

# Novel neutralizing human monoclonal antibodies against tetanus neurotoxin

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## Research Article

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# Abstract

Tetanus is a fatal disease caused by tetanus neurotoxin (TeNT). TeNT is composed of a light chain (Lc) and a heavy chain, the latter of which is classified into two domains, N-terminus Hn and C-terminus Hc. Several TeNT-neutralizing antibodies have been reported, but it remains unclear which TeNT domains are involved in neutralization. To further understand the mechanism of these antibodies, we isolated TeNT-reactive human antibody clones from peripheral blood mononuclear cells. We then analyzed the reactivity of the isolated antibody clones to each protein domain and their inhibition of Hc-ganglioside GT1b binding, which is critical for TeNT toxicity. We also investigated the TeNT-neutralizing ability of isolated antibody clones and showed that Hn-reactive clones protected strongly against TeNT toxicity in mice. Furthermore, combination treatment of Hn-reactive antibody clones with both Hc-reactive and TeNT mix-reactive antibody clones enhanced the neutralizing effect. These results indicated that antibody clones targeting Hn effectively neutralized TeNT. In addition, the use of a cocktail composed of Hc-, Hn-, and TeNT mix-reactive antibodies provided enhanced protection compared to the use of each antibody alone.

## Introduction

Tetanus is a common, fatal disease caused by infection with *Clostridium tetani*, a Gram-positive obligate anaerobe. The bacterium enters wounds in the form of spores that germinate to produce tetanus neurotoxin (TeNT). The toxin blocks the neuronal release of inhibitory neurotransmitters, including acetylcholine, resulting in characteristic symptoms such as spastic paralysis<sup>1</sup>. TeNT is synthesized as a single 150-kDa polypeptide and is subsequently cleaved to generate an active toxin. The active form of TeNT consists of a light chain (Lc, also called fragment A, 50 kDa) and a heavy chain (100 kDa) linked by a disulfide bond, and the heavy chain is composed of two domains, Hn (also called fragment B, 50 kDa) and Hc (also called fragment C, 50 kDa)<sup>2</sup>. Lc works as a protease to cleave molecules such as vesicle-associated membrane protein-2 (VAMP-2), which is a soluble neuronal N-ethylmaleimide-sensitive attachment receptor (SNARE) protein, resulting in the blockade of inhibitory neurotransmitter release<sup>3,4</sup>. Hn plays a functional role in the translocation of Lc into the cytosol of neuronal cells<sup>5</sup>. Hc is indispensable for TeNT binding to target cells through ganglioside receptors such as GT1b<sup>6</sup>.

Human tetanus immunoglobulin (TIG), which is derived from the plasma of vaccine-administrated donors, is an effective tetanus therapy. However, due to the lack of source plasma and the risk of contamination with infectious materials, the TIG should be replaced with a recombinant antibody (rAb) product<sup>7,8</sup>. Compared to mouse antibodies, human antibodies have an advantage as a therapeutic agent because they do not require a difficult humanization process. Several TeNT-neutralizing human antibodies have been reported<sup>9</sup>, but it is still relatively unclear which targeted TeNT domains are required for these antibodies' neutralization activity. In addition, it has been reported that combinations of different antibodies, such as TIG, lead to enhanced neutralizing activity<sup>9</sup>, but it is not well understood

which antibodies in such mixtures contribute to neutralization. To address these questions we isolated TeNT-reactive human antibody clones and assessed their TeNT inhibitory activity.

## Results

### Isolation and characterization of TeNT-reactive lymphoblastoid cell line (LCL) clones

To isolate monoclonal antibodies reactive to TeNT, we isolated peripheral blood mononuclear cells (PBMCs) from the peripheral blood of healthy volunteers, and infected these cells with Epstein-Barr virus (EBV), and established LCLs. Two weeks after EBV infection, we analyzed the reactivity of LCL supernatant to the mixture of Lc, Hn, and Hc proteins (defined as the TeNT mix) using an enzyme-linked immunosorbent assay (ELISA). We performed the experiments twice and obtained 18 and 35 TeNT mix-reactive LCL clones, respectively (Table 1). We also determined the reactivity of LCL clone supernatants to Lc, Hn, and Hc proteins by ELISA, and found that antibodies in the supernatants of four LCL clones bound to the TeNT mix but not to Hc, Hn, or Lc proteins individually (Table 1).

Table 1  
Number of screened PBMCs and isolated clones

Experiment ID	Screened PBMCs	Reactive clones				
		Hc	Hn	Lc	TeNT mix <sup>a</sup>	Total
1	4.7x10 <sup>7</sup>	14	0	3	1	18
2	2.8x10 <sup>7</sup>	17	3	12	3	35
Total	7.5x10 <sup>7</sup>	31	3	15	4	53

a. TeNT mix is composed of Hc, Hn, and Lc proteins. The number of TeNT mix-reactive clones indicates refers to clones that were not reactive to Hc, Hn, or Lc individually but were reactive to the mixture of these three antigens in ELISA.

### 8A7 and 17F7 inhibit the binding of Hc to ganglioside GT1b

Binding of the TeNT Hc domain to ganglioside GT1b plays a key role in TeNT toxicity, and inhibiting Hc-GT1b binding interfered with TeNT toxicity. Thus, we performed an ELISA-based Hc-GT1b binding assay to evaluate the ability of Hc-reactive antibody clones to block Hc binding to ganglioside GT1b. We analyzed 31 supernatants of Hc-reactive LCLs, and antibody clones 8A7 and 17F7 inhibited Hc binding to GT1b in a dilution-dependent manner (Fig. 1). In contrast, clones 8H2 and 25H5 enhanced Hc-GT1b binding in a dilution-dependent manner (Fig. 1).

### TeNT mix-reactive antibody clones strongly protect against TeNT toxicity in mice

To determine the target domain responsible for the neutralizing ability of antibody clones, we performed an *in vivo* TeNT neutralization assay using LCL supernatant prepared in a second antibody clone isolation experiment. The mixture of all LCL supernatants (35Ab mix) completely neutralized TeNT (Fig. 2A). Mixtures of Hc-, Lc-, Hn-, or TeNT mix-reactive clones also protected the mice from TeNT toxicity, and the TeNT mix-reactive mixture had the strongest protective activity (Fig. 2A). In addition, the combination treatment with Hn- and TeNT mix-reactive supernatants increased the protective activity compared to that of each mixture alone (Fig. 2B).

### Immunoglobulin Gene Repertoire Analysis And Characterization Of Recombinant Antibodies

To characterize the isolated monoclonal antibodies, we cloned the immunoglobulin heavy and light chain genes of 8A7 and 17F7, which reacted with Hc and inhibited Hc-GT1b binding, and analyzed their immunoglobulin gene repertoires. Hn-reactive 8D8 and TeNT mix-reactive 16E8 were also analyzed. The V (D) J gene combinations and complementary determining region (CDR) 3 amino acid sequences were differed between each clone, indicating that 8A7, 17F7, 8D8, and 16E8 had different origins (Table 2). We next used ELISA to assess the reactivity of purified recombinant antibodies (Abs). 8A7 and 17F7 reacted with Hc, and 8D8 was reactive to Hn (Fig. 3A-C). All four antibodies bound to toxoid and TeNT mix in a dose-dependent manner (Fig. 3D, E). However, 8D8 and 16E8 showed very weak reactivity to TeNT (Fig. 3F). We also confirmed that purified recombinant 8A7 and 17F7 dose-dependently inhibited Hc-GT1b binding (Fig. 3G).

