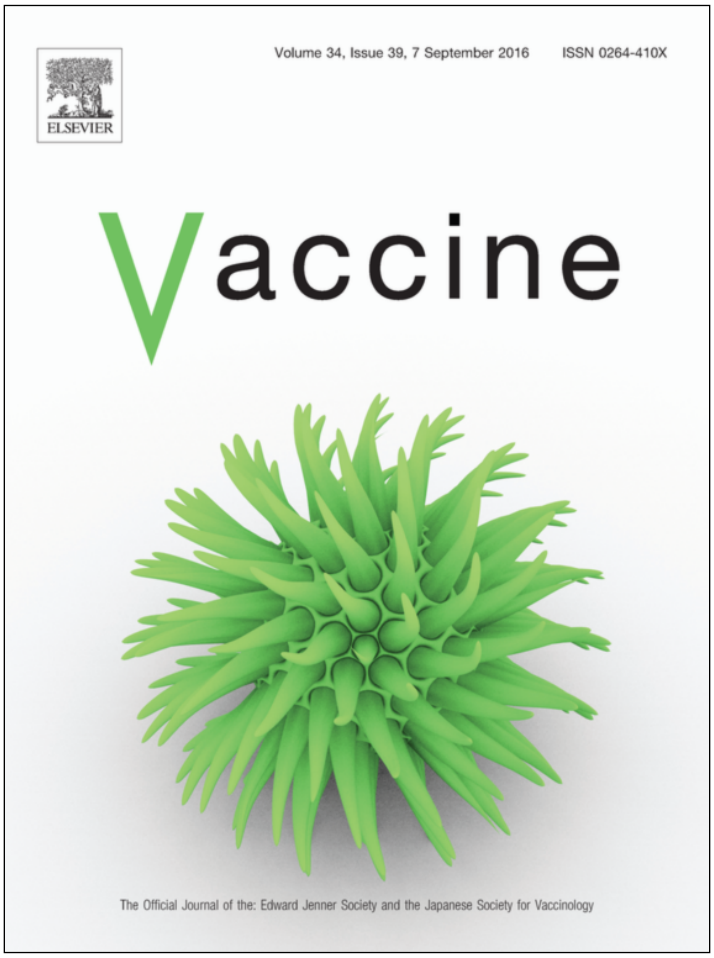

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# Immunization with BLS-Stx2B chimera totally protects dams from early pregnancy loss induced by Shiga toxin type 2 (Stx2) and confers anti-Stx2 immunity to the offspring

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are bacterial pathogens involved in food-borne diseases. Shiga toxin (Stx) is the main virulence factor of STEC and is responsible for systemic complications including Hemolytic Uremic Syndrome (HUS). It has been previously demonstrated that Shiga toxin type 2 (Stx2) induces pregnancy loss in rats in early stage of pregnancy. The main purpose of this study was to determine if an active immunization prevents Stx2 mediated pregnancy loss and confers passive protective immunity to the offspring. For that purpose Sprague Dawley female rats were immunized with the chimera based on the enzyme lumazine synthase from *Brucella* spp. (BLS) and the B subunit of Shiga toxin 2 (Stx2B) named BLS-Stx2B. After immunization females were mated with males. At day 8 of gestation, dams were challenged intraperitoneally with a sublethal and abortifacient dose of Stx2. The immunization induced high anti-Stx2B-specific antibody titers in sera and most important, prevented pregnancy loss. Pups born and breastfeed by immunized dams had high anti-Stx2B-specific antibody titers in sera. Cross-fostering experiments indicated that passive protective immunity against Stx2 was transmitted through lactation. These results indicate that immunization of adult female rats with BLS-Stx2B prevents Stx2-induced pregnancy loss and confers anti Stx2 protective immunity to the offspring.


## 1. Introduction

Shiga toxin (Stx) is the main virulence factor of Shiga toxin producing *Escherichia coli* (STEC), a bacterial pathogen involved in food-borne diseases. Two types of Stx (Stx1 and Stx2) and their variants can be expressed by STEC. However, Stx2 producing strains are more virulent and epidemiologically more relevant than those producing only Stx1 or both [1,2]. Stx2 is encoded by bacteriophages and its production is the major risk factor for the development of Hemolytic Uremic Syndrome (HUS). It is well known that STEC infections comprise mostly children [1,3], but adults can also be involved [4,5]. Since Stx is a transposable virulence factor gene new STEC profiles can emerge [6,7] and susceptible populations not previously considered could be affected. In humans, early pregnancy loss due to infections comprises almost 15% of all recognized pregnancy losses [8,9], and most of the causes of spontaneous miscarriage often remains unexplained [10]. Previous reports support the hypothesis that symptomatic or asymptomatic STEC infections during pregnancy may cause maternal or fetal damage mediated by Stx2 [11–14]. In addition, we have previously demonstrated that Stx2 intraperitoneally (i.p.) injected in rats in the early stage of pregnancy, causes spontaneous abortion by a direct cytotoxic effect in the highly perfused feto-uteroplacental unit [13,14].

