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Dual-route targeted vaccine protects efficiently against botulinum neurotoxin A complex

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Abstract

Clostridium botulinum readily persists in the soil and secretes life-threatening botulinum neurotoxins (BoNTs) that are categorized into serotypes A to H, of which, serotype A (BoNT/A) is the most commonly occurring in nature. An efficacious vaccine with high longevity against BoNT intoxication is urgent. Herein, we developed a dual-route vaccine administered over four consecutive weeks by mucosal and parenteral routes, consisting of the heavy chain (Hc) of BoNT/A targeting dendritic cell peptide (DCpep) expressed by *Lactobacillus acidophilus* as a secretory immunogenic protein. The administered dual-route vaccine elicited robust and long-lasting memory B cell responses comprising germinal center (GC) B cells and follicular T cells (Tfh) that fully protected mice from lethal oral BoNT/A fatal intoxication. Additionally, passively transferring neutralizing antibodies against BoNT/A into naïve mice induced robust protection against BoNT/A lethal intoxication. Together, a targeted vaccine employing local and systemic administrative routes may represent a novel formulation eliciting protective B cell responses with remarkable longevity against threatening biologic agents such as BoNTs.

Keywords

Botulinum neurotoxins; Target vaccine; Dual-route vaccine; B cells; Dendritic cells

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Author contribution

MM directed and designed the experiments, which were executed by BS, NC, MZ, MG, MV and YG. BS and MZ performed the mouse work; NC performed flow cytometry analyses; BS, NC, MZ, JO, CJ and MM analyzed and interpreted the data; MM wrote the manuscript.

Conflict of interest

All authors declare that they have no commercial, or other association that might pose a conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vaccine.2017.11.008>.

1. Introduction

Botulinum neurotoxins (BoNTs) are among the most toxic substances known to man. These toxins are secreted as exotoxins from Gram-positive spore forming *Clostridium botulinum* (*C. botulinum*). *C. botulinum* can be cultivated from the soil, and its toxins can be mass produced as a bioweapon; one gram of aerosolized toxins can potentially eradicate one million individuals [1]. Due to its extremely toxic nature, *C. botulinum* is categorized as a select agent A. *C. botulinum* secretes neurotoxin serotypes (A-H) [2–4]; however, human botulism is commonly associated with toxin sero-types A, B, E, and F. These poisonous BoNTs can lead to death via airway obstruction secondary to muscle paralysis [2–4]. The BoNTs consist of two peptide chains bridged together with a disulfide bond [5]. The heavy chain (Hc) acts as the vehicle for the short chain to facilitate entry into nerve cells. BoNTs are susceptible to gastric enzymes and a low pH; however, they are protected from harsh conditions of the gastrointestinal tract by complexing with a hemagglutinin protein expressed by the bacteria. The short chain of BoNTs possesses zinc (Zn⁺⁺)-dependent protease activity that cleaves soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) to inhibit acetylcholine vesicle-delivery at nerve synapses, leading to complete flaccid paralysis. Of the different serotypes of toxins produced by *C. botulinum*, serotype A is the most common. BoNTs or *C. botulinum* producing BoNTs can enter the host by various routes; however, the most common and natural mode of introduction is the orogastric route. Presently, due to lack of long-lasting and efficacious protective vaccines, the therapeutic and preventative approach(s) against BoNTs comprise the physical elimination of neurotoxins and provision of basic care to sustain life. However, anti-toxin antibody treatment may be considered a prime pathway for therapy [6–8]. More precisely, BoNTs are transcribed as a single polypeptide that is further cleaved into two chains bound to a disulfide link. The Hc of BoNTs binds to a neuronal receptor, SV2, for internalization of the toxins (e.g., BoNT/A), whereupon, neutralizing antibodies against BoNTs, particularly Hc-BoNT/A, prevent the devastating effects of the neurotoxin complex [6]. Thus, it is urgent to generate innovative vaccine strategies using BoNT subunits to significantly confer long-lasting protection against BoNT complex intoxication.

