IMMUNIZATION WITH A CHIMERA BETWEEN THE B SUBUNIT OF STX2 AND BRUCELLA LUMAZINE SINTHASE CONFERS TOTAL PROTECTION AGAINST SHIGA TOXINS

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ABSTRACT

The striking feature of Enterohemorrhagic *Escherichia coli* (EHEC) infection is the production of potent Shiga toxins (Stx) implicated in the development of the life-threatening hemolytic-uremic syndrome (HUS). Despite the magnitude of the social and economical problems caused by EHEC infections, no licensed vaccine or effective therapy is presently available for human use. One of the biggest challenges is to develop an effective and safe immunogen to ensure non toxicity but also a strong input to the immune system to induce long-lasting, high affinity antibodies with anti-Stx neutralizing capacity. The enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly stable dimer of pentamers and a scaffold with enormous plasticity for the display of foreign antigens on its structure. Taking into account BLS advantages and the potential capacity of B subunit of Stx2 (Stx2B) to induce antibodies that prevent Stx2 toxicity by blocking its entrance to the host cells, we engineered a new immunogen by inserting Stx2B at the amino termini of the BLS gene. The resulting chimera demonstrated a strong capacity to induce long-lasting humoral immune response. The chimera induced antibodies with high neutralizing capacity for Stx2 and its variants in mice. Moreover, immunized mice were completely protected against high lethal doses of Stx2-challenge up to ten months after the last immunization. We conclude that this novel immunogen represents a promissory candidate for vaccine or antibody development with preventive or therapeutic ends, for use in HUS endemic areas or during future outbreaks caused by pathogenic strains of Stx-producing *E. coli.*

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) strains are important human food-borne pathogens [1]. The clinical manifestations of EHEC infections range from watery diarrhea, or hemorrhagic colitis (HC), to the most severe outcome, the life-threatening hemolytic-uremic syndrome (HUS)[2]. The infection correlates with ingestion of contaminated meat or vegetables, but is also transmitted by water or even person-to-person contact [3, 4]. Sporadic or massive outbreaks have been reported in several developed countries but, in Argentine, HUS shows an endemic behavior and represents a serious public health problem with high morbidity and mortality values [5, 6].

The striking feature of EHEC infection is the production of potent Shiga toxins, responsible of HUS development [7, 8]. The Shiga toxin (Stx) is formed by a single A subunit, which possesses N-glycosidase activity to the 28S rRNA and promotes protein synthesis inhibition in eukaryotic cells, and five B subunits, which bind to globotriaosylceramide (Gb3) at the surface of host cells [9, 10]. Although two major types (Stx1 and Stx2) and several subtypes (variants) have been described, so far Stx2 and its variant Stx2c are the most frequently toxins found in severe HUS cases among EHEC infected subjects [11, 12]. Stx1 and Stx2 differ significantly both in amino acid sequences and in biological functions [13, 14]. Although some authors report that Stx1 and Stx2 do not provide heterologous protection through their B subunits, this matter remains controversial [15-17]. On the other hand, Stx2c and Stx2d variants are readily neutralized with antibodies against Stx2, but not with antibodies against anti-Stx1 [18].

Despite the magnitude of the social and economical problems caused by EHEC infections, no licensed vaccine or effective therapy is presently available for human use. One of the biggest challenges is to develop an effective and safe immunogen to ensure non toxicity but also a strong input to host immune system to induce long-lasting, high affinity antibodies that ensure a good neutralization capacity in serum. Despite multiple approaches, this goal has not been successfully reached, mainly because the B subunit of Stx2 (StxB), one of the most attractive candidates, is a very poor immunogen [19]. It is known that there is a close correlation between the degree of repetitiveness and thermodynamic stability of an antigen and the efficiency of induction of the B cell response [20]. Several studies have shown that the pentameric