Table 2 Repertoire and CDR3 sequence of cloned immunoglobulins

IgH

ID	VH	DH	JH	CDR3	Somatic mutation (nt)
8A7	IGHV3- 33*03	IGHD6- 19*01	IGHJ4*02	ARDKGYINGWYVPFFDY	30
17F7	IGHV3- 33*01	IGHD6- 13*01	IGHJ3*02	ARESGYASSWYFNGDAFDI	20
8D8	IGHV4- 39*01	IGHD3- 3*01	IGHJ5*02	ARLGVKKITLFGGEVIPRSSWFAP	24
16E8	IGHV1- 46*04	IGHD6- 13*01	IGHJ4*02	ARDRRQQLVFDS	30

## IgL

ID	Vk	Jk	CDR3	Somatic mutation (nt)
8A7	IGKV3-15*01	IGKJ2*01	QQYDNWPPVT	6
17F7	IGKV4-1*01	IGKJ4*01	QQYSSTPLT	11
8D8	IGKV1-33*01	IGKJ5*01	QQYDTLSIT	8
16E8	IGKV4-1*01	IGKJ3*01	QQYYSLSRGLT	14

CDR: complementarity determining region, nt: nucleotide

### **The mixture of several antibodies reactive to different domains exerts strong neutralization activity in mice**

We assessed the TeNT-neutralization ability of purified rAbs in mice. For each antibody, 3.75 ng (TeNT:rAb ratio 1:37.5) exerted protective ability against 1 LD<sub>50</sub> TeNT in mice, and 8D8 showed the strongest protective activity (Fig. 4A). In all cases, 3,750 ng of each antibody (TeNT:rAb ratio 1:37,500) completely protected the mice from 1 LD<sub>50</sub> TeNT (Fig. 4B). We next analyzed the ability of antibody mixtures to enhance the neutralization activity. We assessed mixtures containing Hn-reactive 8D8, TeNT mix-reactive 16E8, and either Hc-reactive 8A7 or 17F7. Both antibody cocktails targeting Hc + Hn + Lc completely neutralized 1 LD<sub>50</sub> TeNT toxicity in mice beginning at 1.25 ng each (3.75 ng in total, TeNT:rAb ratio 1:37.5) (Fig. 4C and D). The neutralizing ability of each antibody was enhanced in combination with other antibodies (Fig. 4A, C, and D).

### **The neutralizing antibody 8D8 binds to the hydrophobic region of Hn**

We determined the binding site of 8D8 in Hn by ELISA. We first tested deletion mutants of Hn. Hn (458–828) showed no binding activity to 8D8, while this activity was retained by Hn (790–864) (Fig. 5A). We also prepared several additional mutants by replacing every three amino acids from 829 aa to 864 aa of the Hn (700–864) mutant with alanine and then examined the antibody's binding ability (Fig. 5B). Hn (700–864) AAA.3, 4, 5, and 7 mutants lost binding to 8D8 (Fig. 5B), indicating that the regions from 835 aa to 843 aa and from 847 aa to 849 aa of Hn are important for 8D8 to recognition of Hn.

For more comprehensive assessment for these observations, we applied a computational approach to predict the variable domain structure of 8D8 and its recognition domain in TeNT. The computational simulation of antibody-antigen docking revealed that the mode of 8D8-Hc binding was similar to that from the analysis of the ELISA-based binding activity (Fig. 5 and Fig. 6).

## Discussion

TIG is widely used to treat tetanus and is known to be effective. This indicates that vaccinated individuals have TeNT-neutralizing antibodies in their peripheral blood. We hypothesized that we would be able to isolate neutralizing antibody clones from the PBMCs of such individuals. In this study, we modified a standard EBV infection method and established LCLs to isolate antibody clones. Then we cloned immunoglobulin heavy and light chain genes from the RNA of the LCLs. Using this strategy, we successfully isolated 53 TeNT mix-reactive antibody clones and identified four TeNT-neutralizing human antibodies, namely 8A7, 17F7, 8D8, and 16E8, from the PBMCs of a healthy individual. Recombinant Hn-reactive 8D8 and TeNT mix-reactive 16E8 antibodies had strong TeNT neutralizing activity, although at high doses all four antibodies completely protected mice from TeNT-induced death. In addition, combination treatment with Hc-, Hn-, and TeNT mix-reactive antibodies enhanced protective ability in mice.

In the present study, we isolated three Hn-reactive clones from 53 TeNT mix-reactive clones. Compared to Hc- or Lc-reactive clones, fewer Hn-reactive clones were isolated. It has been reported that the Hn domain is hidden by the other domains due to the three-dimensional structure of TeNT<sup>10</sup>. Interestingly, Hn-reactive recombinant 8D8 did not bind to TeNT in our study. Botulinum neurotoxin (BoNT) is another highly potent toxin and is composed of three domains responsible for receptor binding, toxin translocation into cells, and proteolytic cleavage of host cell proteins, respectively<sup>11</sup>. It has been reported that most BoNT-neutralizing antibodies recognize Hc, and few antibodies bind to the Hn domain<sup>12,13</sup>. Nevertheless, in this study we found that 8D8 bound to Hn via both Hn (835–843), in which hydrophobic amino acids form a cluster, and Hn (847–849), leading to strong TeNT-neutralizing activity in mice. Considering the reported three-dimensional (3-D) structure of TeNT, the region from 835 aa to 849 aa of TeNT could be relatively antigenic as it is externally exposed<sup>10</sup>. These points suggest that this region of Hn is a good target for neutralizing antibodies despite the low immunogenicity of Hn in humans. The computational simulation results of antibody-antigen docking in this study supports these agreements, because the simulation results gave a closer match to the experimental results of ELISA-based binding assays.

On the other hand, we isolated four TeNT mix-reactive antibody clones, including 16E8, that did not bind with each Hn, Hc, or Lc in ELISA, suggesting that these antibodies recognize the three-dimensional structure of TeNT. In fact, recombinant 16E8 bound to the TeNT mix and to toxoid, but not TeNT, native toxin, suggesting that the three-dimensional structure of TeNT differs from that of TeNT mix and toxoid in our ELISA conditions. However, recombinant 16E8 antibody protected mice from TeNT toxicity as strongly as recombinant 8D8. Volk *et al.* also reported a protective mouse monoclonal antibody derived from tetanus toxoid-immunized mice that reacted with toxoid but not TeNT in ELISA<sup>14</sup>. Moreover, it has been reported that the structure of TeNT is changed by pH<sup>10</sup>, and thus it may be different in an ELISA assay than *in vivo*. These findings indicate that to select neutralizing antibody candidates *in vitro*, it is important

to use ELISA to analyze the reactivity of antibody clones to the mixtures of each domain protein and to toxoid, as well as to each domain protein individually.

