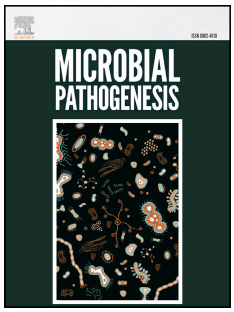
Accepted Manuscript



Different frequencies of memory B-cells induced by tetanus, botulinum, and heat-labile toxin binding domains

Ehsan Rezaie, Hekmat Nekoie, Ali Miri, Gholamreza Oulad, Ali Ahmadi, Mojtaba Saadati, Mahmood Bozorgmehr, Marzieh Ebrahimi, Jafar Salimian

PII: S0882-4010(18)31046-5

DOI: https://doi.org/10.1016/j.micpath.2018.12.003

Reference: YMPAT 3296

To appear in: *Microbial Pathogenesis*

Received Date: 4 June 2018

Revised Date: 18 October 2018

Accepted Date: 3 December 2018

Please cite this article as: Rezaie E, Nekoie H, Miri A, Oulad G, Ahmadi A, Saadati M, Bozorgmehr M, Ebrahimi M, Salimian J, Different frequencies of memory B-cells induced by tetanus, botulinum, and heat-labile toxin binding domains, *Microbial Pathogenesis* (2019), doi: https://doi.org/10.1016/j.micpath.2018.12.003.

Different Frequencies of Memory B-cells Induced by Tetanus, Botulinum, and Heat-labile Toxin Binding Domains

Running Head: Memory B cells frequency and duration of memory

Ehsan Rezaie[a], Hekmat Nekoie[b], Ali Miri[c], Gholamreza Oulad[d], Ali Ahmadi[a], Mojtaba Saadati[b], Mahmood Bozorgmehr[e], Marzieh Ebrahimi[f#], and Jafar Salimian[g#]

Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Science, Tehran, Iran[a]; Biology Research Center, IH University, Tehran, Iran[b]; Human Genetic Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran[c]; Applied Biotechnology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran[d]; Department of Immunology, Avicenna Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran[e]; Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, Tehran, Iran[f]; Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

Please address all correspondence to: **Dr. Jafar Salimian**, Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Vanak Square, Tehran, Iran. (T) 982182482503 (F) 982188211524 (E) jafar.salimian@gmail.com or **Dr Marzieh Ebrahimi,** Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology. (T) 982122306485 (F) 982122413720 (E) Ebrahimi@royaninstitute.org

These studies were equally supported by authors Ebrahimi and Salimian.

Abstract

Along with robust immunogenicity, an ideal vaccine candidate should be able to produce a long lasting protection. In this regard, the frequency of memory B-cells is possibly an important factor in memory B-cell persistency and duration of immunological memory. On this basis, binding domains of tetanus toxin (HcT), botulinum type A1 toxin (HcA), and heat-labile toxin (LTB) were selected as antigen models that induced long-term, midterm and short-term immune memory, respectively. In the present study, the frequency of total memory B-cells after immunization with HcT, HcA and LTB antigens after 90 and 180 days, and also after one booster, in 190 days, was evaluated. The results showed a significant correlation between frequency of total memory B-cells and duration of humoral immunity. Compared to other antigens, the HcT antibody titers and HcT total memory B-cell populations were greater and persistent even after 6 months. At 6 months after the final immunization, all HcT- and HcA-immunized mice survived against tetanus and botulinum toxins, and also LT toxin binding to GM1 ganglioside was blocked in LTB-immunized mice. We conclude the frequency of memory B-cells and their duration are likely a key factor for vaccine memory duration.

Key Words: Memory B-cell, Frequency, Vaccine, Tetanus toxin, Botulinum toxin, Heat-labile toxin

1. Introduction

Immunological memory, the ability to respond more strongly upon re-encounter with the same pathogen by immune system, constitutes the basis of vaccination [1, 2]. In fact, along with robust immunogenicity, an ideal vaccine candi-date should be able to produce long lasting protection. Due to their critical role in maintenance of serum antibody levels, memory B-cells and plasma cells are key cells that contribute to any long-lasting protection [3]. Several factors can affect the magnitude and longevity of produced memory B-cells [4]. Among these, the most important include the frequency of memory B-cells derived from a given specific antigen or/and the intrinsic traits of these antigen-experienced B-cells. As previous studies have demonstrated, various antigens can induce memory B-cells with differences in persistence [4]. Nevertheless, the question remains, how?

Although protein antigens have similar mechanisms for stimulation and induction of immune responses and immune memory, the strength of the response and duration of produced memory may be different. The question that persists is which factor(s) may be influenced by the nature of the antigen? It seems that the frequency of memory B-cells and also the antigen experience inherited by memory B-cells are important factors [5]. To address this concept, a study should be designed wherein different antigens with different potential to induce memory B-cells persistency and /or frequency are utilized. The tetanus toxoid vaccine used for infant immunization [6] and confers a 10-yr protection against tetanus [7] would be one appropriate model for evaluation of antigen induced long-lasting memory. A second appropriate antigen could be botulinum toxin [given as pentavalent botulinum toxoid (PBT)] that can confer a 2-year protection [8]. Tetanus and botulinum neurotoxin (TeNT and BoNT) are structurally similar and consist of two structural heavy and light chains. The 100 kDa heavy chain domain comprises a

50 kDa translocation domain (Hn) and 50 kDa receptor binding domain (Hc). The use of subunit vaccines especially against clostridial is a common approach so that genetically- constructed clostridial vaccines offering the functional nontoxic fragments when administrated into animal. The advantages of these components are included production in a large wide of microbial expression hosts in the form of pure vaccine antigens, having smaller size, maintain immunogenicity of the parent proteins, show little or no toxicity and ability to elicit protective immunity. So, the Hc domain of clostridial toxins are applied in many studies and it has been proved that the Hc domain could be high-performance vaccine providing a complete protection against challenge with the whole toxin [9, 10]. The carboxyl terminal of the heavy chain of tetanus (HcT) and botulinum (HcA), is the main binding domain and because of immunogenicity properties, is considered as a vaccine candidate for tetanus and botulism respectively. The third appropriate antigen could be the heat-labile B subunit toxin (LTB) produced by entrotoxigenic *Escherichia coli* used as a vaccine candidate. This agent is known to confer almost a 6-month protection [11-13].

Accordingly, we used the tetanus Hc-fragment (HcT), the botulinum type A1 Hc-fragment (HcA), and also the LTB as representative antigens that their complete inactivated toxins produce, respectively, long (10 year)-, medium (2 year)-, and short (6 month)-term immunological memory. Specifically, the study investigated both the frequency and duration of memory B-cells (as an index of memory longevity) in mice at 3 and 6 months post-immunization with these recombinant HcT, HcA, and LTB proteins.