*Abbreviations:* ANOVA, analysis of variance; BLS, lumazine synthase from *Brucella* spp.; $CD_{50}$, 50% cytotoxic dose; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; gd, gestation day; Gb3, globotriaosylceramide; HUS, Hemolytic Uremic Syndrome; i.p., intraperitoneally; IgG, immunoglobulin G; IgA, immunoglobulin A; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; Stx, Shiga toxin; Stx1, Shiga toxin type 1; Stx2, Shiga toxin type 2; Stx2B, B subunit of Stx2; Stx2A, A subunit of Stx2; STEC, Stx producing *Escherichia coli*; SD, standard deviation.

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One of the mechanisms of the immune system to fight against toxins or pathogens is the development of neutralizing antibodies. In this regard, studies of two STEC outbreaks associated the seropositivity of anti-Stx2 antibodies with protection to the development of systemic complications [15]. These findings together with the extremely rare occurrence of a second episode of HUS in the same patient, suggest a protective role for anti-Stx antibodies against HUS development [16]. Epidemiological studies demonstrated that people between 20 and 50 years old have high titers of anti-Stx2 [17]. This pattern could reflect the age-related incidence of HUS. Taking these data together, adults without Stx2 antibodies serum titers could be exposed to an increased risk for developing STEC complications mediated by Stx.

It is well known that meanwhile building up its immune system, the infant is supported by the transplacental IgG [18] and by IgA antibodies during breastfeeding [19]. Thus passive transmission of specific immunoglobulins provides the same protection for pathogens that the mother is being protected.

Nowadays no licensed vaccine or effective therapy is available for human use against Stx2. Taking into account this fact a novel immunogen was recently developed by inserting the B subunit of Stx2 at the amino termini of enzyme lumazine synthase from *Brucella* spp. (BLS-Stx2B). Active immunization with this chimera induced high titers of anti-Stx2 neutralizing protective antibodies in mice [20]. In addition, passive immunization of the offspring conferred protection against a lethal challenge with STEC [21]. The main purpose of the present study was to determine if circulating anti-Stx2 neutralizing antibodies generated by an active immunization with BLS-Stx2B in female rats prevent early pregnancy loss mediated by Stx2. We also evaluated if pups breastfeed by immunized mothers were able to acquire passive immunity against Stx2 and obtain protection against a lethal dose of Stx2.

## 2. Materials and methods

### 2.1. Drugs and chemicals

Purified Stx2 was purchased from Phoenix Laboratory, Tufts Medical Center, Boston, MA, USA and it was checked for LPS contamination by *Limulus amoebocyte lysate assay* (Biowhittaker Inc. Maryland, USA). Toxin was diluted with sterile phosphate-buffered saline (PBS) before injection. The final solution contained <10 pg LPS/ng of pure Stx2.

### 2.2. Animals

Sprague Dawley female and male rats (200–280 g; 2–3 months of age) were acquired from the Animal Facility of the School of Pharmacy and Biochemistry. Timed pregnant rats were obtained as previously described [13]. Day 1 of gestation (gd 1) was determined when sperm was observed in the vaginal smear. Animals received food and water *ad libitum* and were housed under controlled conditions of light (12-h light, 12-h dark) and temperature (23–25 °C). This study was carried out in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Committee for the Care and Use of Laboratory Animals of the University of Buenos Aires (CICUAL, Permit Number 2954/10 and 1494/2013).

### 2.3. Experimental protocol

#### 2.3.1. Immunization

Female rats ($n = 7$) were subcutaneously immunized with three doses of 200 μg of BLS-Stx2B chimera [20] with aluminium hydroxide (1:1) at days 0, 15 and 30. Nonimmunized rats received PBS (Control $n = 4$). One day before every immunization, blood samples were collected by venopuncture of the tail vein and serum was obtained allowing clotting for 1 h at 37 °C and then centrifuged for 10 min at 2500 rpm. Serum samples were collected and stored at −20 °C until used. This experimental protocol was repeated twice (total $n$/group = 9–15).