We also isolated 31 Hc-reactive antibody clones, and two of these, 8A7 and 17F7, were the only clones that inhibited the binding of Hc to ganglioside GT1b *in vitro*. On the other hand, we observed enhancement of Hc-GT1b binding both by 8H2 and 25H5. Fitzsimmons *et al.* also reported an antibody that promoted Hc binding to GT1b<sup>15</sup>. This binding enhancement might be due to a conformational change in Hc. Recombinant 8A7 and 17F7 reacted with toxoid and TeNT in ELISA and had TeNT-neutralizing activity *in vivo*. Because the interaction of the TeNT Hc domain with GT1b is a critical to TeNT toxicity, blocking this interaction is thought to be very important for neutralization. To isolate TeNT-neutralizing antibody clones, therefore, it would be useful to examine whether antibody clones inhibit the binding of Hc to GT1b *in vitro*.

The combined supernatants of 35 TeNT mix-reactive LCL clones from our second experiment containing 0.5 ng of each antibody completely protected mice from 1 LD<sub>50</sub> TeNT. Of the various mixtures of domain-reactive antibody clones, the TeNT mix-reactive mixture conferred the strongest protection. The addition of Hn-reactive supernatant to the TeNT mix-reactive supernatant enhanced the latter's protective ability, but unlike the 35Ab mix, it did not provide complete protection. These results suggest that in addition to the Hn- and TeNT mix-reactive antibody mixtures, other domain-reactive antibody clones are required for complete protection in our assay. Aliprandini *et al.* recently reported that the combination of Lc-, Hn- or full-length TeNT-reactive human antibodies conferred complete protection against TeNT in mice<sup>16</sup>. In fact, Hc-reactive 8A7 or 17F7, Hn-reactive 8D8, and TeNT mix-reactive 16E8 completely neutralized 1 LD<sub>50</sub> of TeNT in our mouse experiments.

Finally, we isolated TeNT-neutralizing antibody clones from the PBMCs of a healthy individual using a modified EBV infection method. An *in vivo* TeNT neutralization assay revealed that the Hn-reactive and TeNT mix-reactive antibody clones had a strong protective ability and the combination of rAbs reactive to Hn, Hc, and TeNT mix enhanced TeNT-neutralizing activity. Collectively, the cocktail of the recombinant neutralizing antibodies identified in this study could be of help to the therapy for TeNT-induced infectious diseases instead of TIG.

## Methods

**Cell culture and reagents.** B95-8-ZHT cells which are derived from B95-8 marmoset cells and stably express EBV BZLF1 in a 4-hydroxytamoxifen-dependent manner<sup>17</sup>, were cultured in RPMI1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Merck, Darmstadt, Germany), streptomycin/penicillin, and 2-mercaptoethanol (Nacalai Tesque). LCLs were cultured in RPMI1640 (Nacalai Tesque) supplemented with 20% FBS (Merck), streptomycin/penicillin, 2-mercaptoethanol (Nacalai Tesque), K3 (Ajinomoto Bio-Pharma Services, Osaka, Japan), Ciclosporin (Novartis Pharma, Basel, Switzerland), IL-6, and BAFF (R & D systems, Minneapolis, MN, USA) (LCL medium).

**EBV preparation.** To prepare EBV (B95-8 strain), B95-8-ZHT cells were treated with 400 nM 4-hydroxy-tamoxifen (Merck) for 5 days and then collected the cultured conditioned medium as EBV stock.

**Human blood samples.** The blood donor was a healthy volunteer. Ethical approval for the study was obtained from the Human Research Committee of the National Institute of Health, and informed consent was obtained from all participants. The study was performed in accordance with the guidelines of the Declaration of Helsinki.

**Mice.** Four-week-old ddY mice were purchased from SLC Japan, Inc (Izu, Japan). The mice were used 1 week after purchase.

All mice were maintained in a specific pathogen-free animal facility in accordance with the Osaka University guidelines for animal experimentation.

The authors complied with the ARRIVE guidelines.

**EBV infection and LCL establishment.** PBMCs were separated from whole blood by gradient centrifugation method using Lymphoprep reagent (Abbott Diagnostics Technologies AS, Oslo, Norway). Then IgM<sup>+</sup> B cells were depleted from the PBMCs using Magnetic Cell Sorting-based anti-human IgM Microbeads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The IgM<sup>+</sup> B cell-depleted PBMCs were suspended in EBV stock (10<sup>7</sup> cells/mL) and rotated at 37°C for 1 h. The cells were then suspended in LCL medium (5 x 10<sup>4</sup> cells/mL) and seeded at 200 µL/well in round-bottomed 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA). After 2-week culture, the supernatant and the cells were collected, and ELISA and RNA isolation were performed.

**Purification of recombinant TeNT subunit and domain proteins.** Plasmid DNAs encoding TeNT Lc (1-424 aa), Hn (458–864 aa, C467S), and Hc (865–1315 aa) were purchased from Eurofins Genomics (Luxembourg), and the protein-encoding regions were each inserted into a pCold GST expression vector (Takara, Shiga, Japan) by homologous recombination using the In-Fusion system (Takara) (pCold GST Lc, pCold GST Hn and pCold GST Hc, respectively). BL21 Star (DE3) (Merck) was transformed with each expression plasmid and cultured in Luria Broth medium supplemented with 200 µg/mL ampicillin at 37°C. Expression was induced by the addition of isopropyl β-D-1 thiogalactopyranoside to a final concentration of 0.5 mM at an OD600 of ~ 0.5 with cooling to 15°C. After expression for 24 h, cells were harvested at 4°C by centrifugation for 20 min at 12,000 g and were stored at -80°C.

Frozen cell pellets were resuspended in a 1:5 (w/v) ratio of lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, EDTA-free complete protease inhibitor cocktail tablets [Roche, Basel, Switzerland]), and lysed using an EmulsiFlex-C3 homogenizer (Avestin Inc., Ottawa, Canada), and centrifuged for 30 min at 20,000 g and 4°C. Then the supernatant was filtered using a 0.45-µm syringe filter (Sartorius, Gottingen, Germany) and applied onto two 5-mL His Trap TALON columns (Cytiva, Marlborough, MA, USA). The proteins were eluted by 20 mL of an elution buffer (20 mM Tris-HCl pH 8.0, 200 mM imidazole, 200 mM NaCl). The hexa-histidine and glutathione S-transferase (GST) tags were removed by adding HRV3C protease directly to the eluent, which was then dialyzed overnight against phosphate-buffered saline (PBS). The protease

and cleaved product were removed by a GSTrap HP column (Cytiva) in PBS containing 5 mM 1,4-dithiothreitol (DTT). The unbound fraction was dialyzed twice against 10 mM Tris-HCl pH 8.0 for 3h, and purified by a HiLoad 16/60 Superdex 75 prep-grade column (Cytiva) equilibrated with size-exclusion buffer (10 mM HEPES pH 7.4, 100 mM NaCl).

**Expression of recombinant TeNT Hn mutant proteins.** Expression plasmid vectors for TeNT Hn mutants were constructed using a KOD -Plus- Mutagenesis Kit according to the manufacturer's protocol (Toyobo, Osaka, Japan). The primers used for PCR are shown in Table 3. The method for expressing proteins in *E. coli* is described above. The proteins were extracted using 1% sodium deoxycholate, 5 mM EDTA pH8.0, and PBS as lysates. The cell lysates were used for capture, and ELISA was performed to determine the 8D8 antibody binding region for the Hn protein.

