An oligoclonal combination of human monoclonal antibodies able to neutralize tetanus toxin *in vivo*

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ABSTRACT

The use of antibody-based therapy to treat a variety of diseases and conditions is well documented. The use of antibodies as an antidote to treat tetanus infections was one of the first examples of immunotherapy and remains the standard of care for cases involving potential infections. Plasma-derived immunoglobulins obtained from human or horse pose risks of infection from undetectable emergent viruses or may cause anaphylaxis. Further, there is a lack of consistency between lots. In the search for new formulations, we obtained a series of clonally related human monoclonal antibodies (mAbs) derived from B cells sorted from donors that presented anti-tetanus neutralizing titers. Donors were revaccinated prior to blood collection. Different strategies were used for single-cell sorting, since it was challenging to identify cells at a very low frequency: memory B cell sorting using fluorescent-labeled tetanus toxoid and toxin as baits, and plasmablast sorting done shortly after revaccination. Screening of the recombinant mAbs with the whole tetanus toxin allowed us to select candidates with therapeutic potential, since mAbs to different domains can contribute additively to the neutralizing effect. Because of selective binding to different domains, we tested mAbs individually, or in mixtures of two or three, in the neutralizing *in vivo* assay specified by Pharmacopeia for the determination of polyclonal hyperimmune sera potency. An oligoclonal mixture of three human mAbs completely neutralized the toxin injected in the animals, signaling an important step for clinical mAb development.

1. Introduction

At the end of the 19th century, passive therapy with antibodies was demonstrated to be a powerful tool against infectious diseases. Chemotherapy with antibiotics, when available, represented the main therapeutic approach in clinical practice, even though antibody-based therapies provides immediate immunity (Casadevall and Scharff, 1995), and hyperimmune sera still represents the standard of care for combatting deadly toxins derived from microorganisms or animal venoms. Serotherapy demonstrates its importance in the face of microorganism resistance to antibiotics, lack of available vaccines, lack of large vaccination campaigns, and lack of response to vaccines due to an immunocompromised state from disease or age (Doherty et al., 2016).

Since monoclonal antibodies (mAbs) became available, there has been a drive to substitute hyperimmune sera obtained from animals or human blood. Technological developments have allowed for the humanization of murine mAbs and, more recently, the generation of complete human mAbs. Fully human mAbs can be obtained using display technologies and transgenic animals, but neutralizing human antibodies derived from human B-lymphocyte sequences have the advantage of heavy and light chain pairing selection after *in vivo* antigen driven somatic hypermutation. Nowadays, it is possible to collect specific memory B cells or plasmablasts from peripheral blood after vaccination or disease onset, and obtain human mAbs with neutralizing capacity. This technology is transforming the field of mAb generation, especially for infectious diseases. Tetanus is a case that fits the requirements for

developing human mAbs based on this protocol.

Tetanus is an often fatal neurological disease caused by the toxin of the spore-forming bacterium *Clostridium tetani*, a Gram-positive bacillus widely distributed in soil, rusted instruments, dust, and animal and human feces (Collingridge and Davies, 1982). *C. tetani* spores are long-lived and highly resistant to temperature and radiation (Borick and Fogarty, 1967). The contamination of wounds with *C. tetani* spores under anoxic environments provides the perfect condition for spore germination and toxin production (Ernst et al., 1997; Gibson et al., 2009). Drug addicts are at risk due to injections under the skin, a hypo-aerobic environment (Gonzales y Tucker and Frazee, 2014; Mallick and Winslet, 2004).

Tetanus toxin (TeNT), or tetanospasmin, is one of the most potent toxins known with an estimated human lethal dose of less than 2.5 ng/kg of body weight (Gill, 1982). TeNT is synthesized as an inactive single polypeptide chain of 150 kDa composed of three 50 kDa domains. After proteolytic cleavage, it assembles into two chains linked by a disulfide bond: heavy chain (fragments B and C) and light chain (fragment A) with 100 and 50 kDa, respectively. Fragment C is involved in the interaction with the cell, and fragment B in the internalization of the toxin. Inside the nerve cells, upon reduction, fragment A, a zinc-endopeptidase, is responsible for the specific cleavage of synaptobrevin at a single point. The toxic effect is due to blockage of the release of inhibitory neurotransmitters, glycine and gamma-aminobutyric acid (GABA), in medullar neurons causing hypertonia, a spastic paralysis typical of tetanus clinical manifestations (Bizzini, 1979; Cook et al., 2001; Matsuda and Yoneda, 1974; Schiavo et al., 1992). High-affinity interaction of fragment C with neurons represents the first step of action through a dual-receptor mechanism involving gangliosides and synaptic proteins (Herreros et al., 2000; Yeh et al., 2010). Ganglioside variation is due to the different positions and numbers of N-acetylneuraminic acids around a central polysaccharide structure. Two carbohydrate-binding sites have been described for TeNT, the lactose-binding site (W pocket) and the sialic acid site (R pocket) (Calvo et al., 2012). Regarding the protein receptor for TeNT, besides synaptic vesicle 2, others have proposed a glycophosphatidylinositol (GPI)-anchored protein and nidogens without the molecular details of these potential interactions (Bercsenyi et al., 2014; Masuyer et al., 2017; Munro et al., 2001; Rummel et al., 2003).

The number of reported tetanus cases in the last five years is approximately 300/year in Brazil and 12,000/year in the world (WHO). Tetanus prevention consists of a vaccine composed by the formaldehyde-detoxified toxin called tetanus toxoid (TT). For accidents that involve potential tetanus transmission, treatment with immunoglobulins is recommended to start early in case of clinical suspicion, since immunoglobulins can only neutralize the circulating toxin and are not able to pass through the blood-brain barrier where toxins that are already bound are located (Ataro et al., 2011; Rodrigo et al., 2014). Available treatments involve immunotherapy with administration of polyclonal antibodies produced by immunized horses or collected from human donors. Derived from plasma, both consist of a large mixture of antibodies with no consistency between preparations. Being heterologous, horse serum may cause adverse effects, while antibody compositions obtained directly from human blood, in turn, may represent risk of infection with emergent viruses not yet known or detectable.

Our choice of using TeNT over the toxoid as a target has the advantage of selecting antibodies that are able to interact with epitopes presented by the natural toxin. Based on the toxoid process of production, that includes toxin treatment with formaldehyde, some antibodies raised against it may be immunogenic although not protective. It was shown that some of the mAbs produced by hybridoma cells obtained after immunization with the toxoid did not bind to the toxin, only to the toxoid (Sheppard et al., 1984; Volk et al., 1984). In addition, the use of the toxin allowed us to select B cells that produce antibodies against all its parts and evaluate a broader repertoire of mAbs. Some

authors used fragment C, considered a potential target for toxin neutralization to select anti-tetanus B lymphocytes (Franz et al., 2011). However, antibodies against the light chain of the toxin have proven to be neutralizing as well (Matsuda et al., 1992; Volk et al., 1984). Hence, our use of the entire toxin allowed the evaluation of a broad repertoire of antibodies that resulted in mAb sequences recognizing different regions of the toxin, and the exploration of their contribution for TeNT neutralization.