The commensal, *Lactobacillus acidophilus* (*L. acidophilus*), demonstrated the potential to be an excellent delivery vehicle for oral vaccine subunits, including protective antigen (PA) of *B. anthracis* [9,10]. Such an oral vaccine produced by this bacterium induced protective responses against pathogen challenge [9]. However, two major disadvantages of such employed plasmid-based delivery may account for the risk of losing the plasmid, being the lack of antibiotic selection and delivery of the antibiotic resistance gene to gut commensals. To overcome these potential hurdles, *L. acidophilus* now expresses targeted Hc-BoNT/A to DCpep from a chromosomal location [11]. Oral vaccination using this new vaccine delivery system, when combined with intradermal injection of purified targeted Hc-BoNT/A-DCpep vaccine, elicited robust and long-lasting protective B cell responses against lethal BoNT/A complex intoxication, as well as remarkable longevity of humoral responses that protected mice against subsequent oral BoNT/A complex intoxication nine months later.

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from NCI and Charles River and reared at the animal facility at the University of Florida. Mice (6–8 week) were used in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida under protocol number, 2014107129.

2.2. Vaccination

L. acidophilus strain, NCK2310, was generated, previously [11], which expresses Hc-BoNT/A-DCpep as fusion protein. NCK2310 was grown in static MRS media anaerobically at 37 °C. NCK2310 was used for gavaging BALB/c mice (10^9 CFU/100 µL sterile PBS/mouse).

2.3. Preparation of the vaccine

Hc-BoNT/A-DCpep was isolated from NCK2310 culture supernatants that were first freeze-dried and resuspended in 1/10th of the initial volume. The concentrated material was dialyzed against water for 48 h to remove excessive salt and other media components. The remaining contents were concentrated by a centrifuge concentrator (20 kD cut-off), and confirmed by Western blot analyses using anti-Hc-BoNT/A antibodies (Metabio Inc., Madison, WI).

2.4. Botulinum neurotoxin

Clostridium botulinum neurotoxin serotype A (BoNT/A) complex for mouse oral intoxication, and *Clostridium botulinum* serotype A neurotoxin (BoNT/A) were purchased from Metabio Inc. (Madison, WI) and kept under strict vigilance with appropriate documentation for their use, in accordance with Environmental Health and Safety (EHS) guidelines at the University of Florida (University of Florida Acute toxin registration number AT-4164). Mice were orally intoxicated with BoNT/A complex (1 or 3 µg/-mouse), as indicated in the respective figure legends. BoNT/A was used for ELISA or its neutralization by sera derived from surviving mice.

2.5. In vivo neutralization assay

To test the quality of serum antibodies developed by the vaccination, 100 µL sera isolated from the vaccinated or naïve mice were incubated with 50 picograms (pg) of BoNT/A for one hr at 4 °C before injecting them into naïve BALB/c mice. Mice survival was evaluated every hour for the next six hr and then every 12 h for ten days.

2.6. Flow cytometry

Single cells were isolated from the colon, spleen, mesenteric lymph nodes (MLNs), and the peripheral lymph nodes (PLNs) [12]. After incubation with Fixable Blue Dead Cell Stain (Life Technologies, Carlsbad, CA) and Fc blocking (Miltenyi Biotec, San Diego, CA), cells were stained with antibodies, or reagents and fixed with PFA 4% for 20 min. Flow

cytometric analyses were performed using a BD LSR II Fortessa (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, Version 10.1r7). A list of the antibodies used can be found in the Supplemental Table 1. Gating strategies are depicted in the Supplemental Figs. 1–3.

2.7. Enzyme linked immunosorbent assay (ELISA)

ELISAs were used to determine the anti-Hc-BoNT/A antibodies in the sera (for IgG titer) and fecal samples (for IgA titer) of vaccinated and surviving mice. Standard ELISA protocols were followed to quantitate antibody responses to Hc-BoNT/A. Commercial BoNT/A (5 µg/mL) was used to coat MaxiSorp microtiter plates (Nunc, Thermo Fisher Scientific, Waltham, MA) overnight at 4 °C. Sera or fecal extracts from individual mouse groups were evaluated for anti-BoNT/A antibodies. Mouse fecal suspensions (10% w/v) were generated in PBS with 50 µg/mL soybean trypsin inhibitor (Sigma-Aldrich, Saint Louis, MO). HRP-goat anti-mouse IgA, and IgG (Southern Biotechnology Associates, Birmingham, AL) were used for detection. Endpoint titers were defined as the highest reciprocal of dilution of samples giving an absorbance at OD₄₁₅ above 0.100 OD units above negative controls after 1 h of incubation at 25 °C.

