

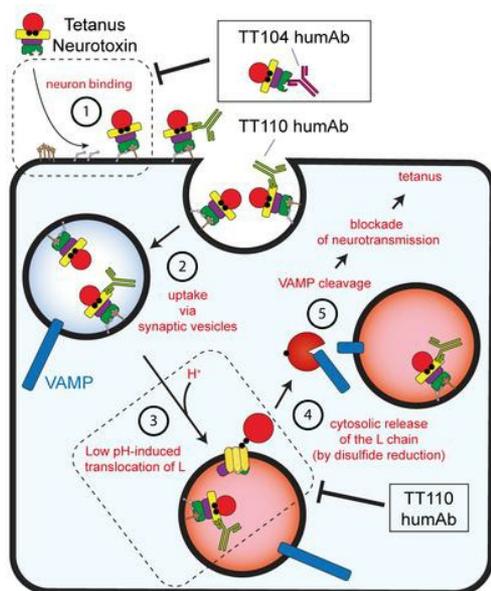
Exceptionally potent human monoclonal antibodies are effective for prophylaxis and therapy of tetanus in mice

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1 **Exceptionally potent human monoclonal antibodies are effective for prophylaxis and therapy of**
2 **tetanus in mice**

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35 **ABSTRACT**

36 Human monoclonal antibodies were used here to study the mechanism of neuron intoxication by
37 tetanus neurotoxin and to evaluate them as a safe preventive and therapeutic substitute of
38 hyperimmune sera for tetanus in mice. By screening memory B cells of immune donors, we selected
39 two monoclonal antibodies specific for tetanus neurotoxin with exceptionally high neutralizing
40 activities, which were extensively characterized both structurally and functionally. We found that
41 these antibodies interfere with the binding and translocation of the neurotoxin into neurons by
42 interacting with two epitopes, whose definition pinpoints crucial events in the cellular pathogenesis
43 of tetanus. This information explains the unprecedented neutralization ability of these antibodies,
44 which were found to be exceptionally potent in preventing experimental tetanus when injected in
45 mice long before the neurotoxin. Moreover, their Fab derivatives neutralized tetanus neurotoxin in
46 post-exposure experiments, suggesting their potential therapeutic use via intrathecal injection. As
47 such, these human monoclonal antibodies, as well as their Fab derivatives, meet all requirements
48 for being considered for prophylaxis and therapy of human tetanus and are ready for clinical trials.

49
50 Keywords: tetanus, tetanus neurotoxin, human monoclonal antibody, tetanus immunoglobulin,
51 tetanus prophylaxis

52

53 **INTRODUCTION**

54 Tetanus neurotoxin (TeNT) is a highly potent exotoxin responsible for tetanus, a life-threatening
55 disease whose major symptoms are muscle rigidity and spasms, spastic paralysis, respiratory deficits
56 and autonomic dysfunctions (1-5). TeNT is produced by toxigenic strains of the anaerobic sporogenic
57 bacterium *Clostridium tetani* (6-8). The few amino acid variations found in the currently known TeNT
58 isoforms do not change their immunogenic properties with respect to the prototypical TeNT
59 (Harvard strain, E88) (9).

60 TeNT consists of a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a single interchain
61 disulfide bond essential for neurotoxicity (8, 10). TeNT is generally described as a protein consisting
62 of three domains, each one serving a different step of its mechanism of neuronal intoxication. The
63 carboxy-terminal domain HC (50 kDa) is responsible for presynaptic binding and consists of two sub-
64 domains; the carboxy-terminal half (HC-C) contains a polysialoganglioside binding site and a nidogen
65 binding site (11-14), whereas the amino-terminal half portion (HC-N), is essential for toxicity though
66 its function is not known (15). HC-N is linked to HN (50 kDa), the domain responsible for the
67 membrane translocation of the L domain into the cytosol. The L domain is a metalloprotease that
68 blocks neurotransmitter release (16).