2. Material and methods

2.1. Blood collection

Donors with neutralizing titers of anti-tetanus antibodies were bled or received a vaccination booster before blood was drawn. All donors signed informed consent forms in accordance with the Human Ethics Committee of the University of São Paulo (CAAE 55454916.2.0000.5467). Peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient with Histopaque (Sigma Aldrich, 10771) then cryopreserved in 90% fetal calf serum (Cultilab, F063) and 10% dimethyl sulphoxide (Sigma Aldrich, D2650).

2.2. Tetanus toxoid and toxin

Both toxoid and toxin were obtained from Instituto Butantan (Brazil), a national producer for DTP vaccine, and checked by SDS-PAGE. The toxin was reconstituted with purified water and aliquots of 100 μL were purified by SEC-HPLC using a TSK gel G3000SWxL column (Tosoh Bioscience, 08541). Phosphate buffered saline (PBS) was used to elute samples in 1.5-mL microcentrifuge tubes. The eluted TeNT was independently labeled with either biotin using the ImmunoProbe™ Biotinylation Kit (Sigma Aldrich, BK101-1KT) or with Alexa Fluor® 647 using the Microscale Protein Labeling kit (Molecular Probes, A30009) according to the supplier's instructions.

2.3. Single cell sorting, RT-PCR, antibody cloning and expression

In single cell sorting of B lymphocytes, B cells enriched from PBMCs using the negative selection approach (B Cell Isolation Kit II. human, Miltenyi, 130-091-151) or total PBMCs were directly stained with 12.5 μg/mL of each of the above labeled TeNT while on ice for 60 minutes and protected from light, as specified in the protocol for single epitope multiple staining (SEMS) (Amanna and Slifka, 2006). After one wash, cells were stained with anti-CD19-PE (clone HIB19, 555413), anti-IgG-FITC (clone G18-145, 555786), and streptavidin-PerCP-Cy5.5 (551419) 1:500 for 20 minutes. Alternately, PBMCs were stained with anti-CD19-APC-Cy7 (clone SJ25C1, 557791), anti-CD27-PerCP-Cy5.5 (clone M-T271, 560612) and anti-CD38-PE (clone HIT2, 555460) for 20 minutes on ice and protected from light. All antibodies used in flow cytometry were purchased from BD Biosciences. The protocol for single cell sorting, RT-PCR, and antibody cloning are described elsewhere (Tiller et al., 2008; Wardemann and Kofer, 2013). Briefly, B-lymphocytes were sorted in 96-well plates, one cell per well containing 4 μL of a lysis buffer [10 mM DTT (Invitrogen, P2325), 8 U RNAsin® (Promega, N2515) in 0.5× PBS] then immediately placed on dry ice. Reverse transcription was performed on the same plate by adding 3.5 μL of a solution containing 150 ng of random hexamer *primers* (Invitrogen, 48190-011), 0.5 μL of 10% Igepal CA-630 (Sigma Aldrich, 10297-018) and 0.15 μL of RNAsin® to each well, followed by incubation at 68 °C for 60 seconds. The plate was then placed on ice and the following components were added to each well in a volume of 7 μL: 3 μL of 5× SuperScript® III reverse transcriptase buffer (Invitrogen, 18080-044), 12.5 nmols of each dNTP (Invitrogen, 10297-018), 100 nmols DTT, 8 U RNAsin®, and 50 μL of SuperScript® reverse transcriptase (Invitrogen, 18080-044). Reverse transcription was performed at 42 °C for 5 minutes, 25 °C for 10 minutes, 50 °C for 60 minutes, and 94 °C for 5

minutes. The cDNA pool was divided across three different 96-well microplates for individual amplification of the variable heavy and κ or λ light chains in a nested PCR reaction (Wardemann and Kofer, 2013). The polymerase chain reaction (PCR) amplified fragments were sequenced and analyzed by IgBlast (http://www.ncbi.nlm.nih.gov/igblast/) for the identification of V(D)J gene segments in comparison to germline sequences. The CDR3 sequence and length were determined by counting the amino acid residues following the last one of the framework 3 up to the conserved tryptophan-glycine motif of the heavy chain and the phenylalanine-glycine motif of the light chains (Tiller et al., 2008) and by IMGT numbering (Giudicelli et al., 2011). Sequences with the same classification of V(D)J gene segments, and same length and similar sequence of CDR3, were considered clonally related. The variable chains genes were reamplified using the first PCR product as template and specific primers, according to the V and J classification, containing restriction sequences, as previously described (Wardemann and Kofer, 2013), and cloned into plasmids containing the constant region of the respective chain (kindly provided by Dr. Hedda Wardemann, German Cancer Research Center). The pair of plasmids cloned with the heavy and light chains corresponding to the original antibody sequence were used for cotransfection of FreeStyle™ 293-F HEK cells (Invitrogen, R790-07). The secreted antibodies were purified by affinity chromatography using protein-A sepharose in an Äkta Purifier System (GE Healthcare). For antibody elution, we used 10mM sodium citrate buffer pH 3.2 and neutralized the eluted antibody with 1M Tris. The concentration of the antibodies was determined by absorbance at 280 nm.

2.4. ELISA

Antibody binding to TeNT, TT and TeNT C-terminal fragment (fragment C) was tested using ELISA. Plates were coated with 100 μL of a 5 μg/mL solution of TeNT or TT in PBS (Dokmetjian et al., 2000) or 2 μg/mL recombinant fragment C (Ribas et al., 2000) (kindly provided by Dr. Elizabeth Martins, Instituto Butantan) in 50 mM carbonate buffer overnight at 4 °C. Blocking was performed with PBS-1% BSA for 2 hours at room temperature. The candidate antibodies were serially diluted in PBS-1% BSA from 50 to 0.4 ng/mL and incubated for 1 hour at 37 °C. Unbound antibodies were washed out prior to the incubation with goat anti-human IgG-HRP (Southern Biotech, 2040-05) and plate development was obtained with tetramethylbenzidine (TMB, Sigma Aldrich, T8768). The serum of a vaccinated donor was used as a positive control.

2.4.1. Antibody-mediated inhibition of the tetanus toxin binding to the ganglioside GT1b

The test was performed as described elsewhere (Fitzsimmons et al., 2001; Yousefi et al., 2014). Briefly, 96-well plates were coated with 100 μL of 1 μg/mL ganglioside GT1b (Sigma Aldrich, G3767) diluted in methanol, incubated overnight at room temperature, and then blocked with PBS-1% BSA. In another plate coated with BSA, the mAbs were serially diluted and mixed in a 1:1 ratio with a 40 μg/mL solution of TeNT. After incubation for two hours at room temperature, the mixture was transferred to the plate coated with GT1b and incubated under the same condition. After washing, the detection of the toxin bound was performed with a horse anti-tetanus serum conjugated with peroxidase (1:1000) and TMB color development. The control consisted of TeNT with no mAb in triplicate to determine the absorbance corresponding to 100% of binding.

2.4.2. Epitope competition ELISA

The assay was done according to Nagata et al. (2004). Briefly, ELISA plates were coated with anti-human IgG-Fc specific (Sigma Aldrich, I2136). After washing, the plate was incubated with mAb#1 overnight at 4 °C. MAb#2 (competitor) was incubated with a fixed dose of TeNT

in a separate tube overnight at 4 °C. This mixture was then added to the plate and incubated for 45 minutes. After washing, the plate was incubated with horse anti-tetanus serum conjugated in-house to HRP and then TMB color developed. A range of TeNT concentration (1–75 ng/ml) was used for the standard curve.