2. Material and Methods

2.1. Expression and purification of recombinant proteins

All experiments were carried out according to protocols outlined in Khalesi et al. [14], Yari et al. [15], and Rezaee et al. [16]. Recombinant genes coding for the tetanus toxin Hc-fragment (HcT) (GenBank: AM412776.1), *C. botulinum* type A1 toxin Hc-fragment (HcA) (GenBank: U22962.1), and *ETEC* heat labile toxin B subunit (LTB) (Genbank: M17874) were separately expressed in pET28a vectors using an Escher*ichia coli* BL21 DE3 host. The HcT and HcA recombinant proteins were purified under native conditions and a nickel-nitrilotri-acetic acid (NTA) matrix; LTB recombinant protein was purified under denaturation conditions.

SDS-PAGE and Western blotting

All three purified recombinant proteins were separately analyzed on SDS-PAGE (12%) and further evaluated by western blotting. Briefly, the separated protein bands were transferred to nitrocellulose membrane using a Mini-Trans Blot electrophoretic transfer cell and then the membrane was incubated for 1 hour at RT. The membrane was incubated for 1 h in 1:2000 dilutions of anti-His Tag HRP- conjugated antibody (Sigma, USA) and developed [17].

2.2. Mice

BALB/c mice (male, 6-8-wk-of-age) were purchased from the Razi Institute (Karaj, Iran). All mice were housed under pathogen-free conditions in facilities maintained at 22°C with a 50% relative humidity and a 12-hr light: dark cycle. All mice had *ad libitum* access to standard rodent chow and filtered water. All experiments were performed in accordance with the Guide-lines from the Animal Care and Research Committee of Baqiyatallah University of Medical Sciences, which itself was in compliance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

2.3. Immunization

For the experiments, mice were randomly allocated into four groups (N = 15/group) to be immunized with the antigen or injection with the vehicle. For this first injection, 25 µg of HcT, HcA, and LTB were separately mixed in complete Freund's adjuvant (CFA; Sigma, St. Louis, MO). For this purpose, the equal volumes of each antigen and CFA were separately mixed using two sterile luer-lock syringes with moving the solution back and forth for several minutes to form an emulsion with maximum stability. Then, each antigen was injected subcutaneously (total volume = 200 µl) into respective mice in each corresponding group. Twenty days later, 20 µg of each antigen was mixed in incomplete Freund's adjuvant (IFA) and then injected into all mice in the corresponding treatment groups. On Days 34 and 48, respectively, mice in respective treatment groups then received booster injections of 15 µg and 10 µg of each antigen in IFA. Throughout, control group mice only received the phosphate-buffered saline (PBS, pH 7.4) injections.

2.4. Antibody titration by ELISA

Antigen-specific IgG levels in isolated serum samples were measured by indirect enzyme-linked immune sorbent assay (ELISA). Blood (300 µl/mouse) was obtained from the retro-orbial plexus of random immunized mice (n =5) in each group on Days 34, 48, 62, 92, 122, 152 and 242 of the experiment. At each timepoint, serum was isolated from the collected blood and placed at -80°C until used in analyses.

For the assays, wells of microtiter plates (Nunc-ImmunoPlates® Maxisorp, Frankfurt, Germany) were coated overnight at 4°C with 5 µg antigen/µl (dedicated wells for each given antigen). After four washes with PBS containing 0.5% Tween-20 (PBST), wells were blocked with PBS-gelatin solution (5% w/v). Sera samples were serially diluted in PBST (1:100 - 1:12,800) and then added to each well. The plates were then incubated for 60 min at 37°C. The

sera was then carefully removed, the wells gently washed with PBST, and then horseradish peroxidase-conjugated anti-mouse IgG-HRP antibody (1:1000 dilution, Sigma) was added to each well. The plates then were incubated 1 hr at 37°C before the well contents were removed and the wells rinsed. Thereafter, tetramethylbenzidine (TMB-H_2O_2, Sigma) solution was added and color development allowed proceeding for 20 min before addition of stop solution (2 M H_2SO_4). Absorbance values in each well were measured at 495 nm using a microplate reader (Bio-Rad, Hercules, CA). Data were expressed in terms of mean OD values.

2.5. Flow cytometry

At 3 and 6 months after the final immunization and 10 days after booster injection, the frequency of total memory B-cells was assessed. At each time point, 3mice/group were euthanized with chloroform and their spleen aseptically isolated at necropsy. Each spleen was processed into a single cell suspension using standard protocols; the liberated cells were collected in PBS-EDTA solution, centrifuged, and then counted in a hemocytometer. Cell viability was routinely ≈ 95%. Aliquots of cells (1.5×10^6) were placed in dedicated tubes and then stained by addition of CD19-peridinin chlorophyll protein (PerCP) (eBiosciences, San Diego, CA), IgD-V450 (BD Biosciences, Heidelberg, Germany), and IgG-fluorescein isothio-cyanate (FITC) (Genetex, Irvine, CA) at manufacturer-recommended antibody levels. The cells were then incubated at 4°C for 40 min in the dark. Thereafter, the cells were centrifuged and washed with PBS. To permit analysis of viability, an additional set of cells was treated with 5 µl of commercial propidium iodide (Sigma) and incubated at 25°C for 1 min in the dark. All cells were then sampled onto a FACSaria flowcytometer (BD Biosciences). A minimum of 800,000 events per sample was acquired. All data was analyzed using FlowJo software (Ashland, OR).

Results were gated on total B-cells ($CD19^+$) and then the frequency of memory B-cells (IgD^- and IgG^+) were evaluated based on the IgD and IgG markers.

2.6. Challenge test

The purified tetanus toxin was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). The LD50 of the toxin was 10^6. The botulinum toxin was semi-purified. Briefly, after culturing *Clostridium botulinum* serotype A for 72 hours, the supernatant was acidified with H_2SO_4. The collected precipitate was washed by distilled water and then extracted with 0.1 M citrate buffer. After centrifugation, the supernatant was partially saturated with ammonium sulfate. The precipitate was resolved in phosphate buffer. The LD50 of the semi-purified toxin was 10^4 [18].

In the remaining 3 mice/group on days 160 and 250, the tetanus toxin (10^4 LD50/ 0.5ml/mice) and the botulinum toxin (10^3LD50/ 0.5ml/mice) were respectively injected intra-peritoneally into the HcT- or HcA- preimmunized mice, and were monitored for 72 hr. The LTB-immunized mice, the efficiency of LTB-specific antibodies to neutralize heat-labile toxin was evaluated in a GM1-binding inhibition assay. Here, serially-diluted serum from LTB-immunized mice was incubated at 37°C for 1 hr with LT toxin (at 2 μg/100 μl). The mixture was then added to wells of microtiter plates that had been pre-coated with GM1 and a GM1-ELISA was then performed as described previously in Yuki et al [19].