Table 3  
Primers used to construct Hn mutant expression vectors

Clone name	Primer 1	Primer 2
Hn (458–828)	TAACTCGAGGGATCCGAATTC	CAGGATGTTTTTTGACTGGG
Hn (700–864)	CATATGTCCCGGGCCCTGGAACAG	ATTATCAAACACTATCGACAACCTTC
Hn (790–864)	CATATGTCCCGGGCCCTGGAACAG	ATGATTAACATCAACATCTTCATG
Hn (852–864)	CATATGTCCCGGGCCCTGGAACAG	AACAAAGTATTCAGTACCCCC
AAA.1	GCAGCAGCAATTAAGCGAACTCCAAATTC	CAGGATGTTTTTTGACTGGG
AAA.2	GCAGCAGCAAACCTCCAAATTCATTGGGATC	GTAAGTGCATCAGGATGTTTTTTG
AAA.3	GCAGCAGCATTATTGGGATCACCGAACTC	CGCTTTAATGTACTGCATCAGG
AAA.4	GCGGCCGCGATCACCGAACTCAAGAACTG	TTTGGAGTTCGCTTTAATGTAC
AAA.5	GCGGCCGCGCTCAAGAACTGGAAAGCAAG	CCCAATGAATTTGGAGTTCGC
AAA.6	GCGGCCGCGCTGGAAAGCAAGATCAACAAAG	TTCGGTGATCCCAATGAATTTGG
AAA.7	GCGGCCGCGAAGATCAACAAAGTATTCAG	TTTCTTGAGTTCGGTGATCCCAATG
AAA.8	GCGGCCGCGAAAGTATTCAGTACCCCCATAC	GCTTTCCAGTTTCTTGAGTTC
AAA.9	GCGGCCGCGAGTACCCCCATACCCTTTTCG	GTTGATCTTGCTTTCCAGTTTC
AAA.10	GCGGCCGCGATACCCTTTTCGTATAGCTAAC	GAATACTTTGTTGATCTTGC
AAA.11	GCGGCCGCGTCGTATAGCTAACTCGAGGGATC	GGGGTACTGAATACTTTGTTG
AAA.12	GCGGCCGCGTAACTCGAGGGATCCGAATTC	AAAGGGTATGGGGTACTG

**ELISA.** The reactivity of rAbs and antibodies in LCL supernatant was assessed against each antigen by ELISA. To detect reactivity against TeNT, 1 µg/mL of Lc, Hn, Hc, TeNT (gifted from Dr. Masaaki Iwaki at the National Institute of Infectious Diseases), TeNT mix (a mixture of Lc, Hn, and Hc proteins), and tetanus toxoid were used for capture, and ELISA was performed as previously described<sup>18</sup>. To measure the concentration of human immunoglobulin in LCL supernatant, 10 µg/mL anti-human IgG (SouthernBiotech, Birmingham, AL, USA) was used for capture and ELISA was performed as previously described<sup>19</sup>. To determine the binding region of 8D8, each Hn mutant protein lysate was used for capture, along with 1 µg/mL of 8D8.

**Hc-GT1b binding assay.** The inhibitory effect of antibodies on Hc-ganglioside GT1b binding was analyzed in an Hc-GT1b binding assay. Fifty microliters of 1 µg/mL ganglioside GT1b (Adipogen Life Sciences, San Diego, CA, USA) were dispensed into 96-well microtiter plates (Thermo Fisher Scientific) and the solvent methanol was evaporated for 8 h at room temperature. After washing three times with PBS-0.1% Tween 20 (PBS-T), the plates were blocked with 2% bovine serum albumin and PBS for 1 h at room temperature. Fifty microliters of 5 µg/mL Hc were mixed with serially diluted 50 µL of LCL supernatant or medium and incubated for 1 h at 37°C. The plate was washed once with PBS-T, and each preincubated Hc/antibody mixture was added to the wells and incubated for 1 h at room temperature. The plates were then washed three times and incubated with mouse anti-Hc (produced in our lab) for 2 h at room temperature. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (SouthernBiotech) was used to detect Hc binding to GT1b.

**In vivo TeNT neutralization assay.** The TeNT-neutralizing ability of all antibodies was analyzed *in vivo*. TeNT (1 LD<sub>50</sub>) was mixed with LCL supernatant containing 0.5 ng of antibody, or with 0.025, 0.125, 1.25, or 1250 ng of purified rAbs and the volume was brought to 400 µL with 2% gelatin, and PBS. After 1 h incubation at room temperature, each antibody was subcutaneously injected into the left femur of four ddY mice (5 weeks of age) and the mice were monitored for symptoms of paralysis.

**Construct of Ig expression vector.** To construct the expression vector for monoclonal antibodies, total RNA was first isolated from LCLs using a miRNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Then nested RT-PCR was performed using a SMART cDNA Library Construction Kit according to the manufacturer's protocol (Takara). The following primers were used for the nested RT-PCR: IgH (IgG1), 1st forward primer (SMART 1st): 5'-AAGCAGTGGTATCAACGCAGAGT-3' and 1st reverse primer: 5'-CGGGGAGCGGGGCTTGCCGGCCGTCGCAC-3'; 2nd forward primer: 5'-GGGGCGCCGCAGAGTGGCCATTACGGCCGGG-3' (SMART 2nd) and 2nd reverse primer: 5'-GGGGAATTCTCATTTACCCGGAGACAGGG-3'; IgL (Igk), 1st forward primer: SMART 1st and 1st reverse primer: 5'-ACTGAGGAGCAGGTGGGGCACTTCTCCCT-3'; 2nd forward primer: SMART 2nd and 2nd reverse primer: 5'-GGGGAATTCCTAACACTCTCCCTGTTG-3'. The PCR-amplified fragment was digested with Not I and EcoR I and cloned into a human EF1α promoter-containing pQEFIP vector or pQEFIN vector that was derived from pQCXIP or pQCXIN vector (Takara), respectively.

**Expression and purification of recombinant antibodies.** Transient expression of recombinant Ig was performed using the Expi293 Expression system (Thermo Fisher Scientific).

Expi293F cells were cotransfected with a mixture of IgH and IgL expression vectors according to the manufacturer's protocol.

Following culture of the transfected cells for 7 days, the culture supernatants were loaded onto a HiTrap Protein G HP Columns (Cytiva). Sample preparation on the column continued according to manufacturer-suggested protocols, resulting in the solubilized preparation of recombinant monoclonal antibodies.

**Analysis of immunoglobulin genes.** Analysis of the immunoglobulin gene repertoire, CDR3 amino acid sequences, and the number of somatic mutations was performed using IMGT/V-QUEST at the web site of the International Immunogenetics Information System ([http://www.imgt.org/IMGT\\_vquest/vquest](http://www.imgt.org/IMGT_vquest/vquest)).

**Antibody structure prediction and antibody-antigen docking.** The 3-D structure of the variable domain of an 8D8 antibody clone was predicted using locally installed Rosetta software (version 2020.37) (<http://www.rosettacommons.org/>)<sup>20</sup>. Briefly, amino acid sequences of light ( $V_L$ ) and heavy ( $V_H$ ) chain variable domains of the antibody clones were used for a BLASTp search against the Protein Data Bank (PDB) database, in order to generate CDR-grafted antibody models as templates.