2.8. Memory B cells transfer

BALB/c mice were orally vaccinated four times with NCK2310 (10⁹ CFU/mouse), once a week, followed by 2 boosts (Fig. 1A). Seven days later, splenic memory B cells were magnetically sorted using the mouse memory B cell isolation Kit (Miltenyi Biotec, San Diego, CA). Briefly, 50 µL each of Anti-IgG1-APC, Anti-IgG2ab-APC and 100 µL Biotin Antibody Cocktail were incubated with 10⁸ isolated splenic cells from the vaccinated mice resuspended in 300 µL PBS containing 0.5% FBS and 2 mM EDTA for 5 min on ice. 200 µL of Anti-Biotin microbeads were added to the mix to remove non-memory B cells using LD columns. Anti-APC microbeads were introduced in the flow through of LD columns to further purify memory B cells from the cellular suspension using MS columns. The isolated cells were counted to prepare a cellular suspension containing 10⁶ cells/100 µL. Memory B cells (10⁶ cells/mouse) were then intraperitoneally transferred into recipient naïve BALB/c mice. One month later, recipient mice were intoxicated with BoNT/A complex (1 µg/mouse). Mouse survival was monitored.

2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism (Version 7.0 for Mac OS X, La Jolla, CA). Mean and S.E.M. values and statistical significance between two variables were determined by two-tailed unpaired *t*-tests. Where appropriate, one-way ANOVA followed by Tukey post-test were performed. Survival curves were analyzed by log-rank (Mantel-Cox) test. Differences were considered to be significant at **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

3. Results

3.1. Oral only vaccination does not fully protect mice against botulinum toxin complex serotype A intoxication

The feasibility of using *L. acidophilus* to orally deliver a targeted vaccine has previously been demonstrated [9,10,13]. To improve the efficacy of such a plasmid-based vaccine delivery, Hc-BoNT/A was genetically fused to DCpep and expressed from a chromosomal location by *L. acidophilus*, NCK2310, as an Hc-BoNT/A-DCpep fusion protein [11]. To evaluate the efficacy of NCK2310 producing Hc-BoNT/A-DCpep to induce protective responses against BoNT/A, BALB/c mice were orally vaccinated with NCK2310 (1×10^9 CFU/mouse) or PBS for four consecutive weeks and two boosts, as described previously [9] (Fig. 1A). These groups of mice were then orally intoxicated with BoNT/A complex (1 μ g/mouse). Approximately 70% of mice orally vaccinated with NCK2310 survived BoNT/A complex intoxication (LD₅₀ 1 μ g), while all mice gavaged with PBS succumbed within 6–30 h (Fig. 1B). Nonetheless, the majority of surviving mice showed significant symptoms of BoNT/A intoxication, including exhaustion, hypothermia, ruffled coat, and impaired physical movement; thus, we concluded that oral vaccination does not robustly protect the mice against lethal BoNT/A complex intoxication and hence, requires further regimen modifications. We wondered whether B cells might play a protective role against BoNT/A complex intoxication in the vaccinated mice [14]. Thus, splenic memory B cells were sorted from the protected mice and transferred into naïve BALB/c mice that were then orally intoxicated with the BoNT/A complex. Data demonstrate partial protection of BALB/c mice, in which the transferred B cells protected 50% of these mice, while all control mice receiving B cells from PBS-gavaged mice succumbed to oral BoNT/A complex intoxication (Fig. 1C).