2.7. Statistical analysis

All data are reported as mean ± SD. Statistical analysis was performed using SPSS v.20 software (SPSS, Chicago, IL). A Tukey's range test was used to compare antibody titers and Ω of memory B-cell frequencies between the groups. The number of surviving animals in different

178 groups was evaluated using a multivariate analysis of variance (MANOVA). A p-value < 0.05
179 was considered statistically significant.

180

181 **3. Results**

182 **3.1. Evaluation of expression and purification of recombinant proteins**

183 Electrophoresis over SDS-PAGE gels was used to confirm the proper expression/purification of
184 all three rLTB, rHcA and rHcT proteins (Figure 1A). Purity of the isolated proteins was
185 confirmed via Western blotting (Figure 1B).

186 **3.2. Antigen-specific IgG titers**

187 Specific IgG titers against each recombinant protein were significantly increased (vs.
188 control mice levels of IgG) after the first injection and seemed to increase for up to 4 weeks after
189 the final immunization (Figure 2). By Month 2 (1 month after that boost), IgG titers in all antigen
190 groups started to decline. The results showed differences in titers among the groups at the
191 various timepoints. Among all the regimens, mice that had been immunized with HcT had the
192 highest IgG titers at each timepoint ($p < 0.05$). Anti-antigen titres in the serum of HcA- or LTB-
193 immunized mice never significantly differed over the entire study period.

194 **3.3. Frequency of memory B-cells after 3 and 6 months**

195 Three months after the final immunization, the frequency of splenic memory B-cells was
196 increased in immunized mice over levels in control mice (Figure 3). In addition, the frequency of
197 IgD^+ B-cells was decreased (relative to control mice values) in the higher memory B-cell
198 frequency groups. The frequency of memory B-cells for the control, LTB, HcA and HcT groups
199 was, respectively, 0.16 [± 0.02], 0.41 [± 0.07], 0.65[± 0.05], and 0.95% [± 0.13]. In parallel, the

incidence of IgD^+ B-cells was seen to be, respectively, 83.8 [± 4.23], 72.1% [± 4.64], 61.5 [± 3.5], and 56.6% [± 5.5] for these groups.

By 6 months after the final immunization, the frequency of memory B-cells had increased to 0.26 [± 0.01], 0.63 [± 0.04], 1.09 [± 0.05], and 1.76% [± 0.18] in, respectively, the control, LTB, HcA, and HcT mice (Figure 4). At this time point, the frequency of IgD^+ B-cells had remained fairly constant, but the frequency of memory B-cells was increased by ≈ 85% in spleens of mice immunized mice with the HcT subunit. As noted in reviews by Walker et al. and Lundgren et al., it is generally believed LTB-derived memory B-cells are completely gone after 6 months [20, 21]. However, it was seen here there were still elevated levels of LTB-derived memory B-cells in the immunized mice at that time point as compared to in the spleens of time-matched control mice (0.63 vs. 0.26%; $p = 0.008$).

3.4. *In situ* re-stimulation of memory B-cells

At 10 days after booster injection of each antigen into the respective immunized groups, the frequency of memory B-cells was dramatically decreased, especially for HcT (Figure 5). Values had gone from 1.76 [± 0.18] to 0.22% [± 0.05] for these mice; values had changed from 0.63 [± 0.04] to 0.33% [0.03] for the LTB mice, from 1.09 [± 0.05] to 0.37% [± 0.26] for the HcA mice, and from 0.26 [± 0.01] to 0.20% [0.01] for the control mice (mice that received antigens for the first time at 6 month time point).

When all flow cytometry data was summarized (Figure 6), it was apparent that despite the presence of different memory B-cells population, the presence of memory B-cells was continually increased up to 6 month after the initial immunization for all the different test antigens. However, among the HcT mice, the changes from Month 3 to Month 6 were the

222 greatest (i.e., 0.70%). The changes were only 0.20% for the LTB mice, 0.42% for the HcA mice,
223 and 0.10% for the control mice.

224 **3.5. Recombinant HcT, HcA, and LTB-specific memory B-cells are long-lived and confer**
225 **protection against tetanus, botulinum and heat-labile toxin.**

226 At 3 and 6 months after the final immunization, all HcT- and HcA-immunized mice were
227 able to survive a challenge with, respectively, tetanus or botulinum toxin [18]. Using a GM1-
228 binding inhibition assay, the data in Figures 7A and 7B shows that LT binding to GM1
229 ganglioside was (A) blocked ≈ 45% by serum collected at 3 months, and (B) by ≈ 30% by serum
230 collected at 6 months after the final immunization with LT protein.

231 **4. Discussion**

232 Generally, upon re-entry into a host of a previously exposed pathogen, memory B-cells
233 rapidly differentiate to antibody-secreting cells; this process may result in a specific rapid
234 immune response against that antigen [22]. Many studies [23-26] focused on the longevity of
235 memory B-cells and demonstrated that they were not phenotypically/functionally similar in
236 responses to different antigens [4]. To address the question, a model was established using three
237 antigens known to impart short-, midterm-, and long-live humoral memory. The study found that
238 after immunization with the antigens, despite the same concentrations and similar conditions,
239 HcT antigen induced higher specific antibody titers. These differences in effect are in line with
240 other studies where antigens with different structures had different abilities to induce humoral
241 immune response and produce different levels of specific antibodies [27]. Despite this fact, the
242 three antigens used in the present study had structural, functional, and even size (for HcA and
243 HcT) similarities, yet still had different abilities to elicit immune responses.

Since a great difference was observed in the frequency of memory B-cells, it is plausible that greater immunogenicity could give rise to larger memory B-cell populations. In response to antigens able to activate more primary B-cells, a higher frequency of primary B-cells could enter secondary follicles, and as a result, a larger population of memory B-cells could be produced. One of the most important factors contributing in B cell development to memory B cell is the affinity of antigen to B cells surface receptors. The affinity of antigen-antibody interaction has a critical role in signal transduction process. It seems that HcT has a greater affinity to B cell receptors to induce more potent signal transduction (higher than the signal transduction threshold) to promote B cell to memory B cells formation. Thus, we surmise that due to a greater affinity, greater signaling potency, and stimulation of a large population of B-cells, HcT antigen could produce a greater frequency of memory B-cells for a longer period of time.