Two-hundred runs of antibody structure modeling were performed using RosettaAntibody software with the generated templates, and the most suitable antibody template was selected according to the modeling results.

For antibody-antigen docking simulation, the 3-D structure of full-length TeNT (PDB ID: 5N0B) composed of Lc, Hn, and Hc domains was downloaded from the PDB website (<https://www.rcsb.org/>)<sup>10</sup>. Two-hundred runs of antibody-antigen docking simulation were performed using SnugDock software with the obtained antibody and TeNT structures, and the most suitable docking model was selected according to the results of the docking simulation and the ELISA-based epitope mapping. The selected docking model was visualized and analyzed using UCSF Chimera with default settings<sup>21</sup>.

## Declarations

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### Author contribution

T.Y. and T.M. designed the study. T.M. K.K. and R.O. performed experiments. T.I. predicted 3-D structure of proteins using a computer. H. A. and K. T. contributed to immunoglobulin gene cloning and expression. T.I. assisted protein expression and purification. R.A.F. and M.S. supervised the study. T.M. and T.Y. wrote and all authors discussed the manuscript.

## Additional Information

### Competing interests

The author(s) declare no competing interests.

### Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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## Figures

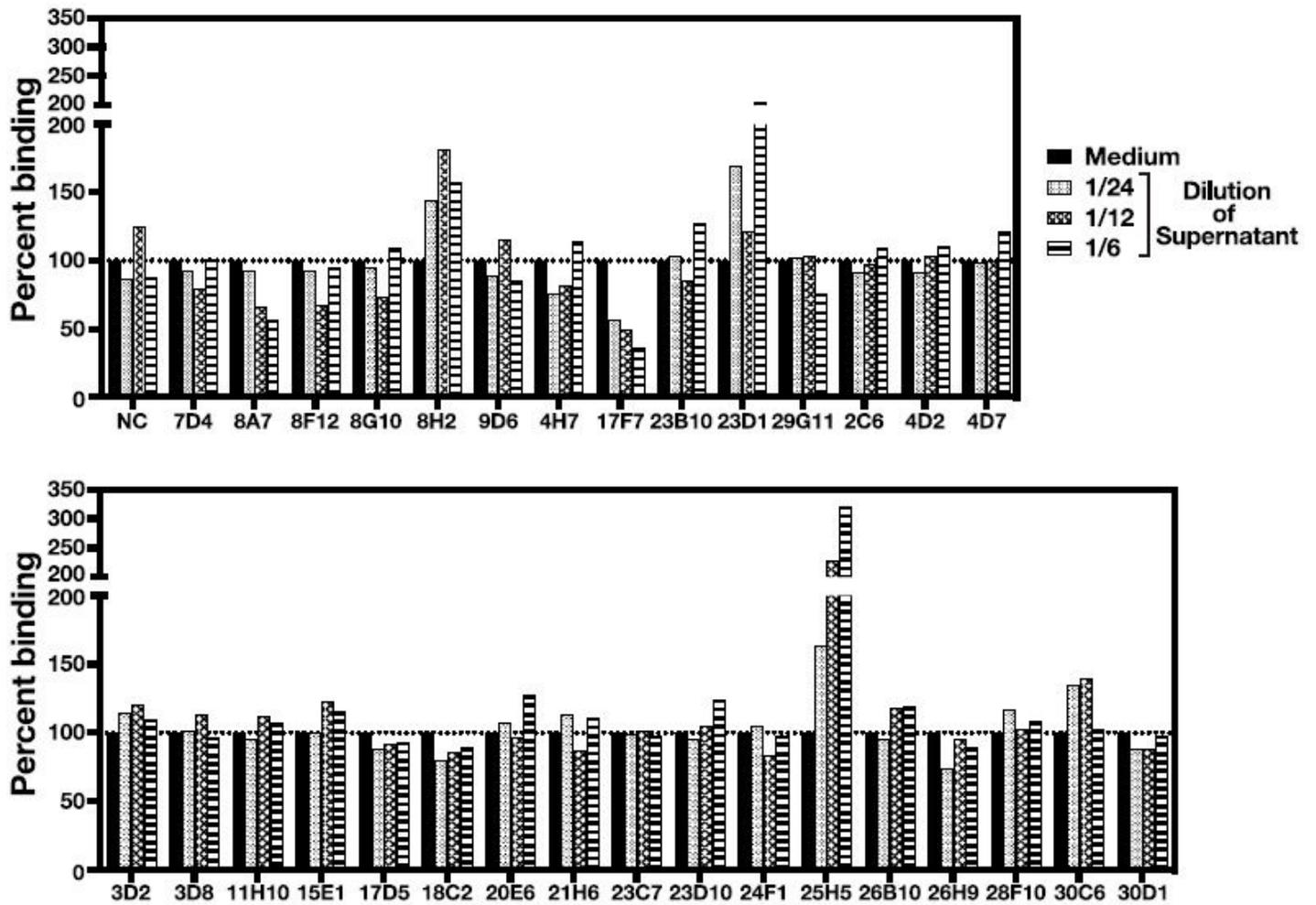


Figure 1

8A7 and 17F7 antibody clones inhibit the binding of Hc to ganglioside GT1b. The inhibition of Hc-GT1b by antibodies was analyzed by an ELISA-based Hc-GT1b binding assay. The supernatants of 31 Hc-reactive clones were serially diluted to 1/6, 1/12, and 1/24. Percent binding of Hc to GT1b is indicated.