#### 2.3.2. Serum Stx2B-specific immunoglobulin determination

Serum Stx2B specific antibodies were analyzed as previously described [20]. Briefly, ELISA plates were coated with 0.5 μg/well with Stx2B. For total specific IgG determination, peroxidase-conjugated goat anti-rat IgG (H + L, 1:5000; Pierce) was used as a secondary antibody. The antigen-antibody reaction was detected with O-phenylendiamine (Sigma, St Louis, MO), and absorbance was read at 492 nm. Results were expressed as end point titers, calculated as the reciprocal values of the last dilution with an absorbance higher than that of the preimmune serum samples +2 standard deviation (SD).

#### 2.3.3. Stx2-neutralization assay

*In vitro* Stx2 neutralizing activity of sera was analyzed on Vero cells. For that purpose, a 50% cytotoxic dose of Stx2 ($CD_{50}$: 0.5 ng/ml) for Vero cells was pre incubated with serial dilutions (1:50 to 1:1600) of serum samples for 90 min at 37 °C and 250 rpm. The mixtures were then added to Vero cells. Culture of cells was performed as previously described with modifications [22]. Briefly, 18.000 Vero cells/well were plated in 96-well plates and grown to confluence in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% of fetal bovine serum (FBS), glutamine 2 mM, penicillin 100 UI and streptomycin 100 μg/ml. Then, cells were incubated under growth-arrested conditions (DMEM medium without FBS) either with Stx2 alone or with the mixture for 72 h at 37 °C and 5% $CO_2$. After treatment, cells were incubated with 0.05 mg/ml of neutral red solution for 2 h at 37 °C and 5% of $CO_2$. After neutral red incorporation, cells were fixed with 4% formaldehyde and 1% $CaCl_2$ and then lysed with 50% ethanol and 1% acetic acid solution. Absorbance was read in a microplate reader (RT-6000, Rayto Life and Analytical Sciences Co. Ltd. China) at 540 nm. Absorbance values from cells incubated under identical conditions but without treatment were considered as 100% of viability. The neutralizing titer was calculated as the last dilution of the serum that was able to completely inhibit Stx2-cytotoxicity.

#### 2.3.4. Pregnancy

After the immunization protocol, Immunized (Imm) and Control dams were mated with males and then i.p. challenged at gd 8 with a sublethal dose of purified Stx2 (0.5 ng Stx2/g of body weight (bwt), 250 μl). Therefore, two groups were performed: Imm + Stx2 ($n = 6$) and Stx2 ($n = 4$), respectively. An additional group of control dams were injected with PBS (Control, $n = 5$). After challenge, dams were housed individually, weighed and controlled until the expected day for delivery. The experiment was repeated twice (total $n$/group = 9–15).

#### 2.3.5. Body weight, pregnancy progression and fostering experiments

After challenge, Control, Stx2 and Imm + Stx2 dams groups were weighed daily for 9 days. Delivery, litter size and pup body weight were registered. To analyze anti-Stx2B antibody titers, serum from pups born from Imm + Stx2 dams were obtained at weaning (21 day-old) and one month after weaning (51 day-old). For cross-fostering experiments, Control ($n = 5$) and Imm + Stx2 dams ($n = 5$) groups remained with 10 pups each one after delivery with the purpose to equal the litters and stimulate similarly the mammary glands. Then, half of the pups born from Imm + Stx2 dams were fostered to Control dams (Imm pups-Control dam,

$n$ = 5/dam) and half of the pups born from Control dams were fostered to Imm dams (Control pups-Imm dam, $n$ = 5/dam). Thus, each dam has 5 own pups (Control or Imm) and 5 fostered pups from a dam belonging to the other group (Imm or Control, respectively). All pups were suckled for 21 days. Then, three randomly selected pups serum from each own and fostered group breastfeed by a Control or Imm dam were pooled to analyze anti Stx2B-antibody titers (total $n$/group = 15). Fig. 1 shows the timeline indicating treatments and sampling during immunization, pregnancy and delivery.

*2.3.6. Milk collection and challenge of pups with a lethal dose of Stx2*

After weaning, 2–4 randomly selected pups (total $n$/pup group = 12–16) from each pup group that were breastfeed by a dam ($n$ = 5) were challenged i.p. with a lethal dose of Stx2 (2.5 ng/g bwt, 100 μl). Survival rates of the pups were registered daily for 72 h. Also after weaning, dams were euthanized and the milk was obtained by dissection of the mammary gland. Milk serum samples were collected after centrifugation for 30 min at 10,000 rpm and stored at −20 °C until used.