3.2. Dual-route vaccine protects fully against BoNT/A complex intoxication

To fortify the protective immune responses against lethal BoNT/A complex intoxication, we sought to vaccinate BALB/c mice by a concurrent dual-route vaccine strategy that has provided significant protection against poliovirus in recent human clinical trials [15]. Since utilizing only the secreted targeted vaccine produced by NCK2310 did not demonstrate any meaningful protection against lethal BoNT/A intoxication, the bacterial supernatants of the NCK2310 strain were processed (Supplemental Fig. 4) and prepared to vaccinate BALB/c with a dual-route vaccine platform. This consisted of oral gavages with the aforementioned NCK2310 (10^9 CFU/mouse) and intradermal injections of purified targeted vaccine Hc-BoNT/A-DCpep produced by NCK2310 that was administrated above the mouse sternum (100 ng/mouse) for four consecutive weeks plus two boosts (Fig. 2A), or for four consecutive weeks (one/week) without boosts (Fig. 2B). Seven days after the last vaccination, mice were orally intoxicated with the lethal BoNT/A complex (1 μ g/mouse). Data demonstrate that mice vaccinated with oral NCK2310 plus intradermal injections of Hc-BoNT/A-DCpep for sextuple (four weeks plus two boosts) (Fig. 2A), quadruple (Fig. 2B) or triple (Fig. 2C) dual-route regimen survived the oral BoNT/A complex intoxication, while all BALB/c mice vaccinated dually once succumbed (Fig. 2A–C) and exhibited significant symptoms of BoNT/A complex intoxication. Although the majority of mice receiving twice the dual-route vaccine survived oral BoNT/A complex intoxication, all

surviving mice showed significant exhaustion, hypothermia, and impaired physical movement (Fig. 2C), indicating inefficiency of the two week-vaccine regimen. Subsequently, sera of surviving mice from the triple, quadruple, and sextuple dual-route vaccinations were collected and analyzed for their reactivity against Hc-BoNT/A as an antigen. Data demonstrate that the optimal titer of anti-Hc-BoNT/A was achieved with the quadruple dual-route vaccine regimen when compared to the triple, and there was no difference in antibody titers among mouse groups from the quadruple or sextuple dual-route vaccine administrations (Fig. 2D). Importantly, surviving mice treated with the quadruple dual-route vaccine regimen did not show any symptomology of BoNT/A complex intoxication that could impact their health.

We then wondered whether the oral gavage combined with intradermal injection might not be necessary for our dual-route vaccine regimen. Thus, we vaccinated mice with only four intradermal injections of the purified vaccine and compared this systemic route of vaccination with our dual-route vaccination. Unexpectedly, 60% of the mice receiving only four intradermal injections of the targeted vaccine succumbed to oral BoNT/A complex intoxication within two days. These animals exhibited serious effects of the lethal BoNT/A complex intoxication (1 µg/-mouse), including weight loss, dyspnea, and loss of eyesight, whereas mice receiving the dual-route vaccination completely survived with none of the aforementioned symptoms (Fig. 3A–B).

The main objective of developing a vaccine against BoNT/A complex was to generate neutralizing antibodies against the toxin. To test whether the induced antibodies against BoNT/A toxin can protect mice, we co-incubated sera (100 µL) obtained from vaccinated and protected mice with approximately 10×LD₅₀ (50 pg) of BoNT/A neurotoxin for 1 h before injecting naïve BALB/c mice via the intraperitoneal route. Mice that received sera from vaccinated and protected mice were fully protected with no apparent symptoms when compared to control mice receiving sera from naïve BALB/c mice that succumbed to intoxication within 18 h (Fig. 3C). These data demonstrate the critical role of B cell-dependent responses that protect against lethal BoNT/A complex intoxication in mice.