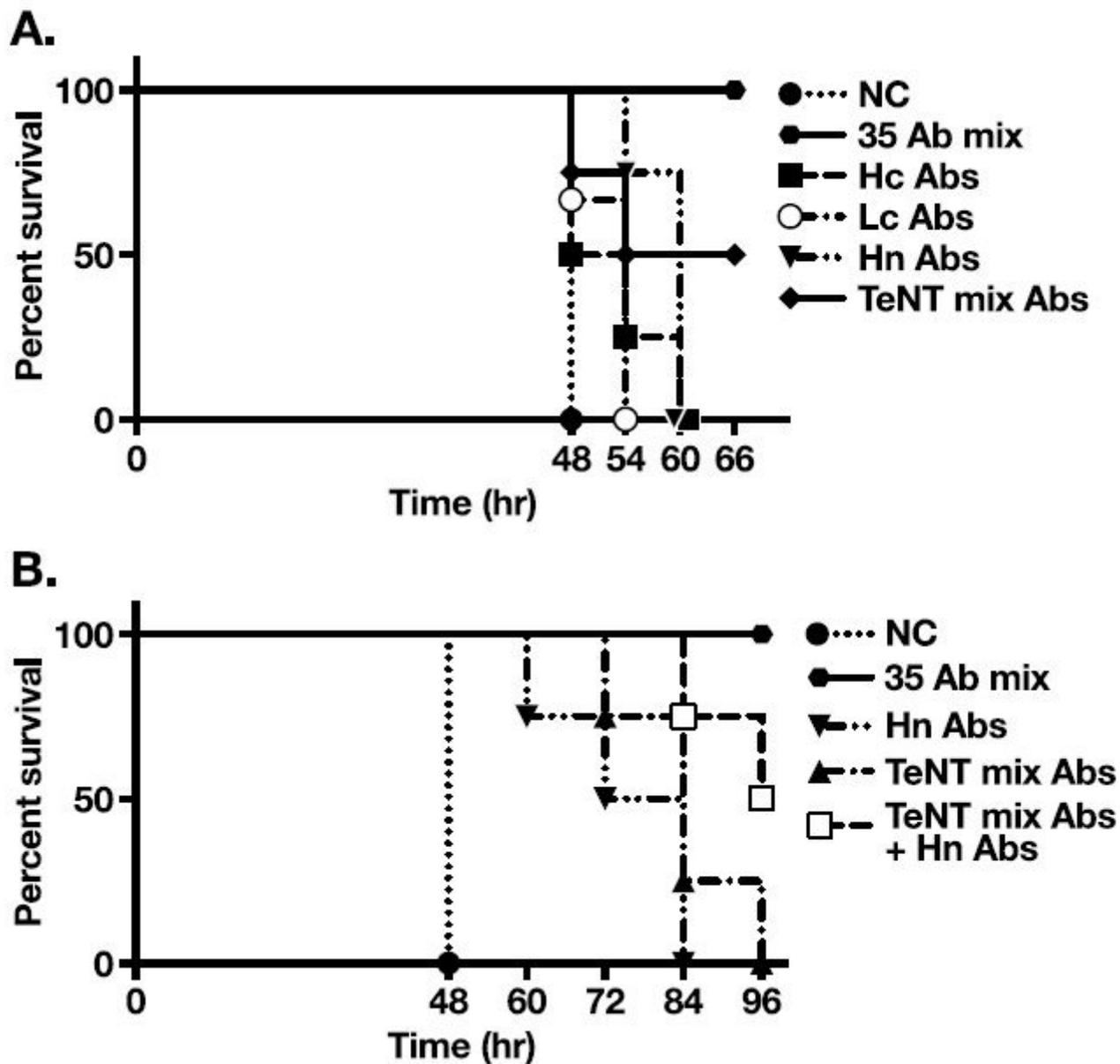


Figure 2

A combination of TeNT mix-reactive antibody clones strongly neutralizes TeNT in mice. The protective effect of isolated antibody clones was analyzed by an in vivo TeNT-neutralization assay. We combined 0.5 ng human IgG-containing LCL supernatants from all 35 clones (35 Ab mix), 17 Hc-reactive clones (Hc Abs), 12 Lc-reactive clones (Lc Abs), 3 Hn-reactive clones (Hn Abs), or 3 TeNT mix-reactive clones (TeNT-mix Abs). Then we mixed the supernatant mixture with 1 LD50 of TeNT and administrated it to ddY mice. Mouse survival rates after administration are shown (n = 4). NC, negative control (medium).

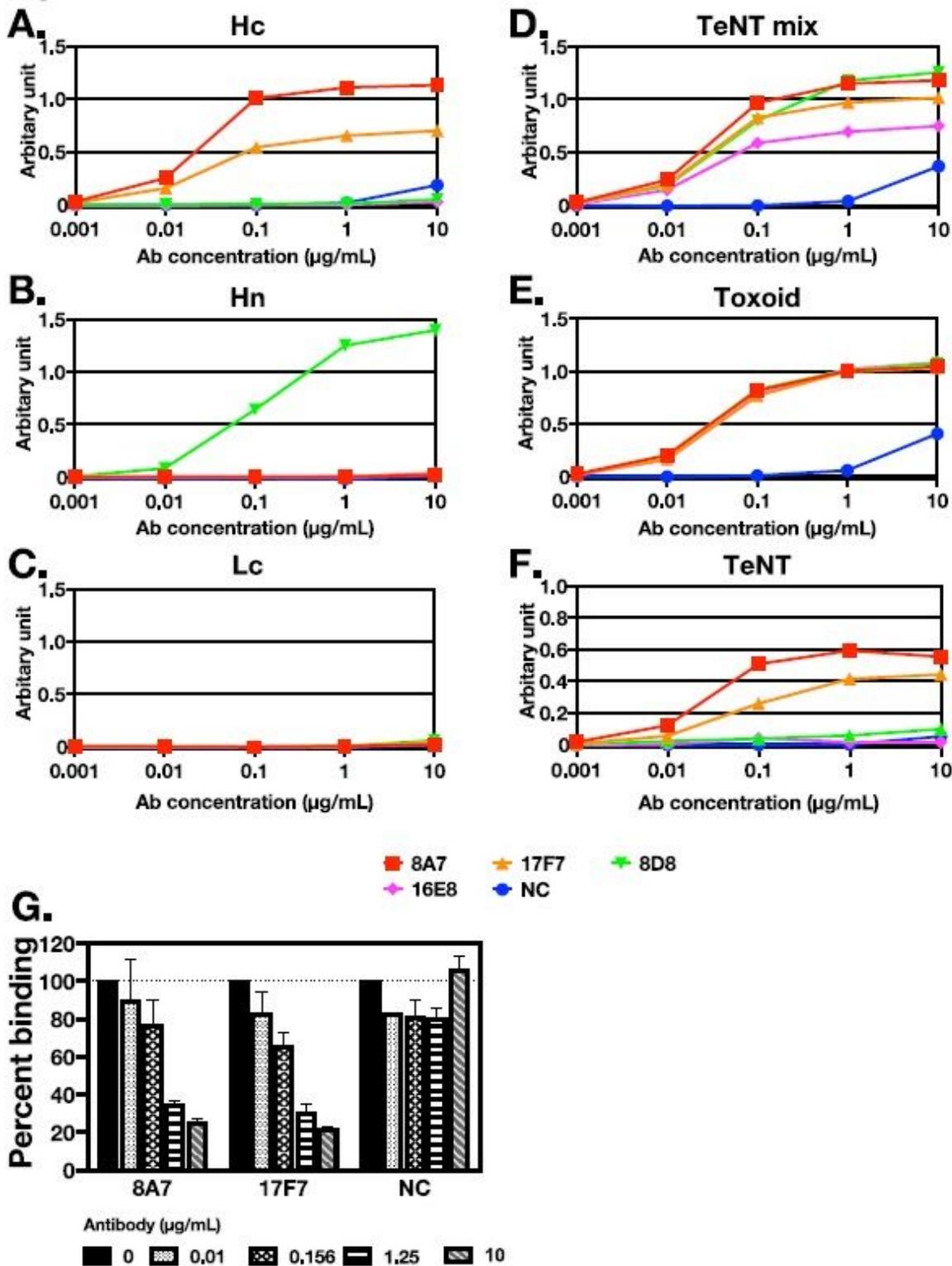


Figure 3

Reactivity analysis of recombinant antibodies by ELISA. The reactivity of purified rAbs (8A7, 17F7, 8D8, and 16E8) against Hc (A), Hn (B), Lc (C), TeNT mix (D), tetanus toxoid (Toxoid; E) and native tetanus neurotoxin (TeNT; F) was analyzed by ELISA in triplicate. The concentration of antibodies was serially diluted from 10 to 0.001 µg/mL. Human IgG purified from human sera was used as a negative control (NC). (G) The inhibitory activity of Hc-GT1b by recombinant 8A7 and 17F7 was analyzed by an ELISA-

based Hc-GT1b binding assay in triplicate. The concentrations of antibodies used for the assay were 0, 0.01, 0.156, 1.25, and 10  $\mu\text{g}/\text{mL}$ . The percent binding of Hc to GT1b is indicated. The symbol or bar in each graph indicates the average. Error bars show the means  $\pm$  s.d.