69 *C. tetani* spores are ubiquitous in the environment and can contaminate necrotic wounds of any
70 kind (burns, ulcers, surgery, tattooing, circumcision and needle injection etc.) where spores may
71 generate vegetative bacteria producing TeNT which diffuses via the blood and lymphatic
72 circulations. TeNT binds to motor, sensory and autonomic presynaptic nerve terminals via at least
73 two independent receptors: a polysialoganglioside and a protein (5, 17). Polysialogangliosides (PSG),
74 including GT1b, GD1b and GQ1b, play a major role in the initial membrane binding of TeNT (11-13),
75 whilst nidogens 1 and 2 (also known as entactin 1/2) were identified as TeNT protein receptors (14).
76 Nidogens and PSG direct TeNT to presynaptic zones that, upon endocytosis, generate signaling
77 endosomes containing neurotrophic factors and their receptors (18). These organelles undergo fast
78 axonal retrograde transport to the perikaryon located in the spinal cord, where they release their
79 content (18-20). TeNT then binds to inhibitory interneurons and is endocytosed into the lumen of
80 synaptic vesicles (SV) (21). Acidification of the SV lumen induces a change in conformation of TeNT
81 whereby the HN domain inserts into the membrane and assists the membrane translocation of the
82 L chain from the SV lumen to the cytosol. Here, the interchain disulfide bond is reduced by the
83 thioredoxin reductase-thioredoxin redox system (22), releasing the metalloprotease activity of the
84 L domain, which specifically cleaves VAMP at a single site (23). Together with SNAP-25 and syntaxin,

85 VAMP forms a complex driving the fusion of SV with the presynaptic membrane with ensuing
86 neurotransmitter release (24).

87 VAMP cleavage in spinal cord inhibitory interneurons prevents the release of inhibitory
88 neurotransmitters, which determines the hyperactivation of the post-synaptic motor neurons with
89 sustained contraction of the innervated skeletal muscles, resulting in spastic paralysis (25, 26).
90 Muscle contractures begin with *trismus* (lockjaw, a cardinal symptom of tetanus) and neck with
91 difficult swallowing and then descend to the thorax, causing respiratory deficit, generalized spasm
92 of opposing skeletal muscles and autonomic dysfunctions that may lead to death (2).

93 Tetanus is prevented by a very effective vaccine based on tetanus toxoid that raises a long-lasting
94 protection due to specific anti-TeNT antibodies, but after the age of forty/fifty the anti-TeNT
95 antibody titer decreases below TeNT neutralization (27). Tetanus is still a major killer in countries
96 where appropriate public healthcare systems are not enforced, and where the infection of the
97 umbilical cord stump or of the birth canal with non-sterile instruments causes *tetanus neonatorum*
98 and/or maternal tetanus (28).

99 Another common medical practice to prevent tetanus in patients presenting necrotic wounds at
100 hospital emergency rooms is the injection of anti-TeNT IgG, known as tetanus immunoglobulins
101 (TIG), isolated from hyperimmune human donors (29). Hyperimmune horse sera are used in low-
102 income countries because of their lower cost but can generate dangerous hypersensitivity reactions.
103 TIG is also administered to patients with symptomatic tetanus, to neutralize circulating TeNT and
104 limit the severity of the disease though this might cause problems (see Discussion) (29). Intrathecal
105 TIG administration is more effective, although this approach is limited by the low percentage of anti-
106 TeNT antibodies present in TIG and by the amount of protein that can be injected in the
107 cerebrospinal fluid (30, 31). All these drawbacks can be overcome by using highly purified human
108 monoclonal antibodies (humAbs), which are already approved for a variety of human diseases (32-
109 38).

110 Here, we report on the identification of two humAbs with extremely high TeNT neutralizing activity
111 and determined the structure of their binding sites. These humAbs provide a long-lasting
112 prophylactic activity against lethal doses of TeNT whereas their Fab prevented tetanus in post-
113 exposure experiments as well as TIG. These findings suggest that these humAbs and their Fab
114 fragments represent appropriate and safe alternatives to TIG.

115

116 **RESULTS**

117

118 ***HumAbs recognize distinct domains of TeNT and display distinct neutralizing capability.***

119 Humabs against TeNT were isolated from memory B cells of immune adult donors who underwent
120 booster vaccinations with tetanus toxoid as described (33). IgG memory B cells were immortalized
121 under clonal conditions and the antibodies produced in the culture supernatant were screened by
122 ELISA for their ability to bind TeNT. The antibody genes from 14 positive clonal cultures were
123 sequenced and recombinant antibodies were produced as IgG1 by transfecting HEK-293 cells.