2.5. Western Blotting (WB)

One microgram samples of TeNT were loaded onto a 10% polyacrylamide gel running under reducing and nonreducing conditions and later transferred to a PVDF membrane (GE Lifesciences, RPN303F) which was blocked with PBS-3% BSA containing 0.1% Tween 20 for 2 hours. A sample of BSA was used as a negative control. Membranes were incubated with the mAbs diluted at 50 ng/mL in PBS-1% BSA for 1 hour at room temperature with mixing. After washing, membranes were incubated with goat anti-human IgG-HRP diluted 1:5000 in PBS-1% BSA for 1 hour. Detection was performed with the use of a chemiluminescent ECL kit (GE Lifesciences, RPN2232) and exposed to X-ray films.

2.6. Epitope mapping

According to PEPperPRINT (Germany), for the linear epitope mappings, the sequence of tetanus toxin (UniProt ID P04958) was elongated by neutral GSGSGSG linkers at the C- and N-terminus to avoid truncated peptides. The elongated antigen sequence was translated into 13 amino acid linear peptides with a peptide-peptide overlap of 12 amino acids. The resulting linear tetanus toxin peptide microarrays contained 1319 different linear peptides printed in duplicate (2638 peptide spots) and were framed by additional HA (YPYDVPDYAG) and FLAG (DYKDDD-DKAS) control peptides (108 spots each). For conformational epitope mapping, the sequence of the tetanus toxin was translated into 7, 10 or 13 amino acid peptides with peptide-peptide overlaps of 6, 9 and 12 amino acids, respectively. After peptide synthesis, all peptides were cyclized via a thioether linkage between a C-terminal cysteine side chain and an appropriately modified N-terminus. The resulting conformational tetanus toxin peptide microarray contained 3966 different cyclic constrained peptides printed in duplicate (7932 peptide spots) and were framed by additional HA (YPYDVPDYAG, 192 spots) control peptides. For the assay, samples of anti-tetanus mAbs were incubated at 1 μg/ml, 10 μg/ml and 30 μg/ml (linear epitope mappings) as well as 0.1 μg/ml and 1 μg/ml (conformational epitope mapping) for 16 hours at 4 °C and shaking at 140 rpm. After washing with PBS, pH 7.4 with 0.05% Tween 20, antibodies were revealed with a goat anti-human IgG (H+L) DyLight 680 (Rockland, 609-144-123) and analyzed by the scanner LI-COR Odyssey Imaging System (LI-COR Biosciences); scanning offset 0.65 mm, resolution 21 μm, scanning intensities of 5/7, 6/7 and 7/7 (red = 700 nm/green = 800 nm). The control staining of the HA epitopes with control antibody mouse monoclonal anti-HA (12CA5) DyLight 800 (PEPperPRINT, PPC.037.002) was done as an internal quality control to confirm the assay quality and the peptide microarray integrity. The prestaining of a linear and a conformational tetanus toxin peptide microarray was done with the secondary antibody goat anti-human IgG (H+L) DyLight 680 and with control antibody mouse monoclonal anti-HA DyLight 800 to investigate background interactions with the antigen-derived linear or cyclic constrained peptides that could interfere with the main readings.

2.7. In vivo neutralization assay

The *in vivo* neutralization assay was performed after approval of the Instituto Butantan Animal Ethics Committee (CEUA no 6203050416). The protocol was the same used by the Quality Control section of Instituto Butantan to evaluate hyperimmune serum potency, according
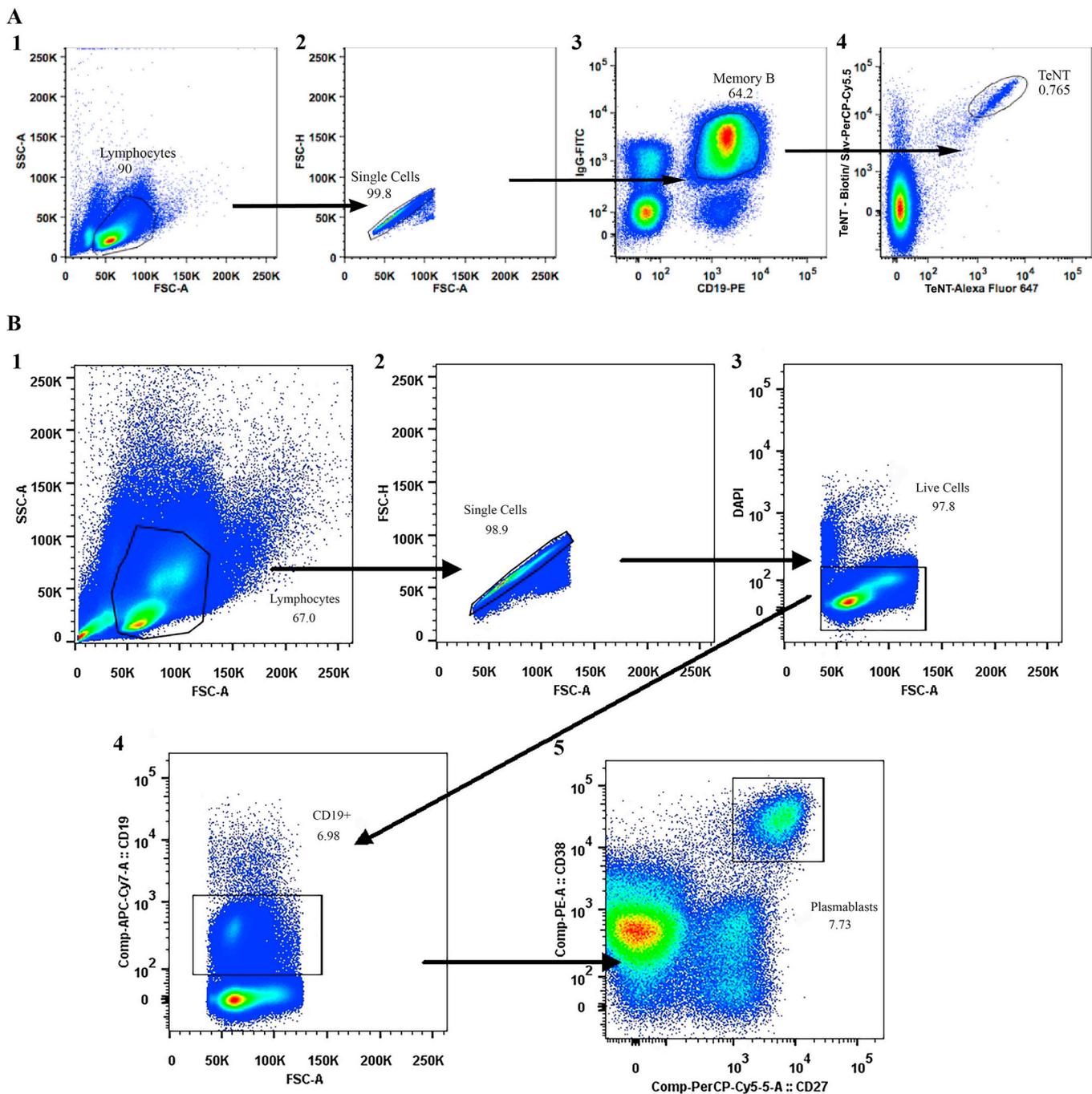
A