*2.3.7. Dot blot*

Dot blot was performed to evaluate the presence of anti-Stx2 antibodies in milk. This method was applied due to of the limited sample volume. Briefly, polyvinylidene difluoride membranes (Bio-Rad Lab, USA) were activated in methanol for 5 min and 1 μg of Stx2 was spotted onto the membrane. Nonspecific sites were blocked with 5% nonfat milk for 2 h at room temperature. Then, the membranes were incubated with the milk from Imm dams (dil. 1:50) for 16 h at 4 °C. Sera (1:50) from Imm and Control dams were used as positive and negative controls, respectively. After that, the membranes were incubated with a secondary antibody HRP-conjugated anti-rat IgG (1:5000, Pierce Biotechnology Inc, USA) for 2 h at room temperature. The reaction was revealed with a diaminobenzidine hydrochloride solution (Sigma-Aldrich Co. St Louis, MI, USA). Specific anti-Stx2 antibodies were detected as brown dots.

*2.4. Statistical analysis*

Statistical analysis was performed using the Graph Pad Prism Software 5.0 (San Diego, CA, USA). Differences in body weight were analyzed by two-way analysis of variance (ANOVA). One-way ANOVA was used to compare anti-Stx2 specific IgG titers and differences in the neutralizing activity of serum samples of pups. In all cases Bonferroni was used as *a posteriori* test. Difference between two groups was analyzed by Mann-Whitney test. Serum IgG titers in Imm pups at weaning and one month after weaning were analyzed by paired $t$-test. Log rank test was used to analyze survival curves. Statistical significance was set at $p < 0.05$.

## 3. Results

*3.1. Antibody response against Stx2B and neutralizing capacity for Stx2*

Immunized rats developed high specific IgG titers against Stx2B in serum that increased gradually after each dose of immunogen (Fig. 2A). Also, serum had a significant neutralizing activity against Stx2 that increased after each booster (Fig. 2B).

*3.2. Body weight of pregnant rats after Stx2 injection*

Imm dams were i.p. injected with a sublethal dose of Stx2 on gd 8 (Imm + Stx2). Control pregnant rats were i.p. injected with either a sublethal dose of Stx2 (Stx2) or PBS (Control). Control dams treated with Stx2 exhibited a decrease in the body weight until 4–5 days after injection as previously reported [13]. On the contrary, Imm dams treated with Stx2 gradually gained weight similarly to Control dams treated with PBS (Fig. 3).

*3.3. Progression of pregnancy in immunized dams treated with Stx2*

Table 1 shows that all the Imm + Stx2 had a normal term delivery with a similar litter (both in number and weight) compared to