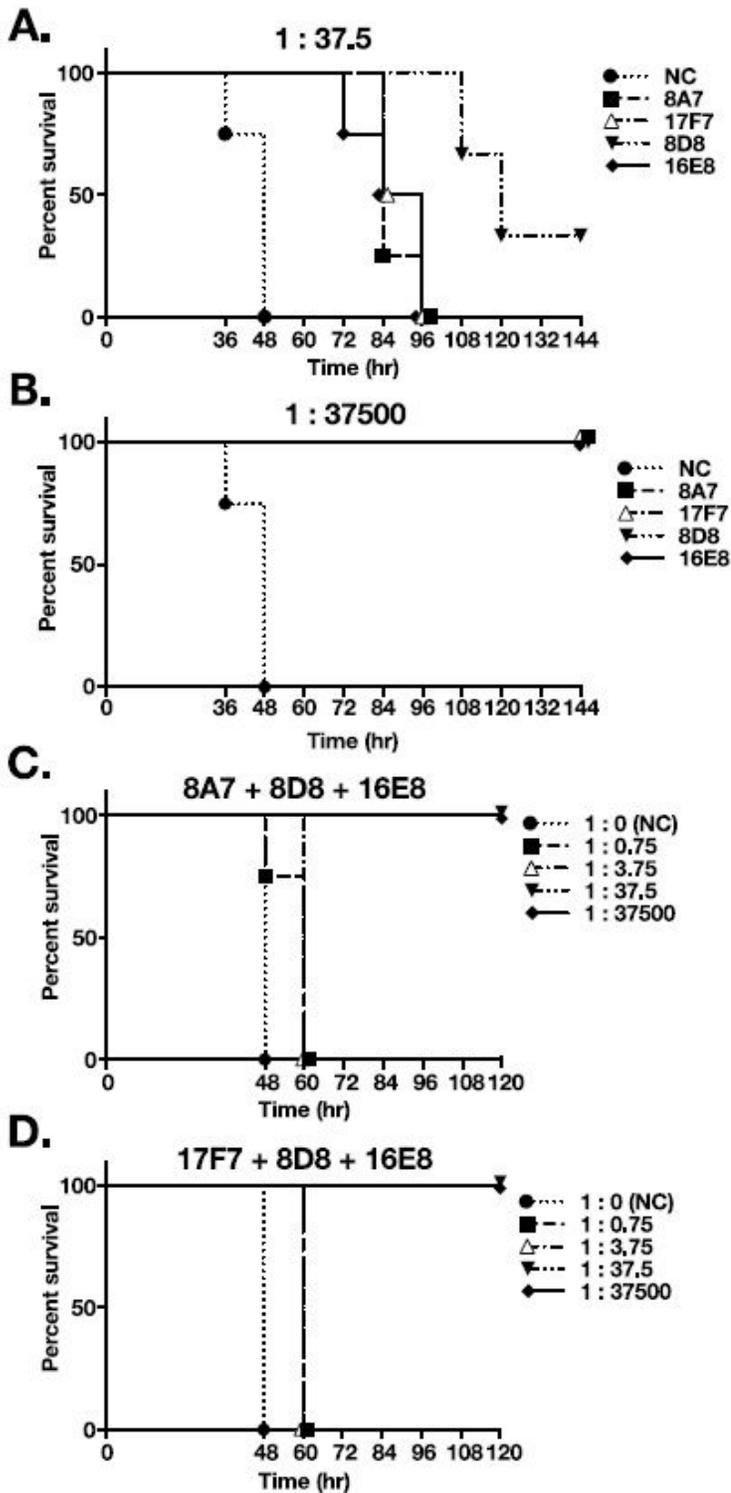
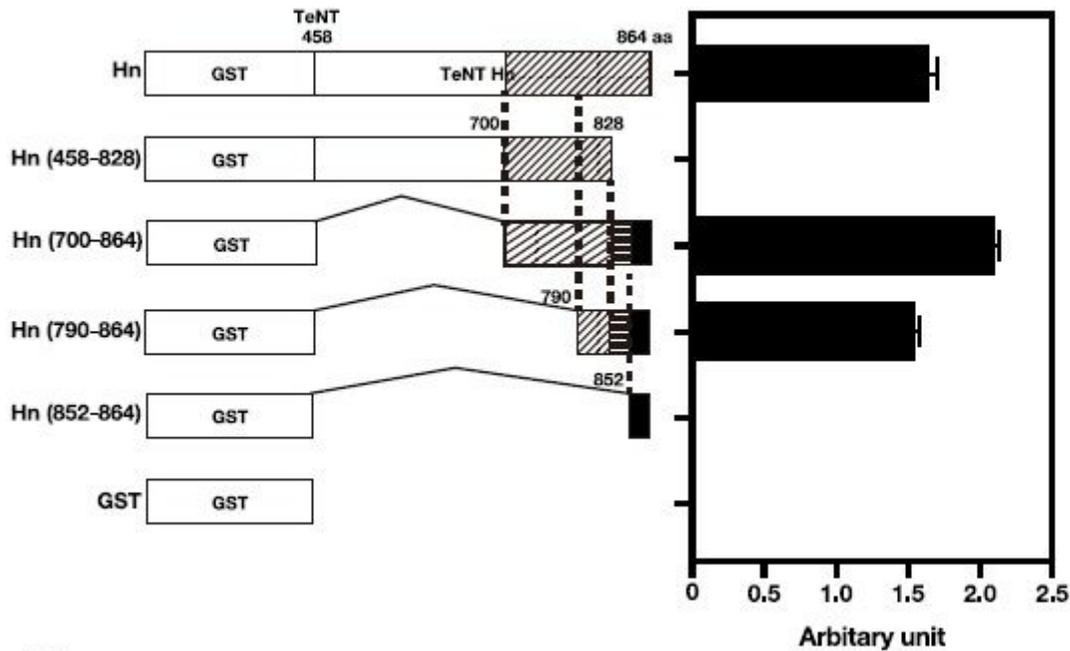


Figure 4

Mixing antibodies reactive to different domains increases neutralization activity in mice. The TeNT-neutralization ability of purified recombinant 8A7, 17F7, 8D8, and 16E8 antibody clones were analyzed by

an in vivo TeNT-neutralization assay. (A, B) 3.75 ng (TeNT:rAb ratio 1:37.5) (A) or 3,750 ng (TeNT:rAb ratio 1:37500) (B) of each antibody was assessed. (C, D) The protective ability of the combination of 8A7, 8D8, and 16E8 antibodies (C) or 17F7, 8D8, and 16E8 antibodies (D) was analyzed. TeNT:rAb ratios of 1:0.75, 1:3.75, 1:37.5, and 1:37,500 were analyzed. Mouse survival rates following administration are shown (n = 4). NC, negative control (PBS).