Since all immunized mice survived after challenge and the specific antibodies were reduced to the lowest level after 6 months, the persistence of memory B-cells (independent of pre-existing specific antibodies) could possibly confer the successful protection. Therefore, the frequency of memory B-cells and longevity might be good indicators of protection. It seems that upon re-exposure of memory B-cells to the same pathogen, the cells either differentiate into short-lived antibody-secreting plasma cells and raises specific antibody levels or they re-enter secondary follicles and produce long-lived plasma cells. This would be accordance with Rosado et al. [28] who reported that 5 years after hepatitis B vaccination, despite a significant reduction in anti-HBS antibody [even below protection cut-off levels], memory B-cells were still able to confer protection. To achieve this, there are likely two mechanisms. First, as many studies have correlated the levels of antibody with the frequency of memory B-cells [29-32], plasma cells maintain levels of antibody through replication, proliferation, and differentiation of the memory

B-cells [33-35]. With this approach, permanent and gradual differentiation of memory B-cells into plasma cells is essential for long-lasting antibodies. The second mechanism is that plasma cells are independent from memory B-cells and following production; they migrate to niches in the bone marrow and maintain the levels of antibody. Along this line, there are studies that have reduced the role of memory B-cells and instead emphasized the function of bone marrow plasma cells for maintenance of antibody levels [3].

Ammana et al. showed that the type of antigen is a key factor in determining the correlation between memory B-cells and maintenance of specific antibody levels [36]. Those investigators assessed this relationship in eight pathogens and found a significant correlation for measles, rubella, and mumps, but not for Varicella-Zoster and Epstein-Barr viruses, and also not for tetanus and diphtheria toxins. This correlation has also been confirmed for cholera toxin [37] and clostridium difficile Toxins A and B [38].

In the case of tetanus, this finding is different from the results of the current study, possibly because here only the binding domain of tetanus toxin was used whereas the earlier study used whole toxoid. Complete toxoid immunization can result in the formation of larger aggregates that can involve a wider range of B-cells. In the same way, lower numbers of epitopes involve a smaller B-cells population and subsequently produce functional memory B-cells that can constantly proliferate and differentiate into plasma cells and thereby maintain specific antibody levels.

Here, it was noted that for all three antigens tested, memory B-cells could maintain their significant levels for up to 3 months after the final immunization. Furthermore, up to 6 months after, the frequency of memory B-cells continued to rise against the three antigens, and so could protect mice against lethal challenges with parent toxins. Despite the fact that by 2 months after

the final immunization, specific antibodies levels gradually decreased for the three antigens, the frequency of memory B-cells was significantly decreased after a boost in Month 6 in all vaccinated groups (but increased in control group that received antigen for first time). Due to a rapid decline in memory B-cells populations 10 days after the boost, it seemed the specific memory B-cells quickly differentiated into antigen-specific antibody-secreting cells to produce specific IgG antibodies against the toxin(s).

As reviewed in Yoshida et al. and Sallusto et al., if a pathogen causes re-infection, specific memory B-cells rapidly respond and differentiate, and then promote antibody-mediated protection [22, 39]. In the current study, 6 months after the final immunization, anti-HcA and anti-LTB specific antibody titers were reduced to minimal levels, and the ability of anti-LT antibodies to prevent LT antigen from binding to its receptor was reduced by 30%. In our previous study it was demonstrated that up to 80% of toxin binding inhibition was seen even 1 month after immunization [17]. However, there was 45 and 30% toxin binding inhibition at 3 and 6 months, respectively. In other words, at 6 months, 70% of the toxin was still effective - meaning no protection. This suggested to us that LTB antigen was most likely not able to confer protective immunity for short times.

4.1. Conclusions

The results of the present study indicated that antibody titers generated against a given antigen were a good indicator to predict the frequency of specific memory B-cells. Accordingly, it is plausible then that increasing the frequency of memory B-cells depends on increasing the immunogenicity of the antigen. The present study also established that sustained frequency of memory B cells in the spleen, independent of that associated with bone marrow-derived memory plasma cells, was a critical factor in antigen-induced memory longevity.

Acknowledgements

The authors would like to thank the flow cytometry technicians in Royan Institute for Stem Cell Biology and Technology, and also Dr M. Tavallaie for kindly gifting a HcA clone. These studies were equally supported by authors Ebrahimi and Salimian. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

References

[1] Campos M, Godson DL. The effectiveness and limitations of immune memory: understanding protective immune responses. International journal for parasitology. 2003;33:655-61.

[2] Zielinski CE, Corti D, Mele F, Pinto D, Lanzavecchia A, Sallusto F. Dissecting the human immunologic memory for pathogens. Immunological reviews. 2011;240:40-51.

[3] Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity. 1998;8:363-72.

[4] Nayak R, Lal G, Shaila M. Perpetuation of immunological memory: role of serum antibodies and accessory cells. Microbes and infection. 2005;7:1276-83.

[5] Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nature immunology. 2011;12:509-17.

[6] Calvo AC, Oliván S, Manzano R, Zaragoza P, Aguilera J, Osta R. Fragment C of tetanus toxin: new insights into its neuronal signaling pathway. International journal of molecular sciences. 2012;13:6883-901.

[7] Koenig MA, Roy NC, Mcelrath T, Shahidullah M, Wojtyniak B. Duration of protective immunity conferred by maternal tetanus toxoid immunization: further evidence from Matlab, Bangladesh. American Journal of Public Health. 1998;88:903-7.

[8] Control CfD, Prevention. Notice of CDC's discontinuation of investigational pentavalent (ABCDE) botulinum toxoid vaccine for workers at risk for occupational exposure to botulinum toxins. 2011;60:1454.

[9] Webb RP, Smith LAJErov. What next for botulism vaccine development? 2013;12:481-92.

[10] Webb RP, Smith TJ, Smith LA, Wright PM, Guernieri RL, Brown JL, et al. Recombinant botulinum neurotoxin Hc subunit (BoNT Hc) and catalytically inactive Clostridium botulinum holoproteins (ciBoNT HPs) as vaccine candidates for the prevention of botulism. 2017;9:269.

[11] Bagheri S, Gargari SLM, Rasooli I, Nazarian S, Alerasol M. A CssA, CssB and LTB chimeric protein induces protection against Enterotoxigenic Escherichia coli. Brazilian Journal of Infectious Diseases. 2014;18:308-14.

[12] Svennerholm A-M, Tobias J. Vaccines against enterotoxigenic Escherichia coli. 2008.

[13] Turner AK, Stephens JC, Beavis JC, Greenwood J, Gewert C, Randall R, et al. Generation and characterization of a live attenuated enterotoxigenic Escherichia coli combination vaccine expressing six colonization factors and heat-labile toxin subunit B. Clinical and Vaccine Immunology. 2011;18:2128-35.

[14] Khalesi R, Sh N, Ehsaei Z, Mansouri M, Amani J, Salimian J, et al. Optimization of gene expression and purification of enterotoxigenic Escherichia coli recombinant LTB protein and antibody production against it. Trauma Monthly. 2010;2010:141-7.

[15] Yari K, Fatemi SS-A, Tavallaei M. High level expression of recombinant BoNT/A-Hc by high cell density cultivation of Escherichia coli. Bioprocess and biosystems engineering. 2012;35:407-14.