B

Fig. 1. Flow cytometry for detection of TeNT-specific B cells (A) or plasmablasts (B). (1) Gating strategy for detection of lymphocytes based on forward scatter (FSC) and side scatter (SSC); (2) Doublets were excluded using FSC height versus FSC area; (A3) Ig class-switched B cells were defined as CD19$^+$IgG$^+$; (A4) TeNT-specific B cells were defined as double-positive TeNT-Biotin/Streptavidin-PerCP-Cy5.5$^+$/TeNT-Alexa Fluor 647$^+$; (B3) Live cells were defined as DAPI$^-$; (B4) B cells were defined as CD19$^+$; (B5) Plasmablasts were defined as CD27$^+$CD38$^+$.

to the Brazilian Pharmacopeia (Brasil, 2010) in accordance with WHO guidelines (WHO, 2013). Swiss mice of 17–22 grams of both sexes were used. A fixed volume of reference TeNT was incubated for 1 hour at 37 °C with serially diluted (2-fold) concentrations of the sample antibody(ies) in a final volume of 2.4 mL in 1% peptone salt solution. For each antibody or mixture of antibodies tested, 50 animals were tested, with 10 per dilution. Each animal received 0.2 mL of the mixture, administered via an intraperitoneal injection, and was observed and recorded daily for death or signs of tetanus for 4 days. Horse hyperimmune anti-tetanus serum, a registered Butantan product, was used as control of the assay. ED$_{50}$ was determined in a completely randomized design and statistical probit model.

3. Results

3.1. Tetanus toxin and toxoid

The treatment of TeNT with formaldehyde eliminates its toxicity and generates the toxoid. Both TeNT and TT were analyzed using SDS-PAGE; TT migrated through the gel as an undefined band, which may indicate the cross-linking by formaldehyde of other proteins to the toxin moiety. In contrast, two sharp bands with approximately 150 kDa were observed in the TeNT sample, corresponding to the entire and the cleaved forms of the protein. In the *C. tetani* cytoplasm, TeNT is synthesized as a single polypeptide chain and, when transported to the
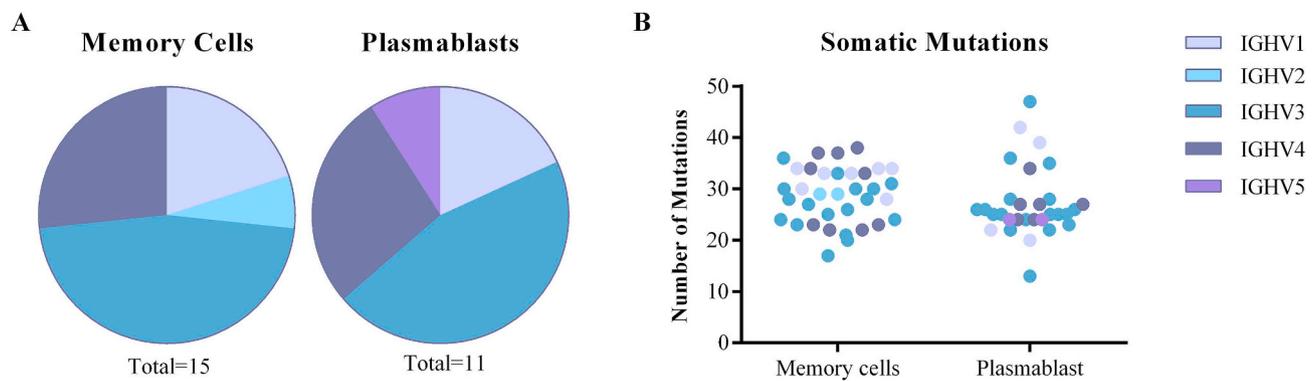
Fig. 2. Graphs showing the repertoire of the VH fragments (A) and the number of somatic mutations (B) in the clonally related sequences. Cells were isolated by two different strategies to stain memory cells (CD19$^+$IgG$^+$TeNT$^+$) and plasmablasts (CD19$^+$CD27$^+$CD38$^+$).

exterior, it is cleaved by enzymes present in the bacterial cell wall resulting in the heavy (100 kDa) and light (50 kDa) chains linked by disulfide bonds (Bizzini, 1979; Matsuda and Yoneda, 1974). As lower molecular weight proteins were present in the TeNT preparation, we performed a SEC-HPLC separation using a GS3000 SWxL column (range separation of 10–500 kDa of globular proteins) and collected peaks identified according to a molecular weight standard.

3.2. Antibody production and identification

To identify memory B cells producing anti-tetanus antibodies, we used SEMS strategy (Amanna and Slifka, 2006), meant for the detection of rare populations, and relies on the interaction of the same protein labeled with different fluorophores. Fig. 1A shows the gating strategy in which we sorted CD19$^+$IgG$^+$ memory cells bound to TeNT stained with two different fluorophores. Since memory cells resulting from tetanus vaccination reside preferentially in lymphoid organs (Cao et al., 2010) and are therefore scarce in circulation, we also used the strategy of isolating plasmablasts from blood after the donors received a tetanus boost vaccination, expecting to increase the frequency of sorted specific B cells (Frolich et al., 2010). Fig. 1B shows the gating strategy on CD19$^+$CD27$^+$CD38$^+$ plasmablasts. Both strategies were used to sort single cells (either CD19$^+$IgG$^+$TeNT$^+$ or CD19$^+$CD27$^+$CD38$^+$) into 96-well plates. After cDNA synthesis, the antibodies' variable regions were amplified by PCR, and the repertoire distribution of the V$_H$ fragments, together with somatic mutations, were analyzed and compared between the two approaches (Fig. 2). From five rounds of sorting through TeNT bait of memory B cells or plasmablasts collected from different donors, we obtained 65 clonally related sequences belonging

to 24 clonal groups. We were able to further process, for cloning and expression in HEK cells, 41 LC-HC pairs. Table 1 shows the sequences of three mAbs that, in combination, neutralized TeNT. Each antibody was expressed by transient cotransfection of the corresponding light and heavy chain vectors into FreeStyle™ 293-F HEK cells. MAbs were purified from the supernatant by protein A resin affinity chromatography and checked by reduced and nonreduced SDS-PAGE.

3.3. Characterization of the monoclonal antibodies

The initial evaluation of mAbs binding capacity was performed by ELISA with different tetanus antigens: TeNT, TT and recombinant fragment C (Fig. 3 and Table 2). We found variation in binding intensity between the 41 expressed mAbs in relation to TeNT and TT on ELISA plates. Only two of them, BUT-TT-117-08 and BUT-TT-140-08 reacted with recombinant fragment C. Interestingly, these two mAbs were the same that partially blocked the binding of the TeNT to its receptor GT1b. None of the other mAbs interfered with the GT1b binding in these experimental conditions (Fig. 3 and Table 2). Next, these mAbs were analyzed by their binding to TeNT by WB after SDS-PAGE under reduced and nonreduced conditions. Table 2 shows the TeNT patterns detected by the mAbs that were chosen for *in vivo* evaluation. We observed that some mAbs recognized the light or heavy chains, while others recognized only the entire TeNT in a nonreducing state (Table 2 and Fig. 4). We also run a competition ELISA between three mAbs that did not react with Gt1b ganglioside and neutralized TeNT, when combined. By this assay, if mAb#1 and mAb#2 bind to the same epitope, mAb#1 does not capture the previously complexed TeNT to mAb#2. The evaluation is shown in Table 3.