arrangement of Stx2B is only marginally stable [21], explaining at least in part this lack of immunogenicity. The enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly immunogenic and stable dimer of pentamers and a scaffold with enormous plasticity to display foreign antigens on its structure [22-26]. The N-termini are displayed at the vertices of a symmetric pentamer at a distance of 40 Ångströms between each other. These are the insertion sites that we used to decorate BLS with the B subunits, taking advantage of the similarity of the oligomeric state of both proteins. We reasoned that BLS would be a perfect scaffold to accommodate and stabilize the five monomers of B subunit, as long as a flexible linker long enough to avoid steric hindrance is used to connect the monomers of both proteins. The pentamer of B subunits folded on top of each of the BLS pentamers would benefit from the high local concentration of the subunits dictated by the covalent attachment to the scaffold, and the high thermodynamic stability of BLS. Effectively, the resulting chimera (BLS-Stx2B) shows a remarkable stability and demonstrated a strong capacity to induce long lasting humoral immune responses. The chimera administered to mice under different formulations and regimens induced antibodies with high neutralizing capacity for Stx2 and its variants. Moreover, mice immunized with BLS-Stx2B were completely protected against high lethal doses of Stx2 challenge i.p. to ten months after last immunization dose. We conclude that this novel immunogen represents a promissory candidate for vaccine or antibody development with preventive or therapeutic ends to be used in HUS endemic areas or during future outbreaks caused by pathogenic strains of Shiga toxin-producing *E. coli*.

RESULTS

Engineering of the chimeric protein BLS-Stx2B

The construction of the chimera was carried out by the same strategy described by Laplagne et al. [26]. The coding sequence of the first eight residues of the N-terminal end of BLS was replaced with the coding sequence of Stx2B and a flexible G/S decapeptide linker (GSGSGSGSGS) that connects both proteins. Both Stx2B and BLS form pentameric structures of the same C5 symetry in which each protomer interacts with other two. Molecular modeling of the chimera (Fig. 1) suggests that the linker used would be long enough to allow the assembly of the Stx2B pentamers onto the pentameric modules of BLS without any steric hindrance. In the decameric BLS particle, two Stx2B pentamers would be displayed in opposite directions at the top and bottom of the structure. It is worth noting that the C-terminal surface of Stx2B (which interacts with the A subunit in the Stx2 holotoxin) would be contacting the BLS scaffold, whereas the opposite surface (that contains the globotriaosylceramide (Gb3) binding sites) would be totally exposed to the solvent.

Expression and characterization of recombinant BLS-Stx2B protein

The plasmid pET-BLS-Stx2B was transformed into competent *E. coli* BL21 (DE3) cells. The inclusion bodies, containing the majority of the BLS-Stx2B protein, were dissolved in 8.0 M urea. As wild type BLS remains folded in those conditions [23, 27, 28], we reasoned that the chimera at 8.0 M urea would have BLS in the folded state whereas the Stx2B subunit would be unfolded. Thus, we decided to dialyze the protein against 1.0 M urea to allow the refolding of Stx2B and the correct assembly of the protein particle. The refolded material was purified by ionic exchange chromatography (see Figure S1).

To confirm the correct folding of the BLS-Stx2B protein, static light scattering (SLS) and circular dichroism (CD) analyses were performed. The similarity between the theoretical MW (254 kDa) and the MW measured for the major peak (234 kDa) (Figure 2A) indicates that BLS folds as a decamer in the chimera. The far UV-CD spectrum of BLS-Stx2B (Figure 2B) was shown to be practically identical to that theoretically calculated for this protein from the combination of the CD signals of the isolated BLS and Stx2B modules. These results altogether indicate that both BLS and Stx2B are

properly folded in the structure of the chimera and that BLS-Stx2B has the expected quaternary structure.

Binding of BLS-Stx2B to Globotriaosyl ceramide (Gb3).

Since the pentameric conformation of Stx2B is required for Gb3 binding, a Gb3-binding ELISA assay was used to functionally confirm that BLS-St2B assembled into the native holotoxin B subunit configuration. We observed a positive reaction in a dose dependent fashion with the chimera and the rStx2 holotoxin, but not with BLS (Figure 2C). From these findings, we conclude that the B subunits on top of the BLS decamer are correctly assembled and maintain their Gb3 binding capacity.