[16] Rezaee E, Saadati M, Salimian J, ovlad g, Miri A, azizi jalilian f, et al. Evaluating and Comparing Immunization Level of the Recombinant Proteins, Binding Domain of Tetanus Neurotoxin and B Subnnit of Heat Labile Toxin of Escherichia coli, and their Relation to Immunological Memory. www sjimu medilam ac ir. 2014;21:266-73.

[17] Salimian J, Salmanian A, Khalesi R, Mohseni M, Moazzeni S. Antibody against recombinant heat labile enterotoxin B subunit (rLTB) could block LT binding to ganglioside M1 receptor. Iranian journal of microbiology. 2010;2:120.

[18] Saadati M. SJ, Ebrahimi F., Bahmani M.K., Gandomi A. Evaluation of antibody Y (IgY) raised against botulinum toxin serotype A in an animal model. Hakim Rsearch J. 2004;7:44 -51.

[19] Yuki Y, Tokuhara D, Nochi T, Yasuda H, Mejima M, Kurokawa S, et al. Oral MucoRice expressing double-mutant cholera toxin A and B subunits induces toxin-specific neutralising immunity. Vaccine. 2009;27:5982-8.

[20] Lundgren A, Jertborn M, Svennerholm A-M. Induction of long term mucosal immunological memory in humans by an oral inactivated multivalent enterotoxigenic Escherichia coli vaccine. Vaccine. 2016;34:3132-40.

[21] Walker RI, Steele D, Aguado T, Committee AHETE. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic E. coli (ETEC) disease. Vaccine. 2007;25:2545-66.

[22] Yoshida T, Mei H, Dörner T, Hiepe F, Radbruch A, Fillatreau S, et al. Memory B and memory plasma cells. Immunological reviews. 2010;237:117-39.

[23] Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. The Journal of Immunology. 2003;171:4969-73.

[24] Cruz SC, Souza SL, Cruz AC, Silva GP, Milagres LG. Human antibody and memory B and T-cell responses after primary and booster immunisation against Neisseria meningitidis B. Vaccine. 2011;29:7387-94.

[25] Hendrikx LH, Felderhof MK, Öztürk K, de Rond LG, van Houten MA, Sanders EA, et al. Enhanced memory B-cell immune responses after a second acellular pertussis booster vaccination in children 9 years of age. Vaccine. 2011;30:51-8.

[26] Jertborn M, Svennerholm A-M, Holmgren J. Immunological memory after immunization with oral cholera B subunit-whole-cell vaccine in Swedish volunteers. Vaccine. 1994;12:1078-82.

[27] Alerasol M, Gargari SLM, Nazarian S, Bagheri S. Immunogenicity of a Fusion Protein Comprising Coli Surface Antigen 3 and Labile B Subunit of Enterotoxigenic Escherichia coli. Iranian biomedical journal. 2014;18:212.

[28] Rosado MM, Scarsella M, Pandolfi E, Cascioli S, Giorda E, Chionne P, et al. Switched memory B cells maintain specific memory independently of serum antibodies: the hepatitis B example. European journal of immunology. 2011;41:1800-8.

[29] Amanna IJ, Slifka MK. Quantitation of rare memory B cell populations by two independent and complementary approaches. Journal of immunological methods. 2006;317:175-85.

[30] Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298:2199-202.

[31] Rohner GB, Snape MD, Kelly DF, John T, Morant A, Yu L-M, et al. The magnitude of the antibody and memory B cell responses during priming with a protein-polysaccharide conjugate vaccine in human infants is associated with the persistence of antibody and the intensity of booster response. The Journal of Immunology. 2008;180:2165-73.

[32] Traggiai E, Puzone R, Lanzavecchia A. Antigen dependent and independent mechanisms that sustain serum antibody levels. Vaccine. 2003;21:S35-S7.

[33] Anderson SM, Tomayko MM, Shlomchik MJ. Intrinsic properties of human and murine memory B cells. Immunological reviews. 2006;211:280-94.

[34] Gatto D, Martin SW, Bessa J, Pellicioli E, Saudan P, Hinton HJ, et al. Regulation of memory antibody levels: the role of persisting antigen versus plasma cell life span. The Journal of Immunology. 2007;178:67-76.

[35] Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dörner T, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. Nature Reviews Immunology. 2006;6:741-50.

[36] Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. New England Journal of Medicine. 2007;357:1903-15.

[37] Patel SM, Rahman MA, Mohasin M, Riyadh MA, Leung DT, Alam MM, et al. Memory B cell responses to Vibrio cholerae O1 lipopolysaccharide are associated with protection against infection in household contacts of cholera patients in Bangladesh. 2012:CVI. 00037-12.

[38] Monaghan TM, Robins A, Knox A, Sewell HF, Mahida YRJPo. Circulating antibody and memory B-cell responses to C. difficile toxins A and B in patients with C. difficile-associated diarrhoea, inflammatory bowel disease and cystic fibrosis. 2013;8:e74452.

[39] Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. Immunity. 2010;33:451-63.

Figure legend

Figure 1. Representative SDS-PAGE gel showing purified recombinant proteins (A) LTB subunit (lane 1), HcA subunit (lane 2) and HcT subunit (lane 3) and molecular weight maker (lane 4). **(B)** Representative Western blot analysis: SM0431 protein size maker (lane 1), LTB subunit (lane 2), HcT subunit (lane 3), and HcA subunit (lane 4). HRP-conjugated mouse anti-His tag antibody (Sigma, USA) was used for detecting of antigens in this test.

Figure 2. A. Time line ruler. Shows all events include injections, blood removal, boosters and challenges at defined timepoints. B. Antigen-specific IgG titres. Blood was collected from all mice at 34, 48, and 62 days (during 4 stages of immunization), and also 1, 2, 3 and 6 months after the final immunization. Random sera from 5 mice/group were then evaluated for levels of IgG antibodies for HcT, HcA, or LTB by ELISA. Data shown are mean [± SD] OD values. **Value significantly different from controls ($p < 0.05$). *Value significantly different from other two regimens at given timepoint ($p < 0.05$).

Figure 3. Memory B-cell frequencies. At about 3 months after the final immunization, splenocytes from HcT-, HcA-, or LTB-immunized and control mice were isolated and stained for memory B-cell markers. On gated CD19 cells, memory B-cells were identified based on the presence or absence of IgG and IgD, respectively. Numbers within the representative plots indicate the relative percentage of memory B-cell population in sample.

Figure 4. Memory B-cell frequencies. At about 6 months after the final immunization, splenocytes from HcT-, HcA-, or LTB-immunized and control mice were isolated and stained for memory B-cell markers. On gated CD19 cells, memory B-cells were identified based on the

455 presence or absence of IgG and IgD, respectively. Numbers within the representative plots
456 indicate the relative percentage of memory B-cell population in sample.