124 The TeNT domains recognized by the 14 humAbs were determined by western blotting of the intact
125 or reduced toxin and of the recombinant sub-domains (Figure 1). The binding specificity of each
126 antibody is shown in Figure S1. 11 antibodies bound to the HC domain, 3 to the HN domain and
127 none to the L chain. 3 out of the 11 HC-specific antibodies recognized the HC-C part, 8 of them
128 bound to the HC-N portion (Figure S1), suggesting that HC-N is the most immunogenic part of TeNT.
129 Recent studies on anti-TeNT humAbs reported that the highest TeNT neutralization was displayed
130 by antibodies recognizing HC, particularly those preventing PSG binding, but did not report on HC-
131 N recognition (39-42). Although the role of HC-N in neuron intoxication is currently unknown, its
132 deletion causes loss of toxicity (15). Therefore, these specific humAbs could be very useful in
133 unravelling the role of HC-N in the molecular and cellular pathogenesis of tetanus. Together with
134 the knowledge that HC-C contains the binding sites for the neuronal receptors of TeNT, these
135 findings strongly suggest that the structure of TeNT is best described in terms of four domains (L,
136 HN, HC-N and HC-C), rather than the three-domain paradigm (L, HN and HC) reported so far (8, 43).

137

138 ***Inhibition of TeNT activity by humAbs.***

139 HumAbs were tested in cerebellar granule neurons (CGNs) for their ability to inhibit TeNT cleavage
140 of VAMP-2, an isoform highly expressed in central nervous system neurons. CGNs were chosen
141 because they are highly susceptible to TeNT, and VAMP cleavage is easily testable by
142 immunoblotting and imaging (44). Figure 2A shows that an overnight incubation of CGNs with 50
143 pM TeNT is sufficient to cleave all the VAMP-2 of these neurons. An initial screening with a hundred-
144 fold molar excess showed that only 4 out of the 14 humAbs tested (TT104, TT110, TT109 and TT39)
145 were able to prevent TeNT action, albeit to different extents. Accordingly, we focused on these
146 humAbs and tested various humAb:TeNT molar ratios to determine their neutralizing potency. As
147 shown in Figure 2B, TT39 and TT109 showed partial neutralization. In contrast, both TT104 and

148 TT110 prevented TeNT intoxication already at a low molar ratio, TT104 being the most powerful.
149 These results were paralleled by immunofluorescence staining of VAMP-2 (Figure 2C).
150 The selected humAbs were then tested for their ability of neutralizing TeNT *in vivo*. Figure 2D shows
151 that preincubation of TeNT with TT104 and TT110 prevented the development of tetanus in mice in
152 a dose-dependent manner, with TT104 displaying full neutralization at equimolar ratio. On a molar
153 basis, TT110 was less effective, but reduced tetanus symptoms at a 2.5:1 molar ratio and completely
154 neutralized TeNT at a 5:1 ratio. TT39 and TT109 did not protect mice from TeNT challenge.
155 Accordingly, TT104 and TT110 were chosen for further structural and functional analyses.

156
157 ***Structure of the ternary complex of TeNT with TT104 and TT110 Fab.***

158 Recombinant Fab fragments of TT104 and TT110 were produced by introducing a stop codon after
159 C_H1 domain (Figure S2). The dissociation constants of the toxin-Fab complexes were measured by
160 surface plasmon resonance using immobilized TeNT as a bait and found to be 6.7×10^{-12} M and 3.0
161 $\times 10^{-9}$ M for TT104-Fab and for TT110-Fab, respectively (Figure S3). The high affinity of these
162 interactions suggested the possibility that stable binary and ternary complexes could be formed
163 between TeNT and the two Fab. Indeed, immunocomplexes were obtained by incubation of single
164 components either in binary (1:1) or in ternary mixtures (1:1:1) (Figure S4). The ternary [TeNT]-
165 [TT104-Fab]-[TT110-Fab] immunocomplex was purified to homogeneity by gel filtration (total mass
166 250 kDa). Attempts to crystallize this complex were not successful and therefore we switched to
167 cryo-EM that produced a model fitting the available structure of TeNT (PDB ID 5n0b) and of the two
168 Fabs modelled using the sequences of TT104 and TT110 Fabs. The overall four-domain folding of
169 TeNT is well conserved in the immunocomplex and superimposes with the structures of isolated
170 domains (Figure 3A and 3B) and of the whole toxin (Figure 3C and 3D) (8, 45-47).