3.3. Induction of protective B cell responses by dual-route vaccine

Production of high-affinity antibodies is crucial to protect the host by neutralizing and subsequently eliminating pathogens [16]. After demonstrating protection of the intoxicated mice with our dual-route vaccine and the induced high titers, we then centered our focus on B cell activation in mice that were vaccinated with the dual-route vaccine for four weeks (quadruple) and were fully protected against BoNT/A complex challenge (Fig. 2B). Therefore, cells were isolated from the peripheral lymph nodes (PLNs) (axillary, prescapular, and inguinal lymph nodes), mesenteric lymph nodes (MLNs), spleens, and colons from all the protected groups of mice and stained for germinal center (GC), pre-memory, memory B cells, and T follicular helper (Tfh) [16–20]. Data demonstrated significant increases in GC B cells in the PLNs, spleens, MLNs, and colons (Fig. 4A and Supplemental Fig. 1). Notably, enhanced GC B cells were observed in PLNs, wherein, almost one-third of activated B220⁺ IgD[−] B cells attained a GC B cell phenotype (Fig. 4A). This dual-route vaccine strategy also induced a smaller but consistent increase in colonic GC

B cells; this may suggest the generation of gut-associated lymphoid tissue (GALT) upon vaccination, a trend that may be important for rapid immune mobilization.

It has been documented that expansion of GC B cells significantly relies on specialized Tfh cells that provide optimal environmental conditions for antigen presentation and expansion of T cell-dependent B cell responses [19,21]. Tfh cells are defined as activated T cells expressing PD-1 along with CXCR5 involved in the recruitment of these cells to the GCs [22,23]. Here, Tfh cells were also significantly increased in PLNs and in the spleen, but not in MLNs (Fig. 5A–B and Supplemental Fig. 2), indicating that these cells are providing cognate help to B cells in order to mobilize humoral immunity by the dual-route vaccine. Moreover, as B cell activation effectively results in antibody production that is critical in protection against pathogens and toxins, we also observed the formation of CD45⁺ B220^{low} CD138⁺ B cells (Fig. 4B and Supplemental Fig. 1). These cells accumulated in the MLNs and in the spleen of protected mice.

The most efficient vaccines induce plasma cell responses maintained by memory B cells, underscoring the significance of protective antibody responses against pathogen challenge [16]. Accordingly, to maintain effective protective responses with significant longevity against BoNT/A complex intoxication, memory B cells must be developed during GC [16]. Here, data revealed significant increases in both pre-memory CD79b⁺ IgD[−] B220⁺ CD43[−] CD24^{hi} CD22⁺ B cell and memory CD79b⁺ IgD[−] B220[−] CD43⁺ CD24^{lo} CD22[−] B cell expansion in the PLNs and spleen (Fig. 6A–B and Supplemental Fig. 3), indicating that the dual-route targeted vaccine efficaciously induces humoral protective responses.

3.4. Dual-route vaccine induces long-lasting protection against lethal BoNT/A complex intoxication

An efficacious vaccine should not only induce protective immunity against pathogen challenge, but it must also offer long-lasting protection against repeated pathogen assaults [24–27]. With this notion in mind, we tested the longevity of our dual-route targeted vaccine, which protected mice against lethal BoNT/A complex intoxication three months later. Importantly, after this period of time and six months later, surviving mice were fully protected against the third lethal BoNT/A complex intoxication using an even higher dose (3 µg/mouse) than the initial intoxication dose (1 µg/mouse) (Fig. 7A–B). Protected mice did not show any symptoms of intoxication. Conclusively, this dual-route targeted vaccine demonstrates an efficacious protection against BoNT/A complex intoxication in surviving mice, highlighting its potency to maintain B cell responses to any further challenge.

4. Discussion

Vaccines are remarkable medical achievements to prevent infectious diseases [28–31]. Despite the protective potency of the vaccines, the concepts of the majority of such vaccines were empirically formulated, leaving little room for understanding immune mechanisms implicated in their protection. Thus, the failure of their efficacy was difficult to discern. Moreover, most earlier vaccine platforms were live attenuated pathogens, and though they were effective in protecting the host, their reversion to virulence was always a potential concern [32]. Conclusively, innovative modern vaccines involving immunogenic pathogen