Table 1

Classification of variable chain fragments of selected anti-tetanus mAbs.

HEAVY CHAIN						
mAb	IGHV gene*allele	IGHD gene*allele	IGHJ gene*allele	Mutations[b]	CDR-IMGT lengths[a]	CDR3 sequence (aa)
243-10	1-69*06	2-8*02	5*02	36	8.8.10	AIALVRWLDP
143-10	3-30*02/3-30-5*02	2-21*01	3*01	27	8.8.15	AKVSVVRAYRYAFDV
120–10	4-39*07	3-10*01	4*02	25	10.7.15	SVGIRRFAVLLAFDY

KAPPA LIGHT CHAIN					
mAb	IGKV gene*allele		IGKJ gene*allele	Mutations[b]	CDR-IMGT lengths[a]	CDR3 sequence (aa)
243-10	3-11*01		4*01	17	6.3.10	QERSGSPPLT
143-10	3-20*01		4*01/*02	26	7.3.9	QQYGNSPLT
120-10	3-20*01		3*01	7	7.3.9	QQYGSSLFT

[a] Number of amino acid residues of the CDRs 1, 2, and 3, respectively.
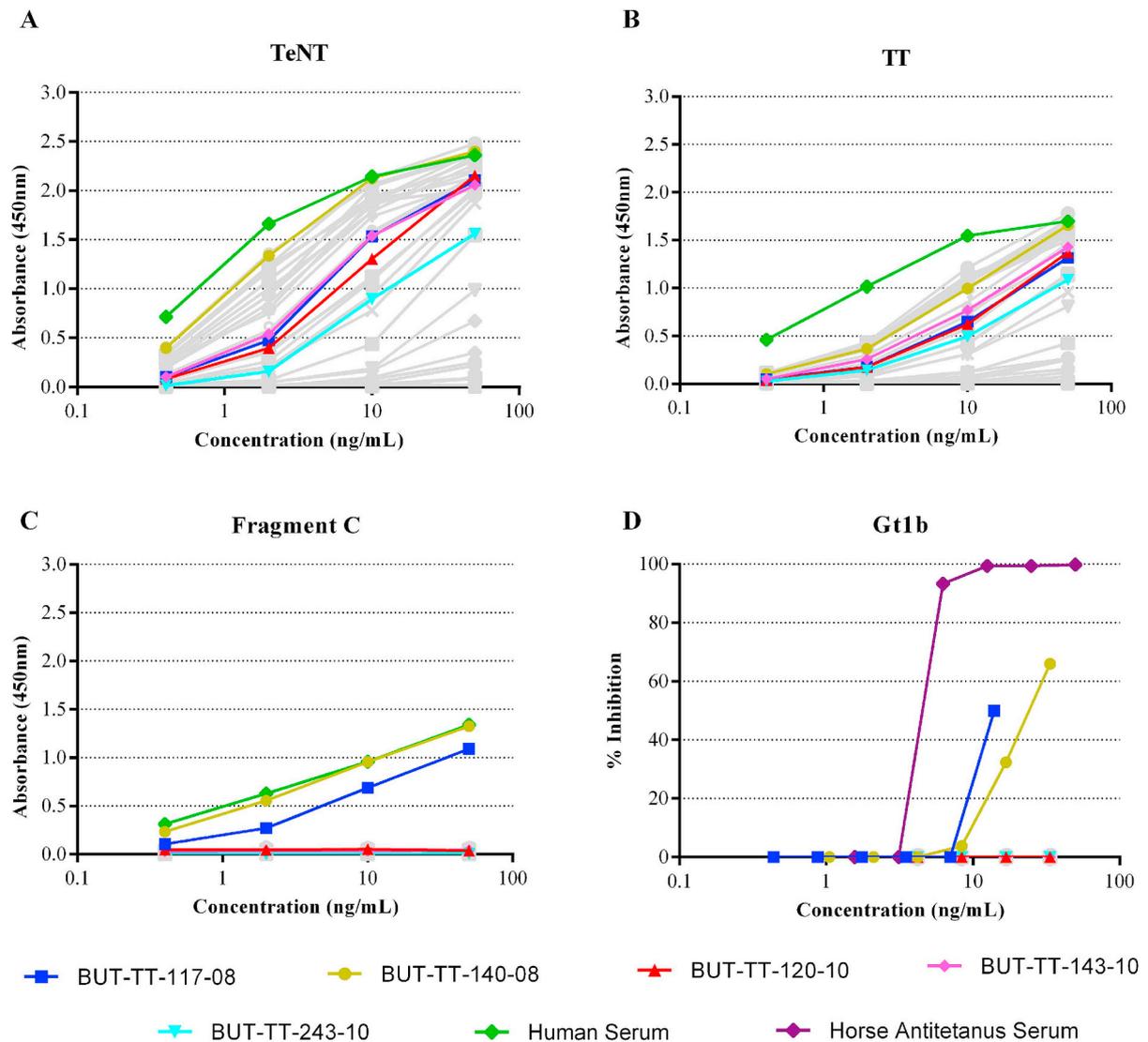[b] Number of mismatches compared to the germline sequence.

Fig. 3. Monoclonal antibodies reactivity by ELISA using 96-wells plates coated with **(A)** 5 μg/mL tetanus toxin, **(B)** 5 μg/mL tetanus toxoid, and **(C)** 2 μg/mL recombinant tetanus toxin fragment C. **(D)** mAbs capacity to inhibit TeNT binding was assessed by incubating GT1b with an appropriate level of TeNT alone or with increasing concentrations of each mAb. The mAbs were tested in the range from 0.4 to 50 ng/mL. Serum of a vaccinated person was used as positive control for A-C; horse hyperimmune serum was used in assay D.

3.4. Toxin neutralization in vivo

On the basis of the antibodies' properties identified by the assays described above, we selected the two mAbs that bound to recombinant fragment C, which were also able to partially inhibit GT1b binding, as well as three other mAbs binding to different TeNT regions, to evaluate their TeNT neutralization capacity *in vivo*. Fig. 4 shows the survival curves of animals injected with a reference TeNT at a fixed concentration and 2-fold serially diluted mAbs administered alone or pooled. The mAbs BUT-TT-117-08 and BUT-TT-140-08, which reacted with recombinant fragment C, were not able to protect the mice from TeNT when administered alone or mixed. Instead, the oligoclonal combination of mAbs BUT-TT-243-10, BUT-TT-143-10 and BUT-TT-120-10 protected all animals from death or any sign of toxicity when administered in a range of 2.5 to 0.3125 μg to each mouse (Fig. 5). The combination of BUT-TT-143-10 and BUT-TT-243-10 also neutralized the toxin, although only at the highest concentration tested. Isolated mAbs did not protect mice from injuries and symptoms caused by TeNT.

Table 2
Binding profile of selected recombinant anti-tetanus mAbs.