171 Similarly to TeNT alone (8), the trimeric complex [TeNT]-[TT104-Fab]-[TT110-Fab] in solution is
172 flexible (Figure S5A). The flexibility is intrinsic to TeNT and not influenced by the binding of the Fabs;
173 it is mainly located around residues 870-875, a loop not resolved in the crystal structure of the toxin
174 and corresponding to the connection between the C-terminal of HN and the N-terminal of HC-N (8).
175 To overcome the flexibility of the complex, the structure was split in two parts analyzed separately
176 and two masks were generated by the multi-body technique to yield two different maps, one
177 relative to [TeNT-L-HN]-[TT110-Fab], and the other one to [TeNT-HC]-[TT104-Fab].

178
179 ***Structure of [TeNT-L-HN]-[TT110-Fab].***

180 Owing to the preferred orientation assumed by the particles in the grid, the analysis of [TeNT-L-HN]-
181 [TT110-Fab] portion provided a resolution of 8.3 Å, insufficient to define their interactions at the
182 atomic level. However, the overall shapes could be clearly distinguished and showed that TT110-
183 Fab binds the HN domain opposite to the L domain (Figure 3A). The buried surface of this epitope
184 was estimated to be 700 Å² and 380 Å² for chains V_H and V_L, respectively, and the interaction area
185 involves the TeNT helices 597-607 and 614-625, extending to the following strand until residue 631
186 and including segment 655-663. Importantly, this latter segment is part of the “BoNT-switch”, a
187 structural module proposed to be a main driver of the low-pH induced membrane insertion of HN
188 in the botulinum neurotoxins (BoNT), a group of toxins sharing the structural architecture and the
189 mechanism of neuron intoxication of TeNT (49). The BoNT-switch is composed of disordered loops
190 and three short helices (α_A , α_B , α_C) that are also present in TeNT (Figure S7); TT110-Fab binds to a
191 region in TeNT corresponding to the α_B of BoNT serotype A1 (BoNT/A1) (Figure S8). At acidic pH, the
192 BoNT/A1-switch rearranges into five β strands (dubbed β_1 – β_5) with α_A flipping out from the toxin
193 structure at the center of an elongated hinge formed by β_2/β_3 hairpins (corresponding to residues
194 I630–Y648 in BoNT/A1 and to V639–Y657 in TeNT). This hinge was shown to insert into the lipid
195 bilayer and to be essential for the subsequent membrane translocation of the L domain of BoNT/A1
196 (49). At the same time, α_B and α_C form the β_4/β_5 hairpin with a hydrophobic surface generated by
197 the structural rearrangement. Considering that TT110-Fab binds to α_B , it is very likely that it
198 neutralizes TeNT by interfering with the low pH driven insertion of HN into the membrane, thus
199 blocking the translocation of the L domain into the cytosol.

200

201 ***Structure of [TeNT-HC]-[TT104-Fab].***

202 The map of the portion of the complex including TT104-Fab and the HC-N and HC-C domains
203 (residues 875-1110 and 1111-1315, respectively) has an overall resolution of 3.9 Å. The area of
204 interaction of the antibody with the HC-C domain is opposite to the HN and L domains and includes
205 portions of polypeptide strands 1140-1145, 1149-1157, 1171-1173, 1202-1204 and 1276-1281
206 (Figure 3B). From the Fab side, the interaction involves mainly the V_H chain whose surface area
207 buried upon the binding is 555 Å², whilst the buried area of chain V_L is 396 Å². This difference is
208 reflected in the number of interactions. V_H residues form one salt bridge and seven potential
209 hydrogen-bonds, whilst V_L contributes to stabilize the protein-protein interaction with additional
210 four hydrogen bonds (Supplementary Table 1, calculation performed with server PISA) (48). The
211 total TeNT buried surface in the complex with TT104-Fab is 792 Å².