457 **Figure 5. Memory B-cell frequencies after *In situ* re-stimulation of memory B-cells.** At Day
458 10 after the booster injection (at 6 months), splenocytes from HcT-, HcA-, or LTB-immunized
459 and control mice were isolated and stained with antibodies against CD19, IgD, and IgG. As
460 shown in the representative plots, the frequency of memory B-cells was decreased in all groups.

461 **Figure 6. Frequency of memory B-cells as a function of time.** Levels were calculated at 3 and
462 6 months after the final immunization, and 10 days after the booster. Results shown are mean ±
463 SD percentages of the cells. **Value significantly different from controls ($p < 0.05$). *Value
464 significantly different from other two regimens at given timepoint ($p < 0.05$).

465 **Figure 7. LT binding to GM1 ganglioside.** Serum from LT-immunized mice was collected at
466 each timepoint indicated in the Methods and analyzed in a GM1 blocking assay. Figure shows
467 outcomes from samples isolated 3 months and 6 months after final immunization. At Month 3,
468 binding was blocked by ≈ 45% with immunized mouse serum; at Month 6, binding was blocked
469 by ≈ 30%, meaning 70% of the toxin was still potentially effect-tive. Sera from control mice and
470 PBS alone were used as negative controls in the assay. Results shown are mean ±SD relative
471 GM1 binding indices. **Value significantly different from controls ($p < 0.05$).

472

473

474

475

476

477

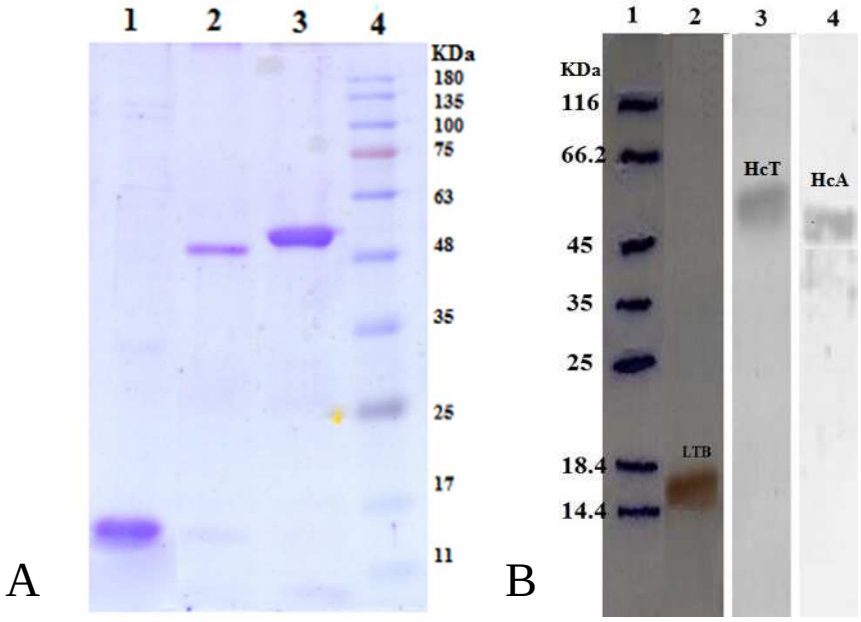


Figure 1. Representative SDS-PAGE gel showing purified recombinant proteins **(A)** LTB subunit (lane 1), HcA subunit (lane 2) and HcT subunit (lane 3) and molecular weight maker (lane 4). **(B)** Representative Western blot analysis: SM0431 protein size maker (lane 1), LTB subunit (lane 2), HcT subunit (lane 3), and HcA subunit (lane 4). HRP-conjugated mouse anti-His tag antibody was used for detecting of antigens in this test.

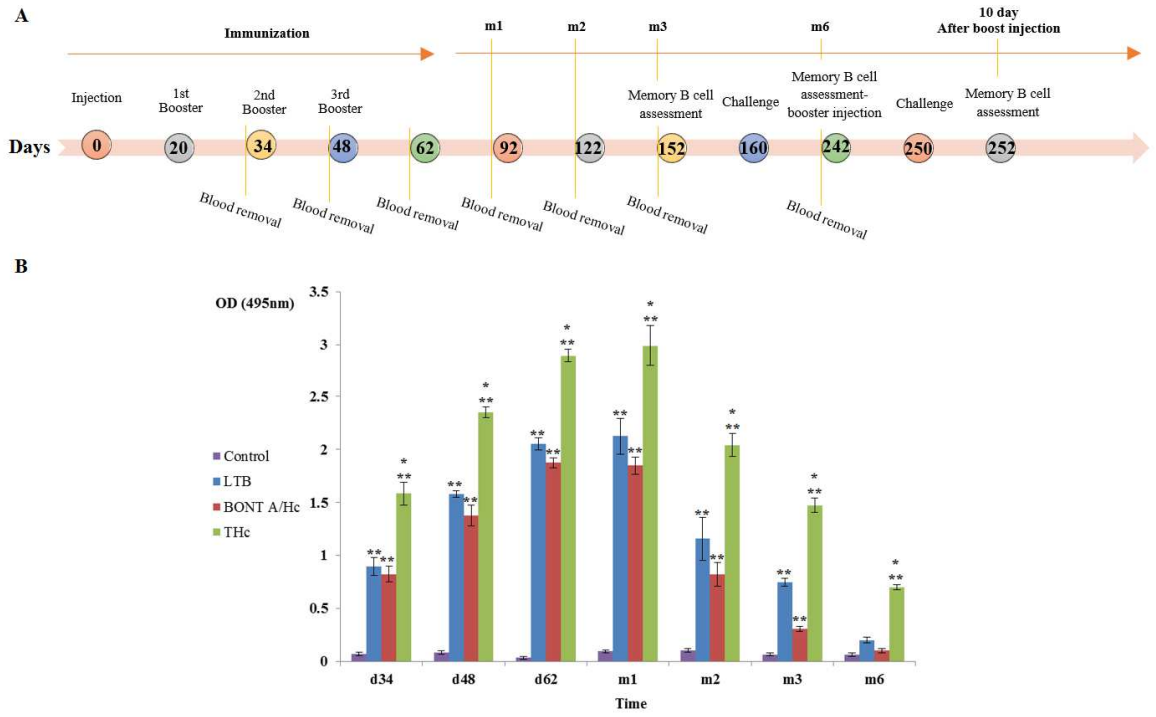


Figure 2. A. Time line ruler. Shows all events include injections, blood removal, boosters, memory B cell assessment and challenges at defined timepoints. **B. Antigen-specific IgG titres.** Blood was collected from all mice at 34, 48, and 62 days (during 4 stages of immunization), and also 1, 2, 3 and 6 months after the final immunization. Random sera from 5 mice/group were then evaluated for levels of IgG antibodies for HcT, HcA, or LTB by ELISA. Data shown are mean [± SD] OD values. **Value significantly different from controls ($p < 0.05$). *Value significantly different from other two regimens at given timepoint ($p < 0.05$).