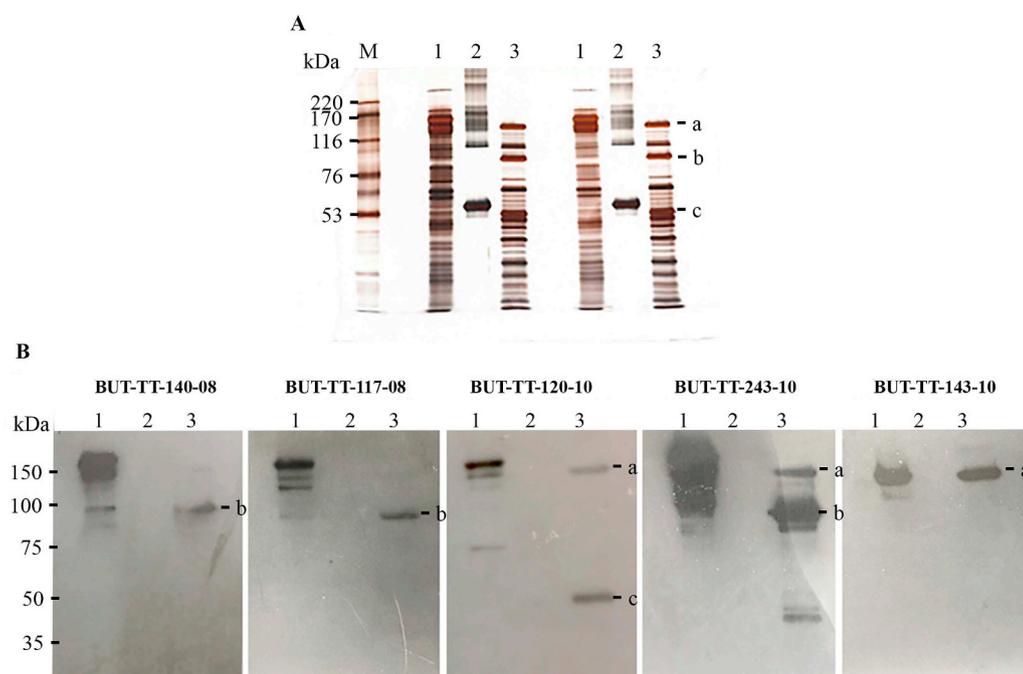| mAb	ELISA			Inhibition of the binding to GT1b	W.B.
	TeNT	TT	Frag C		
BUT-TT-117-08	+	+	+	Partial	Heavy
BUT-TT-140-08	+	+	+	Partial	Heavy
BUT-TT-243-10	+	+	−	−	Heavy
BUT-TT-143-10	+	+	−	−	Non Processed TeNT
BUT-TT-120-10	+	+	−	−	Light

Fig. 4. Western Blotting showing the recognition of different TeNT chains by the mAbs. In (**A**), SDS-PAGE of the TeNT under nonreducing (lane 1) and reducing (lane 3) conditions, showing the entire TeNT (a), the heavy (b), and light (c) chains. BSA (lane 2) was used as negative control. M: protein ladder. In (**B**), results of WB: BUT-TT-140-08 e BUT-TT-117-08 recognized the heavy chain (b); BUT-TT-120-10 recognized the light chain (c) and the entire TeNT (a), which was expected since it also contains the light fragment; BUT-TT-243-10 recognized the heavy chain (b) and the entire TeNT (a); and BUT-TT-143-10 recognized only the non-processed (entire) TeNT.

Table 3
Competition ELISA assay between three mAbs.

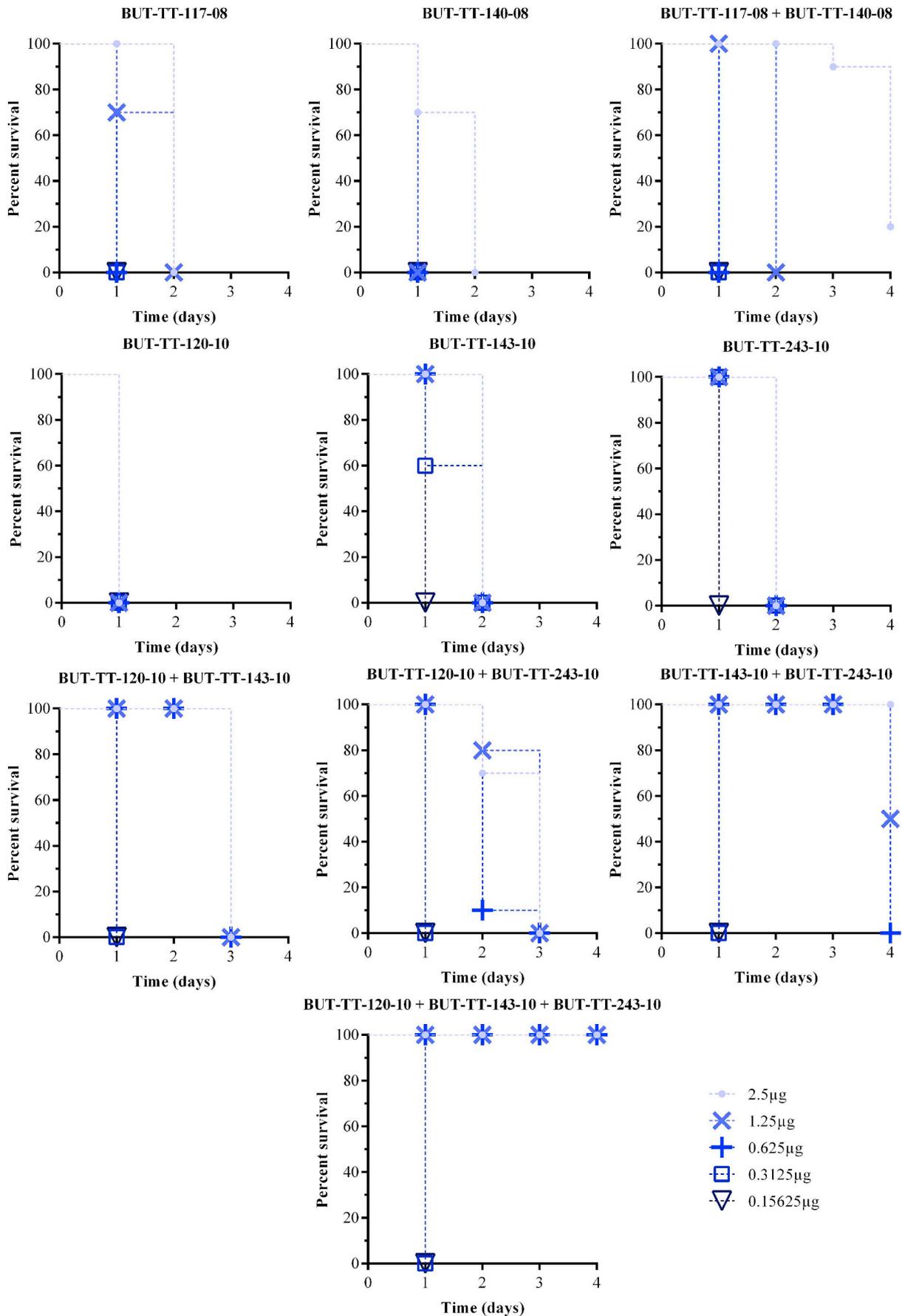| mAb#1	Competition % in the presence of indicated mAb#2 (competitor)		
	BUT-TT-143-10	BUT-TT-243-10	BUT-TT-120-10
BUT-TT-143-10	99	42	16
BUT-TT-243-10	−20	100	−18
BUT-TT-120-10	11	48	101