212 The TT104-Fab binding site is only 12 Å away from the putative nitrogen binding site modelled
213 previously (14) and its proximity suggests a possible interference with the binding of nitrogen to HC-
214 C due to a steric clash (Figure S6). At the same time, this binding might alter the ability of HC-C to
215 interact with the oligosaccharide portion of PSG, which projects out the neuronal plasma membrane
216 and has to be accommodated into the HC-C to mediate binding (17). These possibilities have been
217 tested experimentally (see below).

218

219 ***Model of the overall structure of the immunocomplex.***

220 Owing to the intrinsic flexibility of the complex in solution, it was not possible to obtain a complete
221 3D map of the entire immunocomplex. Nevertheless, thanks to the available crystal structure of
222 TeNT (8, 39), we built a model of the ternary complex by superimposing the L, HN, HC-N and HC-C
223 domains to the TeNT crystal structure (Figure 3C). The flexibility of the TeNT-Fabs complex observed
224 in our images is intrinsic to TeNT (8) and, accordingly, this model represents one of the possible
225 conformations of the toxin with the two Fabs bound. We detected several eigenvalues (Figure S5B
226 and C) that define the dynamics of the motion of the L-HN domains relative to the HC-N and HC-C
227 domains. All the eigenvalues are unimodal, suggesting that these motions are continuous. The first
228 three eigenvalues describe 85% of the variability present in the dataset. The movies of the
229 reconstructed body repositioned along these eigenvectors reveals that the two domains of TeNT
230 have a rocking motion (Supplementary movies 1, 2 and 3). Interestingly, the conformation of TeNT
231 in the immunocomplex is closer to the open conformation of the full-length toxin in which the C869-
232 C1093 disulphide bond was reduced (39) than to that of the non-reduced TeNT (8).

233

234 ***TT104-Fab inhibits the biological activity of TeNT by preventing membrane binding.***

235 TT104-Fab prevents cleavage of VAMP in CGNs and the development of tetanus in mice (Figure 5A
236 and B). The TeNT-TT104-Fab interaction suggests that this Fab should interfere with toxin binding.
237 As detected using a fluorescently labelled HC fragment (A555-TeNT-HC, red signal in Figure 5C and
238 D), TT104-Fab prevents the binding of TeNT both to CGNs and to the neuromuscular junction (NMJ).
239 TT104-Fab inhibition is very specific, as indicated by the lack of interference with the binding of a
240 fluorescent HC of BoNT/A1 (CpV-BoNT/A1-HC, green signal in Figure 5C and D). Of note, the signal
241 of CpV-BoNT/A1-HC is slightly brighter in the presence of TT104-Fab, which sequesters A555-TeNT-
242 HC preventing the competition of the two toxin HCs for PSG binding (green signals in Figure 5C and
243 D).

244 To elucidate the specific mechanism responsible for the TT104-Fab-mediated inhibition of TeNT
245 binding, we assayed the interaction of TeNT with immobilized GT1b, purified nidogen-1 and -2, or
246 their combinations. Figure 5E shows that TeNT binding to GT1b is completely prevented by TT104-
247 Fab, whilst binding to nidogens is strongly reduced. This result is in line with the definition of the
248 surface of the conformational epitope recognized by the idiotype of TT104-Fab. Interestingly, when
249 GT1b and nidogens are immobilized together (Figure 5E, right panel), TeNT binding in the presence
250 of TT104-Fab compares to that reached when only nidogens are present. These findings support the
251 model of nidogen-TeNT interaction occurring at a distinct site with respect to that of PSG (14), and
252 indicate that TT104-Fab interferes with the binding of both receptors. At the same time, these
253 results clearly indicate that membrane binding is the specific step inhibited by TT104 and TT104-
254 Fab, with a consequent strong anti-tetanus activity *in vivo*.