**A.**



**B.**

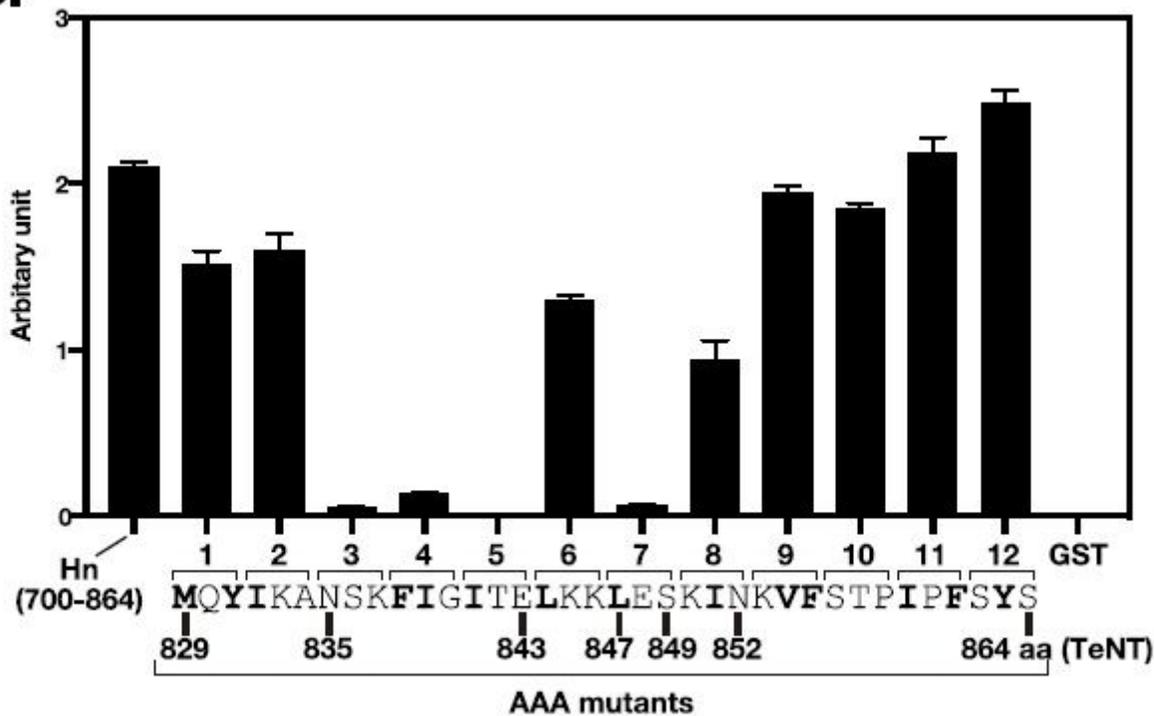
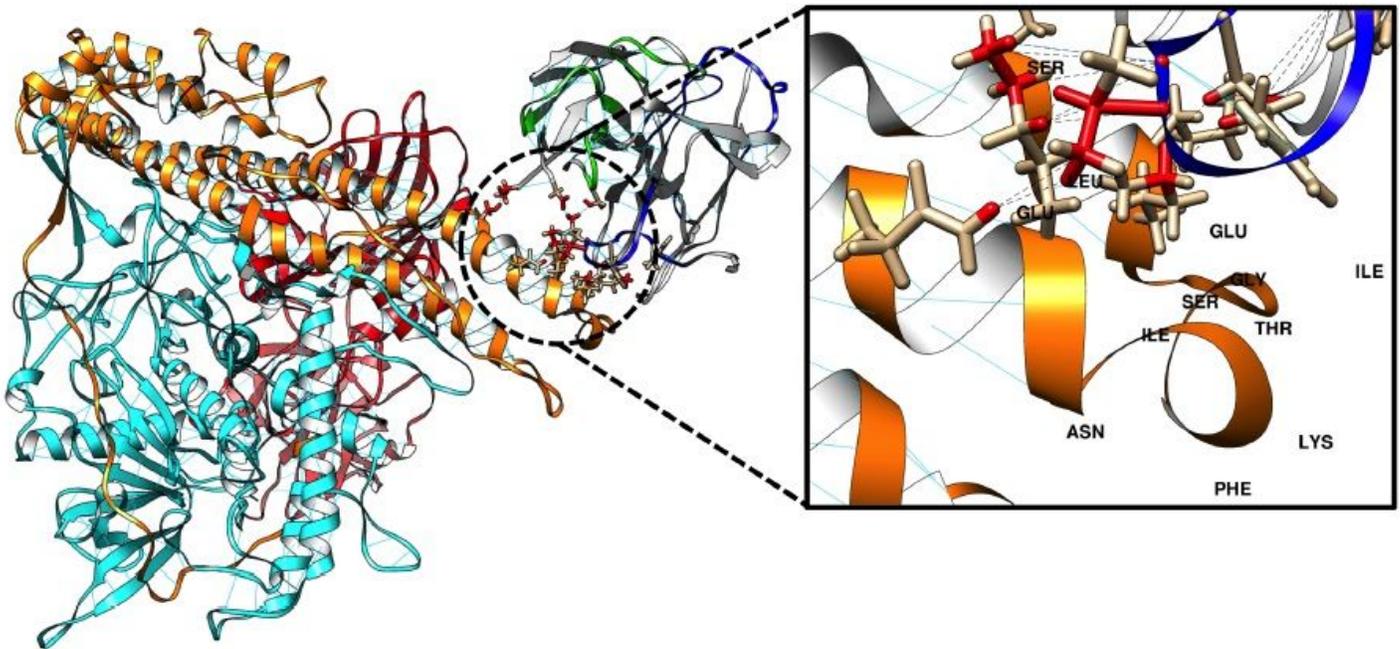


Figure 5

Mixing antibodies reactive to different domains increases neutralization activity in mice. The TeNT-neutralization ability of purified recombinant 8A7, 17F7, 8D8, and 16E8 antibody clones were analyzed by an *in vivo* TeNT-neutralization assay. (A, B) 3.75 ng (TeNT:rAb ratio 1:37.5) (A) or 3,750 ng (TeNT:rAb ratio 1:37500) (B) of each antibody was assessed. (C, D) The protective ability of the combination of 8A7, 8D8, and 16E8 antibodies (C) or 17F7, 8D8, and 16E8 antibodies (D) was analyzed. TeNT:rAb ratios of 1:0.75, 1:3.75, 1:37.5, and 1:37,500 were analyzed. Mouse survival rates following administration are shown (n = 4). NC, negative control (PBS).



**Figure 6**

Docking model of Hn-reactive 8D8 with full-length TeNT. The docking model of Hn-reactive 8D8 with full-length TeNT (PDB ID: 5N0B) was obtained by using SnugDock software. The 3-D structure of the variable domain of 8D8 was predicted by using RosettaAntibody software. A whole structure view and a close-up view of 8D8 and TeNT are shown by ribbon models, and experimentally determined binding sites (NSKFIGITE and LES) are shown by stick models with 3-letter amino acid codes. CDR loops in VL and VH of 8D8 are indicated with green and blue, respectively. Lc, Hn, and Hc domains in TeNT are indicated with cyan, orange, and red, respectively. Hydrogen bonds are indicated with cyan lines. Antibody-antigen interaction sites within a 5 Å distance are indicated with black dashes.