 minutes prior to the procedure. The expected size of the expressed product is 100kD. Identical preparations were examined for the presence of human β -actin using a monoclonal antibody specific for β -actin as a volume loading control.

Figure 3. Schematic representation of the vaccination sequence

A Animal groups, vaccine doses, and vaccine delivery mechanism for subsequent immunological analysis for both BALB/c and CD-1 mice. **B.** Vaccine inoculation sequence, timing of blood harvest, and final toxin challenge for both BALB/c and CD-1 mice.

Figure 4. Toxin-specific antibody responses in BALB/c mice following the vaccination procedures
Serum anti-toxin IgG ELISA responses in BALB/c mice. The data is the result of two independent experiments. Values are representative of two independent ELISA procedures. Group-specific titers were compared using Kruskal-Wallis test for overall significance ($p < 0.0001$). Asterisk (*) represents statistical significance between group titers at $p < 0.05$ following pair-wise one-way ANOVA with Bonferroni correction for multiple comparisons.

Figure 5. Toxin-specific antibody responses in CD-1 mice following the vaccination procedures
 Serum anti-toxin IgG ELISA responses in CD-1 mice representing data from of two independent experiments. Group-specific antibody titers were compared using a Kruskal-Wallis test for overall significance ($p < 0.0001$). Asterisk (*) represents statistical significance between group titers at $p < 0.05$ following pair-wise one-way ANOVA with Bonferroni correction for multiple comparisons. Symbol (§) indicates $p > 0.05$ for the indicated pairwise comparison.

Figure 6. Survival in vaccinated BALB/c mice following challenge with purified *C. difficile* toxin A Kaplan Meier survival curve in vaccinated BALB/c mice following challenge with 300ng of freshly reconstituted toxin A from *C. difficile* in 100μl of sterile saline. The data is the result of two independent experiments. Statistical (p) value represents the likelihood of a significant difference between all groups following pair-wise log-rank analysis between groups.

Figure 7. Survival in vaccinated CD-1 mice following challenge with purified *C. difficile* toxin A
 Kaplan Meyer survival curve in vaccinated CD-1 mice following challenge with 300ng of freshly reconstituted toxin A from *C. difficile* in 100μl of sterile saline. The data is the result of two independent experiments. Statistical (p) value represents the likelihood of a significant difference between all groups following pair-wise log-rank analysis between groups. “NS” indicates differences not significant $p>0.05$ following pair-wise log rank analysis.