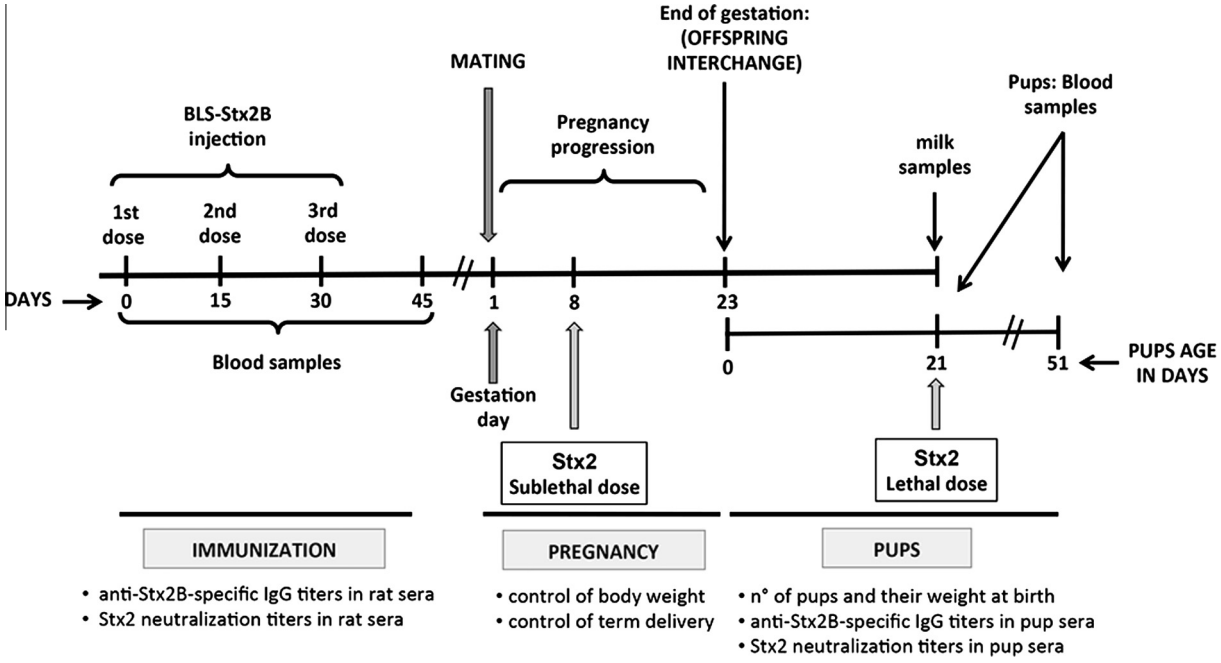


**Fig. 1.** Timeline indicating treatment and sampling during immunization, pregnancy and delivery. Working protocol was divided in three periods: immunization, pregnancy and pups for better explanation.

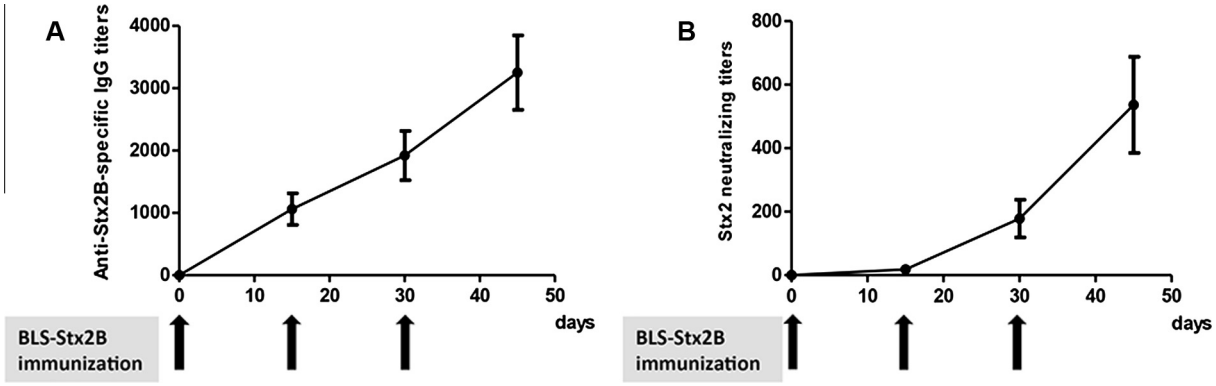


**Fig. 2.** Time course of specific IgG titers against Stx2B and the neutralizing capacity for Stx2 in rat sera. (A) Anti-Stx2B-specific IgG titers were determined by ELISA. (B) Stx2-neutralizing titers were determined by the neutralizing capacity for Stx2 on Vero cell assays. Each time point represents the mean ± standard error of the mean (total $n = 9$–15). Black arrows represent each immunization time point.

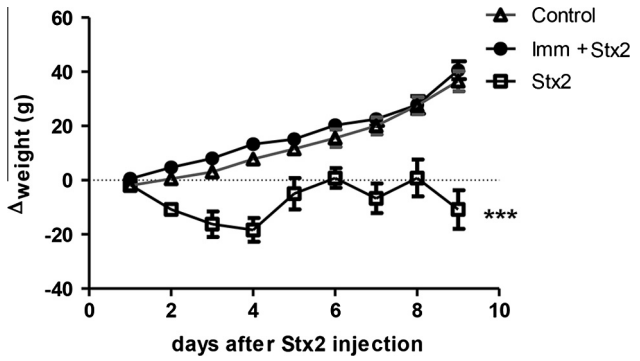


**Fig. 3.** Maternal body weight after Stx2 injection. Immunized pregnant rats ($n = 6$) were challenged with a sublethal dose of Stx2 (0.5 ng Stx2/g bwt) on day 8 of gestation (Inm + Stx2). Control pregnant rats were challenged with either a sublethal dose of Stx2 (Stx2, $n = 4$) or PBS (Control, $n = 5$). Dams were weighed daily until 9 days after injection. Each point of the curves represents the mean ± standard error of the mean. *** $p < 0.0001$ for Stx2 *versus* Control and Imm + Stx2.