subunits can be targeted, and the choice of vaccination route(s) may provide better protection, and are thus attractive possibilities for further fine-tuning in order to enhance their potency against infectious diseases [24,33]. Such targeted vaccines may specifically induce the activation of mucosal phagocytic DCs to specifically direct the recruitment and migration of primed lymphocytes, including B cells, to mucosal sites [34], all of which may result in regulated responses against pathogen challenge [35,36]. The targeted vaccine platform can be employed by *L. acidophilus* to efficiently deliver and simultaneously act as an excellent adjuvant to activate mucosal immunity and subsequent peripheral responses against pathogen challenge [37]. This notion was of significance from various perspectives, including the urgency of generating such a targeted vaccine strategy against acute and rapid intoxication, and whether this subunit vaccine strategy that had already demonstrated to be effective against anthrax [9] would also confer protection against BoNT complex intoxication. To pursue this trend, we considered further investigation with our recently established DC-targeting vaccine [13,38,39] in order to fine-tune the exerted mechanisms that should not only result in efficacious protection, but also in immune regulation to mitigate tissue damage. To proceed with the establishment of our targeted vaccine subunit, we chose to focus on neurotoxin serotype A of *C. botulinum* to be targeted and expressed by *L. acidophilus* [11] in order to demonstrate vaccine potency against acute BoNT/A complex intoxication that results in flaccid paralysis and death. To further develop the subunit vaccine, the DC-targeting peptide and Hc-BoNT/A subunit were combined to be secreted by *L. acidophilus*, NCK2310, from a chromosomal location to avoid the instability of a plasmid based vaccine [11].

Surprisingly, although *L. acidophilus*–expressing anthrax PA elicited protection against experimental anthrax challenge [9], the further developed NCK2310 expressing Hc-BoNT/A provided incomplete protection to orally vaccinated mice. Such incomplete protection may lie in the nature of rapid intoxication that is extremely acute, and such induced immunity may not be sufficient to overcome the pressure of oral intoxication, resulting in inefficacious protection and deteriorating animal health. Therefore, we concluded that our targeted oral vaccine approach required further modification of the administration routes in order to induce efficacious protection against acute intoxication.

To enhance the efficacy of this oral vaccine delivery system, we introduced the intradermal injection of the purified targeted immunogenic subunit to the oral vaccination route. As demonstrated, this dual-route vaccine strategy with no further boosts resulted in full protection against the harsh oral intoxication in the vaccinated mice, whereupon potent and long-lasting B cell responses played critical roles in neutralizing its devastating neurotoxin effects in our mouse model. Interestingly, such a dual-route vaccination also demonstrated efficacious potency to protect against poliovirus [15], highlighting its feasibility and relevance for clinical trials in the near future.

Importantly, our dual-route vaccination also completely protected the mice against the higher dose (3 µg) of oral challenge with the BoNT/A complex. In addition to surviving, vaccinated mice exhibited enhanced formation of GC B cells and memory B cells, suggesting long-term protection resulting from this targeted vaccine platform. Concurrently, this very important feature of our dual-route vaccine demonstrated the extended long-term

protection alone and via administration of the produced neutralizing antibodies into naïve mice that were subsequently orally intoxicated with BoNT/A complex, highlighting the central role of B cell responses against pathogen infection and also against BoNT/A complex intoxication. Furthermore, the synapse of Tfh and B cells, resulting in high-affinity antibody responses and B-cell memory was also induced by our dual-route targeted vaccine, documenting a critical feature of any protective vaccine that should induce the activation of long-lasting memory B cells upon pathogen or toxin challenge [18,40]. This notion is of significance from various perspectives that can highlight the virtual potency of a targeted subunit vaccine platform. This includes the urgency of generating a protective and efficacious vaccine strategy mobilizing long-lasting and regulated immunity against an acute and rapid reaction ravaging immune homeostasis and resulting in devastating health outcomes.

In conclusion, our data demonstrate that this dual-route vaccine strategy can be successfully employed *in vivo*, as it not only induced the activation of B cells, particularly memory B cells, but also protected mice from acute inflammation resulting in deteriorated health and death. Such an induced humoral response with significant longevity is poised to resist any further acute intoxication, an important hallmark that may be seen as an immediate outcome for evaluation of vaccines over time. Together, advances in new vaccine strategies and the choice of their delivery will greatly improve the efficacy of dual-route vaccination that may confer broad and long-lasting protection against pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BoNT	clostridium botulinum neurotoxin
DC_{pep}	dendritic cell binding peptide
Hc	heavy chain of BoNT/A
CL	colon
MLN	mesenteric lymph node
SP	spleen
PLN	peripheral lymph node
GC	germinal center
Tfh	T follicular helper cells

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Fig. 1.