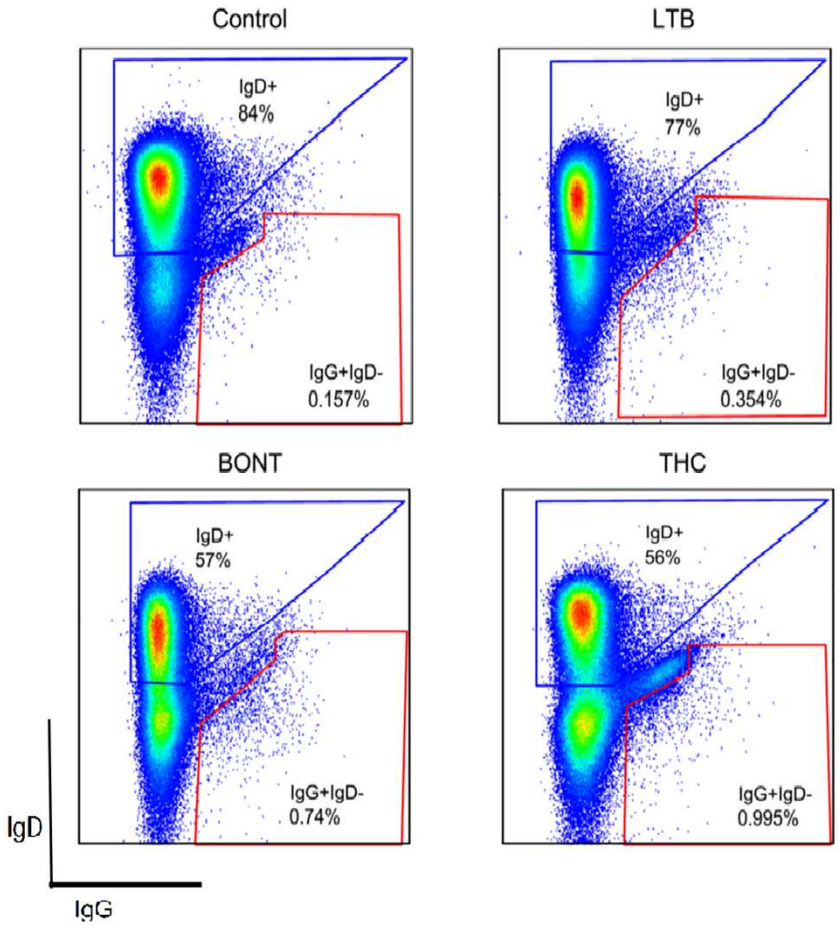


Figure 3. Memory B-cell frequencies. At about 3 months after the final immunization, splenocytes from HcT-, HcA-, or LTB-immunized and control mice were isolated and stained for memory B-cell markers. On gated CD19 cells, memory B-cells were identified based on the presence or absence of IgG and IgD, respectively. Numbers within the representative plots indicate the relative percentage of memory B-cell population in sample.

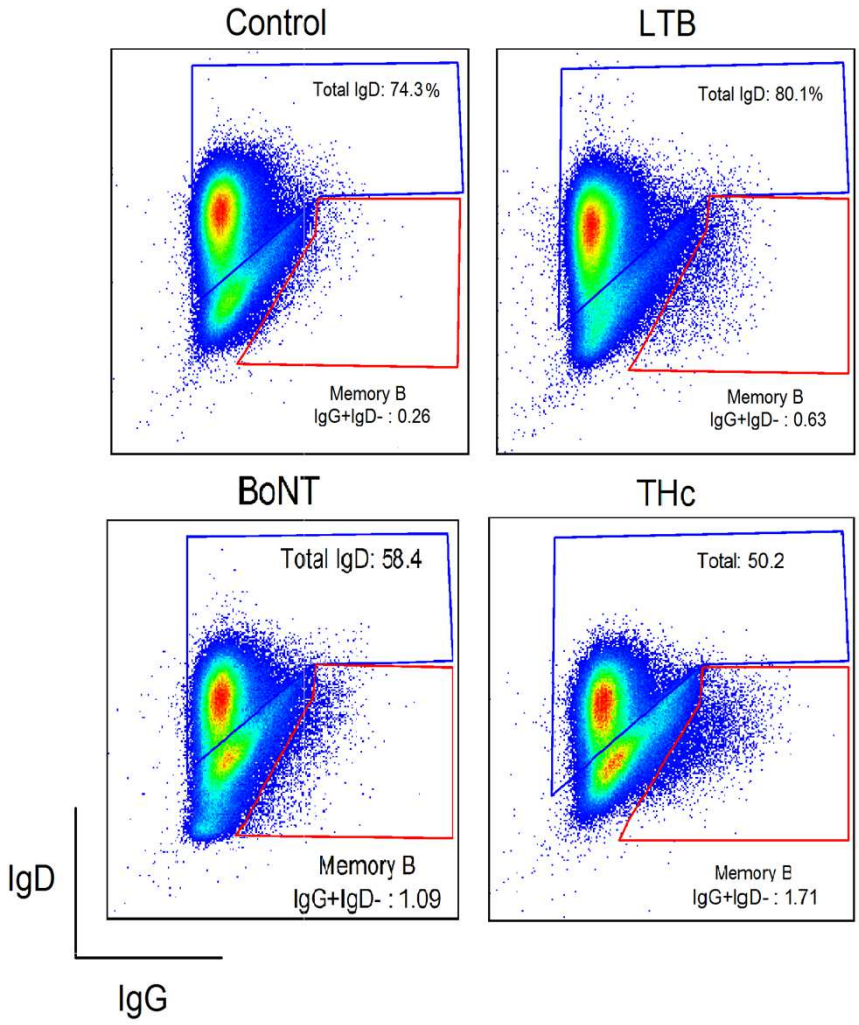


Figure 4. Memory B-cell frequencies. At about 6 months after the final immunization, splenocytes from HcT-, HcA-, or LTB-immunized and control mice were isolated and stained for memory B-cell markers. On gated CD19 cells, memory B-cells were identified based on the presence or absence of IgG and IgD, respectively. Numbers within the representative plots indicate the relative percentage of memory B-cell population in sample.