**Table 1**
Evaluation of pregnancy progression after Stx2 injection.

| | N° of dams | Term delivery | N° of pups (mean ± SD) | Pups weight (g) (mean ± SD) |
|---|---|---|---|---|
| Control | 5 | 5/5 | 12 ± 3 | 6.1 ± 0.5 |
| Stx2 | 4 | 0/4*** | 0 ± 0*** | // |
| Imm + Stx2 | 9 | 9/9 | 12 ± 2 | 6.1 ± 0.3 |

*** $p < 0.001$ *versus* Control. SD standard deviation.

Control dams. On the contrary, all Stx2 dams had 100% of pregnancy loss.

### 3.4. Presence of anti-Stx2 antibodies in the milk

The presence of anti-Stx2 specific antibodies in milk samples extracted from the mammary gland of Imm + Stx2 dams was detected by dot plot. Serum samples from Imm and Control dams were used as positive and negative controls, respectively. Fig. 4 shows that milk from Imm + Stx2 dams presented Stx2-specific IgG.

### 3.5. Anti-Stx2B antibodies in pups at weaning and one month later

To analyze the persistence of maternal antibodies transferred to the offspring, serum samples from pups born from Imm dams

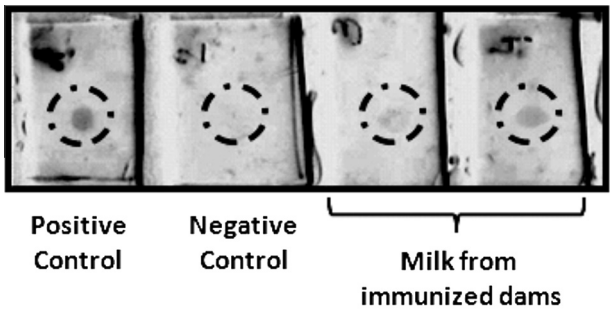


**Fig. 4.** Presence of anti-Stx2 antibodies in the milk of immunized dams. Mammary glands from immunized dams were extracted 21 days after delivery and milk samples were obtained from the dissected tissues. Dot blots were performed using 1 μg of Stx2 per spot. Serum milk samples were incubated (1:50) for 16 h at 4 °C. Serum samples from immunized and control dams (1:50) were used as positive and negative control, respectively. One representative experiment is shown.

breastfed by their own mother were obtained at weaning (21 day-old) and one month later (51 day-old). The anti-Stx2B-specific IgG titers and the Stx2 neutralizing titers were compared as paired samples at both times. High titers of anti-Stx2B IgG antibodies were detected in the serum of the pups at weaning. The antibody titer significantly decreased one month later (Fig. 5A). This fact was also observed in the *in vitro* neutralizing capacity against Stx2, which significantly decreased one month after weaning (Fig. 5B).

### 3.6. Passive immunization through lactation

Pups born from Imm dams (Imm pups) and those born from Control dams but breastfed by Imm dams (Control pups-Imm dam) exhibited high anti-Stx2B IgG antibodies titers (Fig. 6A) with neutralizing capacity against Stx2 (Fig. 6B). On the other hand, pups born from immunized dams but breastfed by control dams (Imm pups-Control dam) exhibited low titers of anti-Stx2B IgG antibodies with low Stx2-neutralizing capacity similarly to pups born from Control dams (Control pups) (Fig. 6A and B).

### 3.7. Challenge of the offspring at weaning with a lethal dose of Stx2

Pups were challenged after weaning with an i.p. lethal dose of Stx2, and survival rates were analyzed (Fig. 7). All Imm pups and Control pups-Imm dam survived to the lethal dose of Stx2. On the contrary, 94% of control pups died between 2 and 5 days after Stx2 injection. Surprisingly, 80% of Imm pups-Control dam

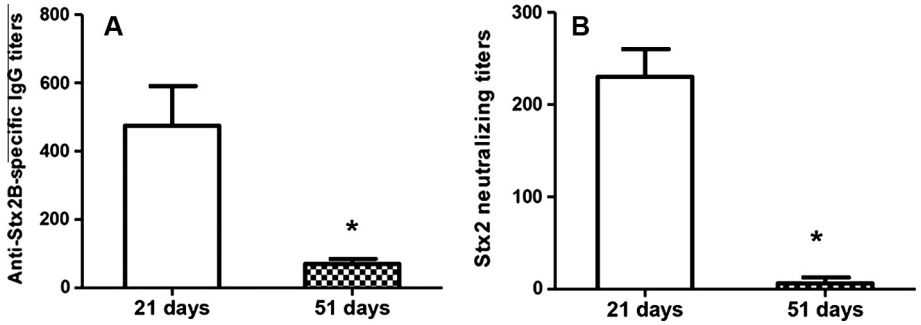


**Fig. 5.** Titers of specific anti-Stx2B antibodies and Stx2-neutralizing capacity in pups sera. (A) Titers of anti-Stx2B specific IgG antibodies were analyzed in pups (Imm pups) born from immunized dams (Imm + Stx2) at weaning (21-day old) and 30 days after weaning (51-day old). (B) Stx2-neutralizing titers were determined by the neutralizing capacity for Stx2 on Vero cell assays. Three randomly selected sera from each group breastfeed by the same dam ($n = 6$) were pooled for analysis. Each point of the curves represents the mean ± standard error of the mean $^{*}p < 0.05$.