Immune response

Time course of the antibody-titers during the immunization

To assess the ability of BLS-Stx2B to improve the humoral immune response compared to isolated recombinant purified Stx2B subunit, groups of mice were i.p. injected with equimolar amounts of the chimera or Stx2B in the presence of Freund Adjuvant (FA). We examined specific IgG antibody in sera from vaccinated mice during more than two months. IgG titers specific to Stx2B, were elicited in both vaccinated groups (Figure 3A). ELISA titers of the BLS-Stx2B group rose during the first three vaccinations and peaked at 14 days post the third vaccination. However, Stx2B-immunized mice did not show specific antibody response before 45 days after the first vaccination dose, with high titer variability between individuals. At all analyzed times, the antibody titers generated by BLS-Stx2B were significantly higher than those of the Stx2B group ($P < 0.005$) (Figure 3A). No Stx2B specific antibodies were detected in BLS-immunized group.

Antibody-titers under different immunization protocols

To evaluate BLS-Stx2B capacity to stimulate the immune system and to generate antibodies under different formulations or vaccine regimens, we immunized groups of mice with BLS-Stx2B protein with Freund Adjuvant (FA) or Aluminum hydroxide (AH), without any adjuvant, or following a DNA-protein prime-boost schedule. Serum samples were collected at different times after vaccination and titers of specific IgG were determined by ELISA. The results indicate that BLS-Stx2B stimulates the immune response under all protocols tested, even without any exogenous adjuvant (Figure 3B).

However, differences in the intensity and/or kinetic of the humoral response among different protocols were observed. In particular, immunization with BLS-Stx2B formulated in AH or FA was kinetically similar in the development of specific antibody, but mice immunized with FA reached significant higher titers (Figure 3B). On the other hand, the DNA–protein prime-boost regimen induced Stx2B specific antibodies lately (35 days after the protein boost), but reached at this time point the same titer than BLS-Stx2B with AH. The protein in absence of adjuvant induced the lowest humoral response, in terms of maximal titers and time to reach them. In fact, mice from this group showed anti-Stx2B specific antibodies after the third immunization and the antibody titer was 10 fold lower than mice immunized with BLS-Stx2B in HA.

Antibody subtyping

IgG1 is associated with the Th2-mediated humoral immune response, and IgG2a is associated with Th1-mediated cellular response. To determine which type dominates the immune response, we examined the titers of the IgG subtypes (IgG1 and IgG2a) in mouse sera from all BLS-Stx2B groups at 45 days after the last immunization dose. BLS-Stx2B antigen induced higher anti-Stx2B specific IgG1 than IgG2a titers ($P < 0.001$) for all protocols analyzed except in prime-boost schedule, in which there was not significant differences between both IgG subclasses (Figure S2).

Affinity

Since neutralizing and protective capacity of sera is closely dependent on the titer but also on the affinity of antibodies for Stx2, we evaluated sera affinity from the different vaccinated groups by ELISA test upon dissociation with ammonium thiocyanate. Results shown in Table 1A indicate that sera from mice immunized with BLS-Stx2B formulated with FA or AH, displayed antibodies with higher affinity for Stx2B than sera from mice immunized with purified Stx2B in FA. Similar affinity results were observed in sera collected from mice immunized with BLS-Stx2B without adjuvant or in prime-boost regimens.

Neutralization titers against recombinant Stx2 (rStx2)

A more correlative *in vitro* indicator of the protective immune response is the neutralization in the Vero cells cytotoxicity assay. In the neutralization test against rStx2, sera from mice immunized with BLS-Stx2B formulated with FA showed the

highest neutralizing titer (P<0.001). In contrast, sera from mice immunized with isolated Stx2B, even when formulated in FA, showed the lowest neutralization activity (Table 1A). Sera from non-immunized or BLS-immunized mice did not show neutralization activity.