3.5. Epitope mapping

Five mAbs were analyzed in microarrays of TeNT peptides to identify their binding epitopes by an algorithm that calculates average median foreground intensities and spot-to-spot deviations of spot duplicates followed by the generation of an intensity map. Of the four mAbs tested on linear peptides, the BUT-TT-140-08 mAb responded strongly to a single epitope-like spot pattern with > 50.000 a.u. (fluorescence intensity). The consensus identified motif was A^{1231}PGIP, which is present in fragment C in close proximity to the residues R^{1226} and Y^{1229} of the sialic acid binding site, which are involved in interactions with the sialic acids of gangliosides (Fotinou et al., 2001). The prediction of epitope binding by BUT-TT-140-08 in Fig. 6A shows the surface structure of TeNT and its accessibility to the medium. The BUT-TT-117-08 mAb showed a very weak response (260 a.u.) against a single epitope-like spot pattern formed by overlapping peptides with the consensus motif G^{1119}NPLRYDTEY, which is also present in fragment C, but distant from the two ganglioside binding sites, the sialic acid binding site (R pocket) and the lactose binding site (W pocket) (Chen et al., 2009; Rummel et al., 2003) (Fig. 6B). The BUT-TT-120-10 mAb showed a weak but clear response of 400 a.u. against a single peptide with the consensus motif L^{1225}RVGYNAPGI (Fig. 6C), which is part of the sialic acid binding site. However, this mAb was seen to bind to the light chain (amino acids 1–457) of the toxin in WB and did not react with fragment C by ELISA. The incubation with BUT-TT-243-10 mAb showed a weak response (600 a.u.) against a single epitope-like spot pattern formed by overlapping peptides with the consensus motif V^{671}LLEY that likely corresponded to the epitope of this antibody. When analyzing the response to each individual peptide, we could see that the signal decreased before and after the V^{670}VLLLEY sequence, a

pattern characteristic of an epitope. It is located in the translocation domain of TeNT (Fig. 6D).

The BUT-TT-143-10 mAb was analyzed by cyclic peptide arrays as conformational epitope binding was expected because it only bound to the nonreduced TeNT in WB. This mAb produced a strong and complex response against epitope-like spot patterns formed by overlapping peptides with consensus motifs at good signal-to-noise ratios; the strongest signals (15.000–20.000 a.u.) were obtained with 7 and 10 amino acid peptides with very similar motifs such as EYYD or DTEY. The three recognized epitopes, E^{67}GASEYYD, A^{751}IKYIDYEY and P^{1121}LRYDTEY, are located in different domains of the toxin, fragments A, B and C, respectively (Fig. 6E). In the ELISA assay, BUT-TT-143-10 mAb did not bind to fragment C. In WB, it only bound to the non-reduced toxin.

4. Discussion

Monoclonal antibodies represent an important and modern area of the biotechnological industry for their specificity and production consistency. Human mAbs are especially important because of their reduced immunogenicity. Thus, human mAbs against TeNT could be an important resource for treatment of infection or suspected infections after accidents. Detection of antigen-specific circulating B cells may be a challenge due to the small number of specific clones among lymphocyte populations. The frequency of antigen-specific B-lymphocytes may range from 0.05 to 0.005% of circulating cells (Smith et al., 2017). There are different methods of enriching antigen-specific B lymphocytes, such as flow cytometry as used in this work, or antigen-coated magnetic beads (Egeland et al., 1988). Other groups have immortalized B-lymphocytes from immunized subjects and then proceeded to screen every clone (Smith et al., 2014). Our group had access and experience with single cell sorting and therefore chose this method for isolating antigen-specific B cells. We found that approximately 0.765% of the circulating memory B cells were antigen-specific. A recent study with HIV-infected elite controllers has shown that among the HLAB*B57$^+$ individuals, the frequency of IgG$^+$ specific B cells frequency ranged 0.13–0.24%, depending on the antigen (Rouers et al., 2017). However, looking at a mixed cohort of HIV infected individuals, another group described that 0.09% of the B-lymphocytes were specific for HIV envelope antigens (Doria-Rose et al., 2009). These results illustrate the

Fig. 5. *In vivo* tetanus toxin neutralizing assay. The *in vivo* neutralizing capacity of TeNT-specific mAbs was evaluated using Swiss mice in accordance to the potency test used for approval of hyperimmune horse sera. A fixed amount of reference TeNT was incubated with the serially diluted mAbs (alone or mixed) and injected subcutaneously into 10 mice for each dilution. The values indicated in the legend correspond to the amount of mAb injected in each one of 10 animals. After four days of observation for any signs of tetanus the survival rates were calculated according to a probit statistical model.

broad range of B cell responses against a natural infection like HIV, and show that the frequency of antigen-specific B lymphocytes found in our study is similar to the frequencies found by others. The problem of low frequency circulating B cells in primary vaccinated donors (Cao et al., 2010) was solved in this work by collecting blood 6–14 days after a boost immunization, which offered a short period where the number of circulating B cells increased and allowed for their identification and separation. To identify sequences of mAbs targeting potential epitopes of the natural toxin, we chose to use the tetanus toxin over the toxoid, allowing us to select B cells that produce antibodies against all its parts and evaluate a broader repertoire of mAbs.

From a variety of expressed mAbs we selected a few for *in vivo* testing, since covering the toxin fragments A, B or C by WB analysis. The *in vivo* neutralization assay demonstrated the inability of the tested mAbs to protect mice when used alone. Even though demonstrating the capacity to partially block binding of the toxin to the ganglioside, as demonstrated *in vitro* by mAbs BUT-TT-117-08 and BUT-TT-140-08, they were insufficient for protection, at least in the concentrations tested. These mAbs showed binding to recombinant fragment C by ELISA, which was consistent with the WB and peptide array results. The high binding affinity of TeNT to gangliosides depends on the ganglioside occupancy of two sites (R and W) (Luo et al., 2012). Even though BUT-TT-117-08 and BUT-TT-140-08 mAbs do not bind to these sites, inhibition may occur by the toxin conformational change upon binding with the mAbs, or by steric hindrance of the mAb (Luo et al., 2012). The non-occupation of these sites may explain why the inhibition by ELISA is only partial at the tested concentrations, and the lack of *in vivo* protection.