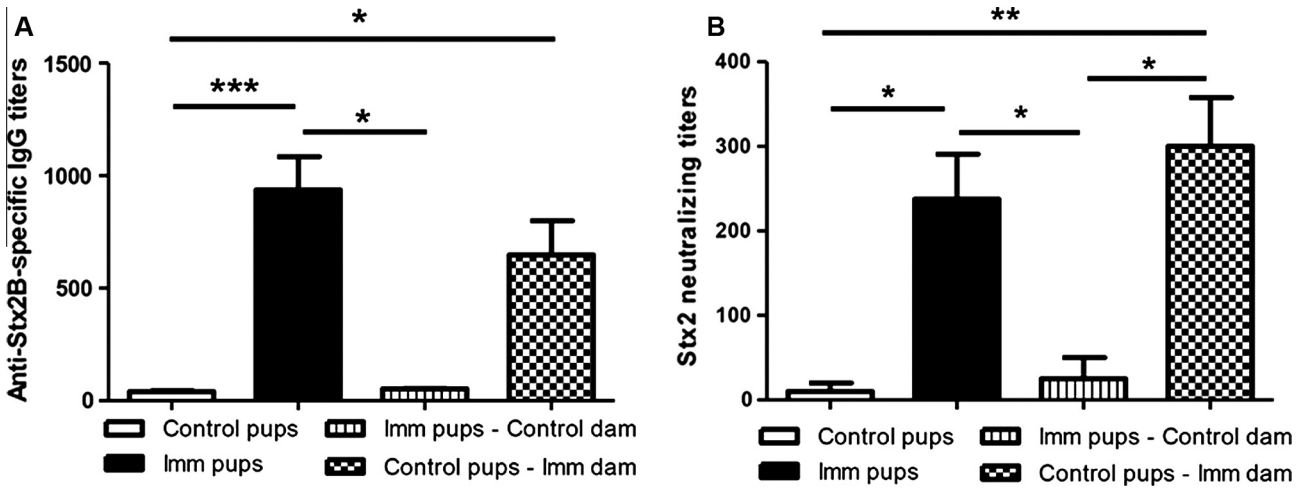


**Fig. 6.** Anti-Stx2B-specific IgG response in sera from pups after fostering experiments. Half of the pups born from immunized or Control dams were interchanged at day 1 post partum, and the other half of the pups remained with their own dams as described in M&M. All the pups (5 pups/group/dam; 25 total pups/group) suckled for 21 days. Three randomly selected pups serum from each own and fostered group breastfeed by a Control or Imm dam were pooled to analyze anti Stx2B-antibody titers (total $n$/group = 15): (A) Anti-Stx2B-specific IgG titers by ELISA. (B) Stx2 neutralizing titers on Vero cells. Each bar represents the mean ± standard error of the mean. Multiple comparisons were performed, statistical differences were observed only when indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

survived to the lethal dose of Stx2 suggesting that specific IgG antibodies against Stx2 passively transmitted through placenta and/or during breastfeeding just after birth are enough to protect pups against a lethal dose of Stx2.

## 4. Discussion

In the present study we evaluated if the immunization with BLS-Stx2B was able to induce specific anti-Stx2 antibodies on female rats and in addition, if this immunization was able to prevent Stx2 mediated pregnancy loss. Also, we assayed if anti-Stx2 immunity could be passively transferred and we found that it was able to protect the offspring against a lethal dose of Stx2.

We have previously shown that Stx2 i.p. injected in rats in the early stage of pregnancy induced pregnancy loss [13], through a mechanism involving hypoxia and inflammation [14].