Cross-reactivity of mouse sera

Then, we evaluated neutralizing activity against wild toxins produced by pathogenic EHEC strains. For this purpose, supernatants from human-isolated EHEC strains producing Stx2, or Stx2c or Stx2d variants were incubated with sera from immunized mice and toxicity on Vero cells was evaluated. We also evaluated neutralization capacity against recombinant Stx1. Sera from mice immunized with BLS-Stx2B strongly neutralized wild-type Stx2 and its variants, and also Stx1. In sharp contrast, only the serum sample harvested from one out of six mice immunized with purified Stx2B was able to weakly neutralize wild-type Stx2, and none of them neutralized Stx2 variants or Stx1 (Table 1B).

Protection of immunized mice against rStx2 challenge

Ex vivo rStx2 neutralization activity

In addition, the anti-Stx2 neutralization activity was evaluated *ex vivo* in mortality curves pre-incubating 1 LD_{100} of rStx2 with one neutralizing unit of each pool of sera from different experimental groups. As indicated in Figure 4A, pre-incubation of rStx2 with sera harvested from mice immunized with BLS-Stx2B under different protocols fully abrogated rStx2 toxicity. On the other hand, sera collected from mice immunized with Stx2B or BLS did not prevent rStx2-toxicity.

In vivo Stx2 neutralization activity

As a final demonstration of the protective efficacy of the novel BLS-Stx2B vaccine, we challenged all groups of immunized mice with 1 LD_{100} of rStx2 fifty days after the last immunization. Figure 4B shows that 100% of the BLS-Stx2B vaccinated mice and 33% of the Stx2B vaccinated mice survived the rStx2 lethal challenge. According to the rStx2 dose used, 0% of the animals immunized with BLS or non-immunized survived the challenge (Figure 4B).

Long–term evolution of immune response

Sera from surviving BLS-Stx2B immunized mice were long term harvested to study the duration of specific antibody response (Figure 5A). Although all BLS-Stx2B regimens induced long-lasting immune response evaluated as anti-Stx2B antibodies by ELISA, the prime-boost schedule was the protocol that displayed highest anti-Stx2B titers after 10 months since the last immunization. In spite of this, all BLS-Stx2B vaccinated mice survived the challenge with $2LD_{100}$ at eight months after the last immunization dose (Figure 5B), and the challenge with 3 LD_{100} at 11 months after the last immunization dose (Figure S3).

DISCUSION

The major finding in this report is that the engineering of a chimera between BLS and Stx2B is highly efficacious as a vaccine against the potent cytotoxins Stx2, Stx2-variants and also Stx1. The use of genetic toxoids of Stx-type toxins is not a novel vaccine strategy [16, 17, 29, 30]. However, the genetic construction of a chimeric toxoid engineered with the scaffold protein BLS decorated with 10 copies of the B subunit of Stx2, is a new approach to circumvent the lack of immunogenicity of this binding subunit [19].

Both components of the chimeric toxoid were selected based on their particular properties. The B subunit of Stx2 was selected because, among the Stx family, Stx2 is the most pathogenic toxin. Since cross reactivity between B subunits is controversial [15-17], a Stx2B-based immunogen would protect against the Stx most related to HUS development. In addition, the B subunit represents the binding unit of the toxin and is non-toxic for mammalian cells [9, 10]. Antibodies able to block the binding process to the specific receptor (Gb3) in mammalian cells should prevent the first step of the toxicity cascade [31]. In addition, a Stx-based vaccine against HUS would not only protect against known EHEC strains, typically O157 and non-O157 serotypes, but it would also be useful against new or rare pathogenic strains of Shiga-toxin-producing *E. coli*, as was the case of the recent large outbreak of HUS caused by the O104:H4 strain [32, 33].

The other component of the chimera, BLS, was chosen because of its advantages as carrier for antigen delivery [24-26]. BLS is especially suited since it arranges as a dimer of pentamers that share the same C5 symmetry as the pentameric Stx2B antigen. The N termini of the pentameric modules of BLS are located at opposite sites at the top and bottom of the decameric particle. These are suitable insertion sites for the display of target proteins through recombinant fusion. However, the display of non-monomeric proteins in the context of BLS may be problematic because of inter-particle crosslinking and aggregation through homomeric interactions of the target subunits. This phenomenon may be critical when protomers remain free because of uneven stoichiometry between the target oligomer and BLS. By contrast, we reasoned that BLS would represent a perfect scaffold for the assembly and stabilization of a pentameric protein if flexible peptide linkers of convenient length are used for the attachment of both proteins to avoid steric hindrance. The results presented herein indicate that the

attachment of the Stx2B subunits to the BLS scaffold promote the pentamerization of the toxin and stabilize its structure due to the high local concentration of the subunits and the influence of the high thermodynamic stability of BLS.