The synergistic effect of the mixture of three mAbs (BUT-TT-120-10, BUT-TT-143-10, and BUT-TT-243-10) was able to guarantee complete toxin neutralization, even when the concentration of each antibody in the mixture was 25-fold lower than the highest concentration used in the individual assays. When only two mAbs targeting the heavy chain (BUT-TT-243-10) and the uncleaved whole toxin (BUT-TT-143-10) were combined, mice showed no symptoms of TeNT-related toxicity during the duration of the assay, when the highest amount of mAb was used (1.25 μg of each). This result compromises their neutralizing capacity by the Pharmacopeia criteria, which acceptance requires the survival of all mice over two consecutive antibody dilutions. Perhaps higher mAb concentrations would fit the requirements. It should be noted that a comparison with the horse hyperimmune serum is not direct, since mAbs are purified and quantified by UV absorbance in μg/mL while serum potency is measured by IU/mL. According to the peptide array, these two mAbs recognize epitopes present in the ganglioside binding domain (fragment C) and in the translocation domain, both in the heavy chain. Since the function of these two regions is relevant for the internalization of the toxin, the predicted binding of the mAbs to these regions seems pertinent for protection. The BUT-TT-120-10 mAb presented conflicting results in WB, ELISA and epitope mapping. The epitope array was constructed with linear, fixed peptides and might not have represented the binding to the toxin in its actual conformation. Most likely, this mAb recognizes a peptide present in the light chain of the toxin, as clearly demonstrated by the WB after SDS-PAGE of the reduced toxin. To rule out any misinterpretation, the expression of this antibody was repeated, as well as the WB. Therefore, even if not proven, we favor that mAb BUT-TT-120-10 binds to an
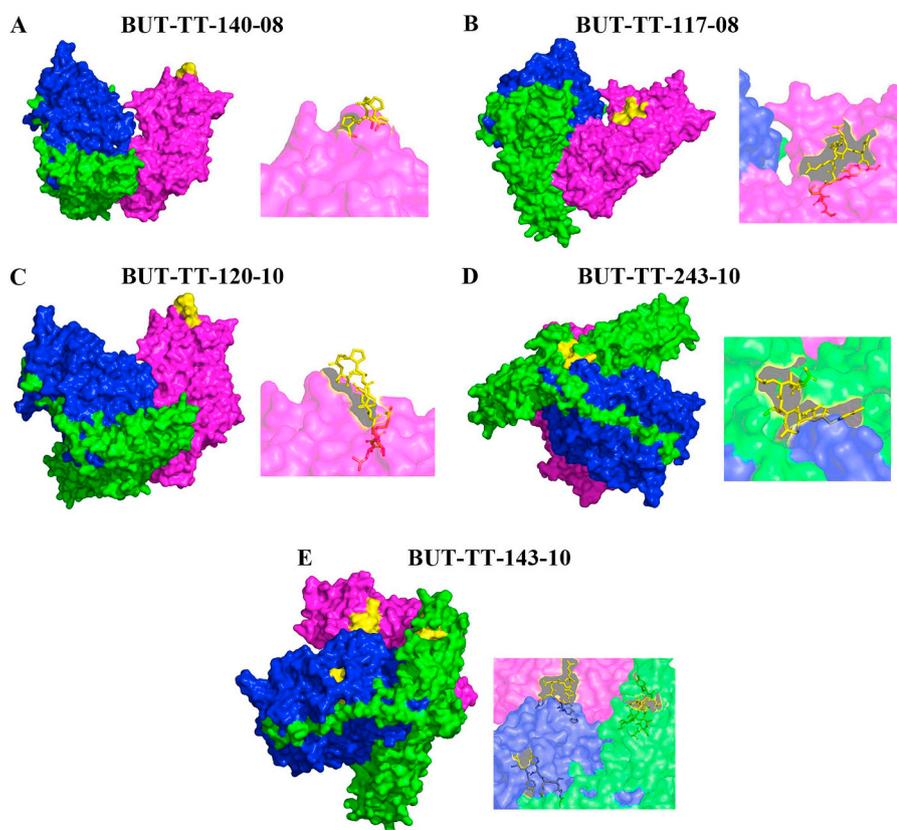

A BUT-TT-140-08   **B** BUT-TT-117-08

C BUT-TT-120-10   **D** BUT-TT-243-10

E BUT-TT-143-10

Fig. 6. Representations of Tetanus Toxin (TeNT) structure and mAbs epitopes detected by epitope array. Light chain (active domain) is marked in blue, heavy chain is marked in green for Translocation domain and in pink for Binding domain (Fragment C). The epitopes recognized by each mAb in the peptide array are shown in yellow. Full view: Surface representations showing epitope accessibility. Close view: epitopes are shown in stick representations. The representations were generated based on the Crystal structure of TeNT (PDB: 5n0b). The figure was produced using PyMol software.

epitope in the light chain of TeNT in its conformational structure, as this is also the case for another clonally related mAb. This also explains its contribution to *in vivo* protection within the oligoclonal mixture.

The conformational epitope mapping of the BUT-TT-143-10 mAb showed a strong and complex response against three main epitope-like spot patterns formed by overlapping peptides with all peptide lengths. There are common similar motifs formed by the residues EYYD or DTEY, with a clear sequence similarity and preference for peptide with D, E and Y. The absence of reaction of this mAb against any of the reduced toxin chains in the WB suggested that it may recognize a discontinuous epitope formed by residues from both chains which is present in the right conformation of the whole toxin when the disulfide bond is not reduced or when the toxin is in its natural form with only one chain (nonprocessed toxin). The expectation of a conformational peptide for this mAb was the reason for mapping cyclic peptides. It is also possible that this mAb binds to a preferential epitope and cross-reacts with others that display similar amino acid patterns. Jayaraman et al. showed an interaction of the tripeptide YEW with the TeNT binding domain that recognizes the R pocket, suggesting that TeNT can bind to a protein as well as to a ganglioside in the same domain (Jayaraman et al., 2005).This tripeptide was identified in proteins associated with signaling endosomes involved in the action of TeNT (Bercsenyi et al., 2014; Munro et al., 2001). As this motif is present in the endosomes' lumen, corresponding to the extracellular domain of these proteins in the plasma membrane, peptide construction variants of YEW were explored to identify putative TeNT receptors, which led to the identification of nidogens (Bercsenyi et al., 2014). We cannot rule out that neutralizing antibodies inhibit the binding of TeNT not only to the well-described ganglioside sites but also to the not yet clearly determined protein interactions (Bercsenyi et al., 2014; Calvo et al., 2012; Masuyer et al., 2017; Munro et al., 2001; Rummel et al., 2003; Yeh et al., 2010). In case the mAbs do not bind to an active site in the TeNT, the interference exerted by them in the toxin functionality may be of allosteric nature, or due to conformational changes of TeNT after mAb binding. To rule out that the three mAbs present in the oligoclonal combination are not-overlapping we run a competition assay defining that competition values > 50% for two mAbs in mutual manner is considered a significant competition and the two mAbs are considered to bind to topographical epitopes (Nagata et al., 2004). The partial competition and the negative values (binding enhancement) may be of allosteric nature or electrostatic change after the competitor binding. The three mAbs chosen for the *in vivo* test recognized different regions of the toxin by WB and this pattern was confirmed by the competition assay.

In this work, we showed the possibility of sorting memory B cells producing specific anti-tetanus antibodies when immunophenotyping blood mononuclear cells by the protocol for CD19$^+$IgG$^+$TeNT$^+$ and plasmablasts by the immunophenotyping protocol for CD19$^+$CD27$^+$CD38$^+$ cells. Due to animal testing constraints, we did not evaluate all the repertoire of specific mAbs *in vivo*. Of the mAbs generated in this study, we chose for the *in vivo* neutralization assay those that bound to fragment C and inhibited GT1b binding. We also tried mixing mAbs that recognized different parts of the toxin and were thus expected to target different epitopes. Our results of *in vivo* neutralization of TeNT by the oligoclonal mixture of three human mAbs in the standard animal assay recommended by Pharmacopeia are promising and warrant further studies.

Conflict of interest

Acknowledgments

Abbreviations

GT1b	GT1b ganglioside
PBMC	peripheral blood mononuclear cells
RT-PCR	reverse transcriptase polymerase chain reaction
SEC-HPLC	size exclusion chromatography-high performance liquid chromatography
SEMS	single epitope multiple staining
TeNT	tetanus toxin
TT	tetanus toxoid

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