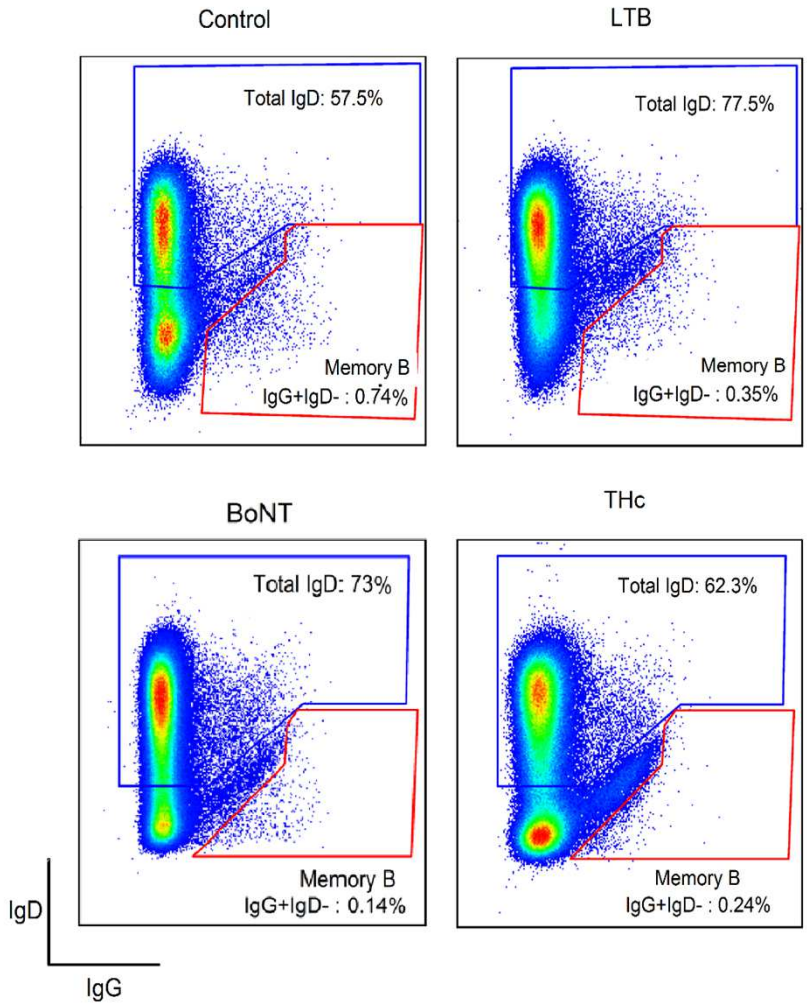


Figure 5. Memory B-cell frequencies after *In situ* re-stimulation of memory B-cells. At Day 10 after the booster injection (at 6 months), splenocytes from HcT-, HcA-, or LTB-immunized and control mice were isolated and stained with antibodies against CD19, IgD, and IgG. As shown in the representative plots, the frequency of memory B-cells was decreased in all groups.

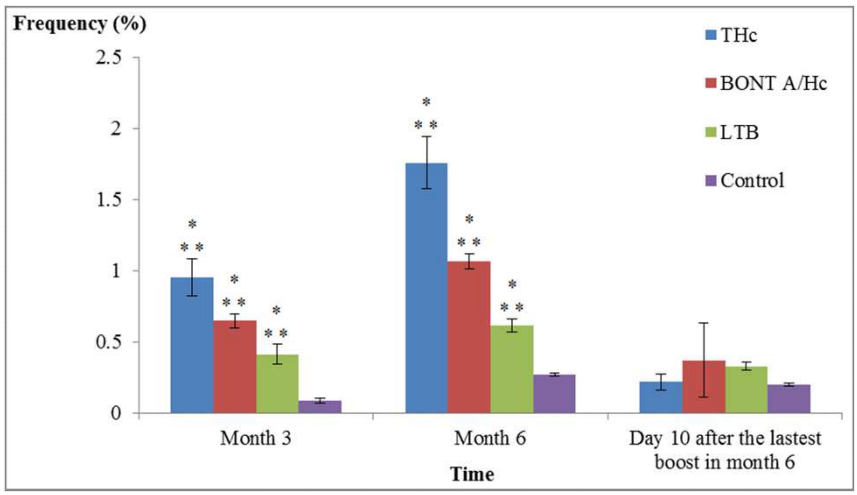


Figure 6. Frequency of memory B-cells as a function of time. Levels were calculated at 3 and 6 months after the final immunization, and 10 days after the booster. Results shown are mean ± SD percentages of the cells. **Value significantly different from controls ($p < 0.05$). *Value significantly different from other two regimens at given timepoint ($p < 0.05$).

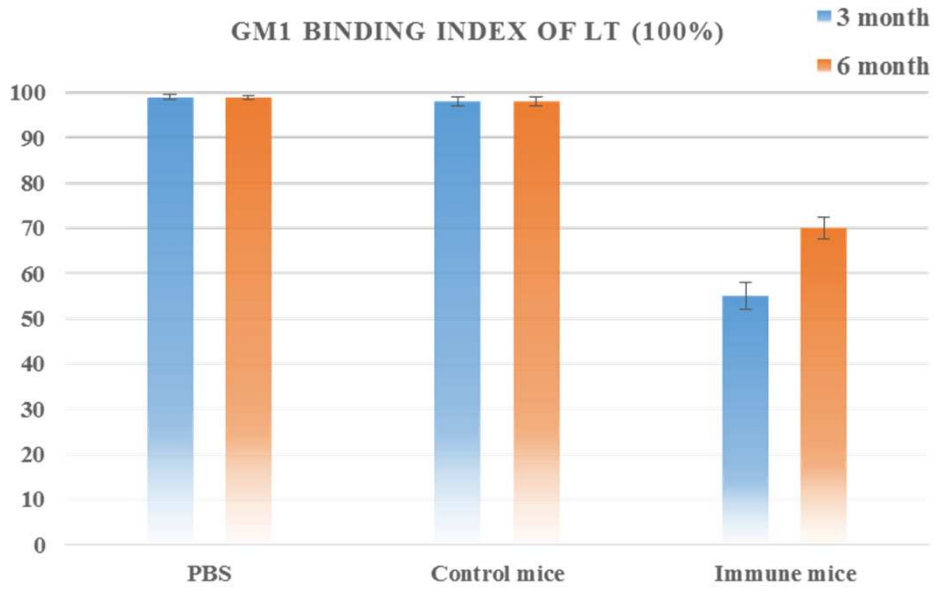


Figure 7. LT binding to GM1 ganglioside. Serum from LT-immunized mice was collected at each timepoint indicated in the Methods and analyzed in a GM1 blocking assay. Figure shows outcomes from samples isolated 3 months and 6 months after final immunization. At Month 3, binding was blocked by ≈ 45% with immunized mouse serum; at Month 6, binding was blocked by ≈ 30%, meaning 70% of the toxin was still potentially effect-tive. Sera from control mice and PBS alone were used as negative controls in the assay. Results shown are mean ±SD relative GM1 binding indices. **Value significantly different from controls ($p < 0.05$).

- The relationship between antibody titers and memory B cells against the HcT, HcA, and LTB antigens is shown.
- The great difference was observed in the frequency of memory B-cells in respond to HcT, HcA, and LTB antigens.
- The sustained frequency of memory B cells was a critical factor in antigen-induced memory longevity.