The strong B cell response elicited when mice were immunized with BLS-Stx2B could be explained by this stabilization and also by the ability of BLS to target and activate dendritic cells [34]. Specific ELISA titers and neutralization activity of the antibodies elicited by BLS-Stx2B were significantly improved when compared to those elicited by Stx2B, when both immunogens were administered in the same formulation (Freund's adjuvant). The most likely explanation is that the Stx2B pentamer is only marginally stable in the absence of the A subunit, thus, when used as immunogen, is not able to raise specific antibodies against conformational epitopes that are located mostly at the interfaces between monomers of the pentamer. In constrast, the display onto BLS strongly increases the stability of Stx2B, allowing the development of antibodies against the native conformation of the pentamer as it is in the holotoxin. These results also confirm that the low immunogenicity of the B subunit of Stx in mice is not due to its adverse effect to germinal center B cells has been previously suggested [35].

More significantly, the antibodies induced by the chimera show similar neutralizing capacity to the wild-type Stx2, its variants and also Stx1. It has been previously demonstrated that all Gb3 binding sites in this family of toxins are located on the same face of the B pentamer, opposite to the A subunit. Two of the three binding sites are formed by residues contributed by neighboring monomers, which requires the right assembly of the pentamer [31]. Thus, the sera capacity to neutralize different Stx family members would be mainly due to antibodies recognizing these binding sites that are conserved in all members of the Stx family [36], and only present when B subunit adopts the spatial pentameric conformation [37]. This fact is of great importance for prophylaxis or therapeutics of HUS, because antibodies showing broad reactivity against Gb3 binding sites should be highly effective on preventing the damage caused by the whole Stx family.

Effective vaccines rely on two key factors 1) an Ag against which adaptive immune responses are generated, 2) an immune stimulus or adjuvant to signal the innate immune system to potentiate the Ag-specific response [38]. Highly purified Ags offer potential advantages over traditional vaccines, including their safety and the capacity to elicit highly specific immune responses [39], but in general they need to be coadministered with additional immunostimulant substances (adjuvants) because they are poorly

immunogenic [38, 40, 41]. In the present study, the immunization with BLS-Stx2B chimera formulated in Freund's adjuvant was the most effective schedule to induce high titers of specific anti-Stx2B antibodies. However, because Freund's adjuvant is only for experimental approaches, we tested the immune response and protection induced by the chimera formulated in aluminum (HA) and without exogenous adjuvant. Interestingly, all BLS-Stx2B immunization regimens, even in the absence of adjuvant, induced a clear protection against the toxin both in *in vitro* and *ex vivo* experiments. Most importantly, all mice vaccinated with BLS-Stx2B chimera were protected against recombinant Stx2 challenge, in spite of the low ELISA antibody titers observed in some cases. These results encouraged us to propose that this immunogen could be used as a vaccine against HUS.

In line with previous data [42, 43], we found that the DNA-protein prime- boost regimen resulted in a long-lasting production of anti-Stx2B specific antibodies with neutralizing capacity. In fact, 9 months after the last immunization dose mice vaccinated with the DNA-protein prime-boost regimen, showed specific antibody ELISA titers similar to their highest values, while sera titers from mice vaccinated with Freund adjuvant declined to 25% of their maximum values. It is important to highlight that all vaccinated mice remained healthy for 10 months, and were protected even after injection of recombinant Stx2 at a dose two-fold (8 months) or three-fold (10 months) the LD100.