Oral only vaccination does not fully protect mice against BoNT/A complex intoxication. (A) Schematic of vaccine schedule. (B) BALB/c mice were orally vaccinated (10^9 CFU/mice) with NCK2310 expressing Hc-BoNT/A-DCpep ($n = 23$ mice) or group of BALB/c mice were gavaged with PBS ($n = 13$ mice) and subsequently challenged with BoNT/A complex (1 µg/mouse). The protection was evaluated by mouse survival post oral intoxication. (C) Memory B cells from mice vaccinated and intoxicated, as described in (A), were magnetically isolated and transferred into recipient BALB/c mice. Recipient mice were then

orally intoxicated with BoNT/A complex (1 µg/mouse). The protection was evaluated by mouse survival post oral intoxication. Mouse survival curves were plotted and analyzed using Log-rank (Mantel-Cox) test; * $P < .05$, **** $P < .0001$.

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Fig. 2.

Dual-route vaccine protects fully against BoNT/A complex intoxication. BALB/c mice were gavaged (G, 10^9 CFU/mouse) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein (100 ng/mouse). One group of mice was gavaged and ID injected with PBS. These mice were orally intoxicated with a lethal dose of BoNT/A complex (1 μ g/mouse). Mouse survival curves were plotted. (A) Schematic of vaccine schedule for 6 gavages and 6 ID injections and survival curve of 6 dual-route vaccines ($n = 6$ mice/group and representative of 7 independent experiments). (B) Schematic of vaccine schedule for 4 gavages and 4 ID injections and survival curve of 4 dual-route vaccine ($n = 6-7$ mice/group and representative of three independent experiments). (C) Schematic of vaccine schedule for 1, 2 or 3 gavages and ID injections and survival curve of 1, 2, or 3 dual-route vaccine ($n = 5-7$ mice/group and representative of two independent experiments). (D) Fecal IgA titer and serological IgG titer from mice vaccinated with 3, 4, or 6 dual-route vaccine ($n = 10$ mice/group) or left unvaccinated (Control: naïve unvaccinated mice, $n = 6$ mice) and intoxicated with a lethal dose of BoNT/A complex (1 μ g/mouse). Mouse survival curves in (A–C) were plotted and analyzed using Log-rank (Mantel-Cox) test and antibodies titer in (D) were analyzed using a one-way ANOVA t test followed by a Tukey post-test, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

Fig. 3.

Quadruple dual-route vaccine protects fully against BoNT/A complex intoxication. BALB/c mice were gavaged (G, 10^9 CFU/mouse) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein (100 ng/mouse). These vaccinated mice were orally intoxicated with a lethal dose of BoNT/A complex (1 μ g/mouse). (A) Schematic of vaccine schedule for 4 gavages plus 4 ID injections or 4 ID injections only. (B) Survival curve of 4 dual-route or of 4 single ID route vaccine ($n = 5-8$ mice/group, data are representative of 2 independent experiments). (C) 100 μ L of serum

either derived from vaccinated or naïve mouse were co-incubated with 50 pg of BoNT/A neurotoxin for 1 h at 4 °C before injecting into groups of naïve mice by an intraperitoneal route. Survival curve of serum protection developed as an effect of dual-route vaccine (n = 5 mice/group and representative of 2 independent experiments). Mouse survival curves in (B) and (C) were plotted and analyzed using Log-rank (Mantel-Cox) test ^{**} $P < .01$.

Fig. 4.