In the present work we demonstrated that the immunization of female rats with BLS-Stx2B previous to pregnancy induced high titers of IgG antibodies against Stx2B. Moreover, sera from immunized rats were able to neutralize the cytotoxic activity of Stx2 on Vero cells. The immunity developed against Stx2 protected dams from early pregnancy loss mediated by Stx2. One of the most characteristic effects of Stx2 i.p. injected in early pregnant rats is the body weight loss [13]. In this study, we demonstrated that female

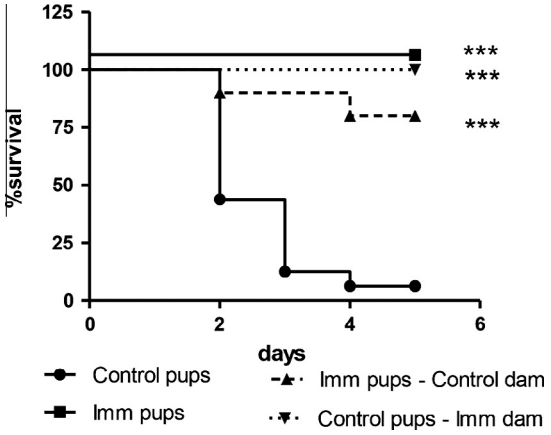


**Fig. 7.** Survival rates of pups in response to a lethal dose of Stx2. Survival rates were analyzed from the different groups of pups. Pups from Control dams (Control pups, $n = 16$) or Imm + Stx2 (Imm pups, $n = 16$), and pups from Imm + Stx2 dams breastfed by Control dams (Imm pups-Control dam, $n = 12$) or pups from Control dams breastfed by Imm + Stx2 dams (Control pups-Imm dam, $n = 12$). Multiple comparisons were performed with Log Rank test. Statistical differences were observed when indicated in: Imm pups, Imm pups-Control dam, and Control pups-Imm dam *versus* Control pups. $^{***}p < 0.001$.

rats immunized with BLS-Stx2B and challenged with a sublethal and abortifacient dose of Stx2 during early pregnancy, gained weight progressively. Furthermore, all immunized dams challenged with Stx2 reached term delivery, and the offspring born was similar to controls, both in number and body weight.

The levels of specific anti-Stx2B IgG antibodies in pups sera at weaning were comparable to those levels of specific antibodies found in the sera from immunized dams. However, the titers declined considerably one month after weaning. These results indicated the passive transfer of specific antibodies from dams to their offspring. Since maternal specific anti-Stx2 antibodies can be transferred by lactation, we analyzed the route of this passive transfer. Pups born from immunized and control dams were interchanged at day 1 postpartum and were left to breastfeed until weaning. Our results showed that all pups breastfed by immunized dams, either born from them or from Control ones had significant anti-Stx2B-specific IgG titers with Stx2 neutralizing capacity. The presence of anti-Stx2 antibodies in the milk from immunized dams supports the conclusion that the passive immunity against Stx2 can be transferred by breastfeeding. These antibodies conferred total protection against a lethal dose of Stx2 to all pups breastfed by immunized dams.

Interestingly, 80% of the pups born from immunized dams and breastfed by Control dams survived. This result suggests that transplacental antibodies could exert passive immunization. Although we cannot discard that suckling even little doses of colostrum immediately after birth was enough to transfer protection against Stx2 to the pups. Nevertheless, in this group of pups, the specific Stx2B IgG titers were low and the *in vitro* neutralizing capacity was not statistically different compared to Controls. Considering this result it is reasonable to think that very low antibody titers are still able to protect pups against the lethal dose of Stx2, probably due to a high affinity of the antibodies against this toxin. In this regard it was previously shown that mice immunized with BLS-Stx2B are completely protected against Stx2 [20] even when low anti-Stx2B antibody titers and a low Stx2-neutralizing activity on Vero cells were detected. Future studies will be necessary to estimate the threshold of antibody concentration necessary to neutralize Stx2 *in vivo* and to characterize the toxin-antibody interaction.

In conclusion, our results demonstrated that the immunization with BLS-Stx2B chimera totally protects rats from early pregnancy loss induced by Stx2 and confers anti-Stx2 immunity to the offspring. Although there are no reports of Stx2-mediated fetal damage or fetal death in humans, we speculate that humoral immunity against Stx2 in pregnant women could prevent possible Stx2 damage to the fetus, as consequence of STEC infections during pregnancy. Additionally, it could benefit neonates by conferring anti-Stx2 antibodies passively transmitted by lactation.

## Conflicts of interest

None.

## Author contributions

Conceived and designed the experiments: FS, MPM, MSP, CI. Performed the experiments: FS, MPM, ACB, RSA, MMA. Analyzed the data: FS, MPM, MMA, MSP, CI. Contributed reagents/materials/analysis tools: FS, MPM, ACB, RSA, MMA, MSP, CI. Wrote the paper: FS, MPM, MSP, CI.

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