In conclusion, considering that Stx2 is the necessary pathogenic factor associated with HUS development, our results demonstrate that BLS-Stx2B should be a useful candidate for the development of subunit vaccines against HUS, or for development of therapeutic anti-Stx neutralizing antibodies to be used during future outbreaks with enterohemorragic *E. Coli*, or new strains carrying Stx genes.

MATERIALS AND METHODS

Engineering, expression and purification of BLS-Stx2B

The *stx2b* coding sequence was amplified by PCR with primers: *Forward* 5´-ATCAACATGCATGCGGATTGTGCTAAAGGT-3´ (NsiI site underlined) and *Reverse10* 5'-TAAAATCTTAAG**AGAACCAGAACCAGAACCAGAACCAGAACC**GTCATTATTAAACTGCAC-3' (AflII site underlined). Primer *Reverse10* included the coding sequence for a 10-aminoacid linker (GSGSGSGSGS, in bold letters). The PCR product was cloned upstream to BLS gene, in a pET11a vector previously generated which contains the BLS sequence [26], generating plasmid pET-BLS-Stx2B. To ensure that the plasmid construct was intact and functional, it was sequenced across the gene insert.

The plasmid was transformed into *E. coli* BL21 (DE3) competent cells for expression of the recombinant protein. Inclusion bodies containing BLS-Stx2B were solubilized by overnight incubation in 8 M urea, 50 mM Tris/HCl, 5 mM EDTA, pH 8 buffer and dialyzed against 1 M urea, 50 mM Tris/HCl, 5 mM EDTA, pH 8.5 buffer. The solubilized proteins were purified by anion exchange chromatography in a Q-Sepharose (Pharmacia, GE Healthcare Life Sciences) column using a HPLC apparatus (Gilson model 320). Elution was performed using a linear gradient between 0 and 1M NaCl in a 1 M urea, 50 mM Tris/HCl, pH 8.5 buffer.

Protein was dialyzed against phosphate-buffered saline (PBS) previous to each immunization.

pCI-BLS-Stx2B DNA vaccine

In order to develop a DNA vaccine coding for BLS-Stx2B (pCI-BLS-Stx2B), the BLS-Stx2B DNA sequence was amplified by PCR using pET-BLS-Stx2B as template and sub-cloned in the pCI-neo vector (Promega, Madison, WI, USA) with primers pCIForward 5´-GTTTAA**GAATTC**GAAGGAGATACC**ACCATG**CATGCGGAT-3' (EcoRI site underlined, KOZAK consensus sequence in bold letters) and pCIReverse 5'-TGTCCACCAGTCATGCTAGCTCAGACAAGCGCGATGC-3' (NheI site underlined). To ensure that the plasmid construct was intact and functional, it was sequenced across the gene insert.

Plasmid was amplified in *E. coli* DH5α cells and isolated using "maxi prep" plasmid isolation columns (Qiagen Inc., CA, USA) following manufacturer's instructions.

Immunization protocols and sample collection

Adult BALB/c mice were immunized with 3 doses of BLS-Stx2B, with Freund's adjuvant (FA, DIFCO-BD, Detroit, Michigan, USA) (intraperitoneal, i.p.); Aluminum hydroxide (AH) (subcutaneous, s.c.) or with no adjuvant (N/A) (i.p.) on days 0, 15 and 30. Groups of mice were also i.p. immunized with BLS and Stx2B in FA. The doses of BLS and BLS-Stx2B were corrected by their molecular weights to inoculate equimolar amounts of each protein compared to that of Stx2B (the dose was equivalent to 20 µg of Stx2B).

For prime boost immunization, mice were injected with 100 µg of pCI-BLS-Stx2B on days 0, 14 and 28 by intramuscular (i.m.) route into the rear legs, followed by a final i.p. booster performed with BLS-Stx2B in incomplete FA at day 40.

Details of the structural analysis of BLS-Stx2B (Circular Dichroism, Light Scattering, Gb3 binding), analysis of antibody responses (Stx2B ELISA, Affinity ELISA, *in vitro* and *in vivo* neutralization) and statistical analysis are provided in SI Materials and Methods.