Dual-route vaccination induces germinal center B cells and plasma cells differentiation against BoNT/A complex intoxication. BALB/c mice ($n = 5$) were gavaged (G) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein four times. These vaccinated (4G + 4ID) mice were then orally intoxicated with a lethal dose of BoNT/A complex ($1 \mu\text{g}/\text{mouse}$). Naïve unvaccinated BALB/c mice ($n = 4$) were used as control. The peripheral lymph nodes (PLN), the spleens (SP), the mesenteric lymph nodes (MLN), and the colon (CL) were analyzed by flow

cytometry. Gating strategy related to the dot plots depicted in this figure is detailed in the Supplemental Fig. 1. (A) Representative histograms of germinal center B cells (PNA⁺Fas^{hi}) among IgD⁻ B220⁺ B cells 14 days after challenge. (B) Representative histograms of plasma cells defined as B220^{low} CD138⁺ cells. Data are represented as mean $\pm$ S.E.M and representative of 3 independent experiments. The P value was determined by a two-tailed unpaired *t* test, **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

Fig. 5.

Dual-route vaccination induces T follicular helper (Tfh) cells against BoNT/A complex intoxication. BALB/c mice (n = 5) were gavaged (G) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein four times. These vaccinated (4G + 4ID) mice were then orally intoxicated with a lethal dose of BoNT/A complex (1 µg/mouse). Naïve unvaccinated BALB/c mice (n = 4) were used as control. The peripheral lymph nodes (PLN), the spleens (SP), the mesenteric lymph nodes (MLN), and the colon (CL) were analyzed by flow cytometry. Gating strategy of the dot

plots depicted in this figure is detailed in the Supplemental Fig. 2. (A) Representative dot plots and histograms of Tfh cells defined as CD4⁺ CD8⁻ CD44^{hi} CD62L^{lo} CXCR5⁺ PD1⁺ B220⁻ cells 14 days after challenge with 4 dual-route vaccine regimen. (B) Representative dot plots and histograms of germinal center Tfh cells defined as GL7⁺ CD4⁺ CD8⁻ CD44^{hi} CD62L^{lo} CXCR5⁺ PD1⁺ B220⁻ cells 14 days after challenge with 4 dual-route vaccine regimen. Data are represented as mean $\pm$ S.E.M and representative of 3 independent experiments. The P value was determined by a two-tailed unpaired *t* test, **P* < 0.05, ***P* < 0.01.

Fig. 6.

Induction of pre-memory and memory B cell responses by dual-routed vaccination. BALB/c mice (n = 5) were gavaged (G) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein four times. These vaccinated (4G + 4ID) mice were then orally intoxicated with a lethal dose of BoNT/A complex (1 µg/mouse). Naïve unvaccinated BALB/c mice (n = 4) were used as control. The peripheral lymph nodes (PLN), the spleens (SP), and the mesenteric lymph nodes (MLN) were analyzed by flow cytometry. Gating strategy of the dot plots depicted in this figure is detailed in the Supplemental Fig. 3. (A) Representative dot plots and histograms of pre-memory B cells defined as CD24^{hi} CD22⁺ CD79b⁺ IgD⁻ B220⁺ CD43⁻ cells 14 days after challenge. (B) Representative dot plots and histograms of memory B cells defined as CD24^{lo} CD22⁻ CD79b⁺ IgD⁻ B220⁻ CD43⁺ cells 14 days after challenge of mice. Data are

represented as mean $\pm$ S.E.M and representative of 3 independent experiments. The P value was determined by a two-tailed unpaired *t* test, **P* < .05, ***P* < .01.

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Fig. 7.

Dual-route vaccine protects fully against BoNT/A complex intoxication over time. BALB/c mice were gavaged (G) with NCK2310 and intradermally (ID) injected with the corresponding purified Hc-BoNT/A-DCpep fusion protein. These vaccinated mice were then orally intoxicated with a lethal dose of BoNT/A complex (1 μ g/mouse) on day 0, or 3 μ g/mouse on day 90 and day 270. (A) Schematic of vaccine schedule. (B) Survival curve of long-lasting effect of dual-route vaccine ($n = 6$ mice/group and representative of 2 independent experiments). Mouse survival curves were plotted and analyzed using Log-rank (Mantel-Cox) test, *** $P < .001$.