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LEGENDS

Figure 1. *Molecular modeling of BLS-Stx2B*

(A) Lateral view of BLS-Stx2B. BLS decamer is colored cyan and each Stx2B pentamer is colored green. The flexible decapeptide linker is colored blue. Relevant aminoacids for Gb3 binding in site 1 (magenta) and 3 (red) are colored to highlight the importance of the pentameric assembly achieved in BLS-Stx2B.

(B) Top view of BLS-Stx2B. Stx2B pentamer could assemble on top of the BLS pentamer without steric hindrance.

Figure 2. *Structural analysis of BLS-Stx2B*

(A) Size exclusion chromatography coupled to SLS analysis of BLS-Stx2B chimera.

(B) Comparison of the far UV-CD spectra of BLS, Stx2B and BLS-Stx2B. The theoretical spectrum of BLS-Stx2B was calculated from the combination of the Stx2B and BLS spectra.

(C) Assessment of BLS-Stx2B assembly by the Gb3-binding ELISA assay. Serially diluted BLS-Stx2B, BLS, rStx2 or *E. coli* BL21 lysate (used as negative control for nonspecific binding) were added to Gb3 coated plates. Binding was determined as detailed in SI Materials and Methods.

Figure 3. *Time course of specific IgG titers against Stx2B*

(A) Stx2B-specific IgG titers in sera from mice immunized with BLS-Stx2B or Stx2B, both formulated in FA, were determined by ELISA as detailed in SI Materials and Methods. Each time point represents the mean ± SEM of 4-6 mice/group. ** $P < 0.005$ vs the same time point of Stx2B group.

(B) Stx2B-specific IgG titers in sera from mice immunized with Stx2-BLS in different formulations or regimens. Each time point represents the mean ± SEM of 4-6 mice/group. * $P < 0.05$ vs the same time point of BLS-Stx2B with no adjuvant (N/A) group; ** $P < 0.005$ vs the same time point of all others groups.

Black arrow-heads indicate protein immunizations; Grey arrow-heads indicate DNA immunizations.

Figure 4. *Mortality curves*

(A) *Ex* vivo neutralization of rStx2 toxicity ($1LD_{100}$) with sera from immunized mice. Pools of sera from each immunized group (4-6 mice/pool) (45 days post last immunization) or non-immunized mice, were diluted according to the *in vitro* neutralization titers as indicated in the figure's reference. ** P< 0.005 vs all other groups.

(B) Protection of immunized mice against a lethal challenge of purified rStx2. Immunized mice with Stx2B + FA, different formulations of BLS-Stx2B or BLS + FA (4-6 mice/group), were i.v. challenged with 1 LD100 rStx2 50 days after the last immunization. * P<0.05 vs Stx2B + FA group, and P< 0.005 vs non-immunized or BLS + FA groups.

Figure 5. *Long term specific IgG abtibody response and protective immune response.*

(A) Titers of specific IgG against Stx2B assayed by ELISA up to 9 months after the last immunization dose. * P<0.05 and ** P<0.005 compared to BLS-Stx2B with no adjuvant (N/A) or AH immunized mice

(B) Mice surviving the first rStx2-challenge received a second challenge with $2LD_{100}$ of rStx2 at the time indicated in part A (2nd challenge). ** P<0.005 compared to non-immunized mice.

Table 1. *Antigen affinity and rStx2-neutralization capacity of sera from immunized mice*

(A) Antibody affinity and rStx2-neutralization capacity of mouse sera from immunized mice (45 days post last immunization) was assayed as described in SI Materials and Methods. Results are expressed as the mean ± SEM of 4-6 mice/group.*P < 0.05 vs Stx2B + FA group; *** P<0.001 vs all other groups.

(B) Neutralization titers against purified rStx1, wild Stx2 and its variants. Sera from immunized mice with Stx2B or BLS-Stx2B, both formulated with FA (45 days post last immunization), were incubated *in vitro* with 1CD50 of rStx1 and EHEC-produced Stx2 variants. Vero cytotoxicity was assayed as detailed in SI Materials and Methods. Each value represents the mean ± SEM of 6 mice/group.