255
256 ***TT110-Fab inhibits the low pH induced conformational change of TeNT.***

257 Although less powerful than TT104-Fab, also the HN-specific TT110-Fab effectively prevents the
258 cleavage of VAMP by TeNT in cultured neurons (Figure 6A) and protects mice from a TeNT challenge
259 when the toxin and the Fab are pre-incubated together before i.p. injection (Figure 6B). As discussed
260 before, the structure of the immunocomplex suggests that TT110 interferes with the HN-dependent
261 membrane translocation of the L chain driven by acidification. After neurotransmitter release, SV
262 are endocytosed and acidified by the vacuolar ATPase, a process necessary for neurotransmitter re-
263 loading. TeNT inside SV in central neurons (21) exploits this physiological process to change
264 structure with exposure of hydrophobic patches on HN that mediate its membrane insertion in such
265 a way as to assist the membrane translocation of the L domain (50). As this process cannot be
266 experimentally accessed from the outside of the cells, we took advantage of a method previously
267 devised to induce the low pH membrane translocation of the L chain directly from the plasma
268 membrane into the cytosol (50-53). As schematized in Figure 6C, after TeNT binding to neurons at
269 0° C, membrane translocation is induced by replacing the cold medium with acidic medium at 37°C
270 for few minutes. Neurons are then incubated in control medium in the presence of bafilomycin A1,
271 a v-ATPase inhibitor preventing toxin entry through the canonical route, and L domain translocation
272 is assessed by determining VAMP-2 cleavage. Figure 6D shows that upon exposure to pH 5, the L
273 metalloprotease enters the cytosol and efficiently cleaves VAMP-2. In contrast, when TeNT is pre-
274 incubated with TT110-Fab, VAMP-2 is no longer cleaved, consistent with a block of translocation.

275 To test the possibility that TT110-Fab prevents the low pH-induced structural change of TeNT (51,
276 54, 55), we performed a fluorometric assay based on the binding of the lipophilic dye ANS to
277 hydrophobic protein patches (56). Figure 6E shows that TT110-Fab prevents TeNT from undergoing
278 the low pH-driven conformational change with exposure of hydrophobic surface patches. This result
279 supports the hypothesis that TT110 blocks the “BoNT/TeNT-switch” of HN and prevents the
280 occurrence of the low pH-driven insertion of HN and the following membrane translocation of the
281 L domain. Considering these results and the available literature, we propose that the protonation
282 of Asp618, Asp621, Asp622, Glu626, Glu658, Glu666 of TeNT (Figure S8), is likely to be the key initial
283 event of the low pH conformational transition of HN leading to its membrane insertion. Future
284 mutagenesis experiments targeting these residues will provide conclusive evidence on the role of
285 these acidic sites in the molecular pathogenesis of tetanus.

286 Collectively, the above experiments demonstrate that TT110-Fab and TT104-Fab act on two
287 different steps of the process of TeNT nerve terminal intoxication, predicting that they should
288 display additive effects in preventing experimental tetanus *in vivo*.

289

290 ***Prophylaxis and therapy of experimental tetanus by TT104, TT110 and their Fabs.***

291 The most frequently used protocol of testing the neutralizing activity of anti-TeNT antibodies
292 involves their pre-incubation with TeNT. This procedure does not match the conditions of injured
293 patients where IgG anti-TeNT are used as tetanus prophylactic agents before the toxin is produced
294 by *C. tetani* in necrotic wounds. Therefore, we performed a set of experiments in which mice were
295 pre-treated with a single intraperitoneal injection of TT104 and TT110, alone or in combination,
296 before inoculating 5 MLD₅₀ of TeNT at delayed time points. Since tetanus usually develops with
297 incubation times ranging between 2 and 15 days after injury (1, 2), animals were pretreated with
298 the TT104 and TT110 alone (400 ng/kg each) or in combination (200 ng/kg+200 ng/kg) 7 and 15 days
299 before TeNT injection (Figure 7A). As a control, we pretreated a cohort of animals with TIG (7 IU/kg,
300 corresponding to the standard prophylaxis with 500 IU used in a human of 70 kg). Remarkably,
301 TT104 provided full protection of mice from tetanus, even when injected 15 days before TeNT
302 (Figure 7B). TT110 was less effective since it was fully protective only when challenge was performed
303 on day 7 (Figure 7C). The combination of 200 ng/kg of TT104 and of 200 ng/kg of TT110 provided a
304 full protection of mice for 15 days, as TIG did (Figure 7D).

305 Once the metalloprotease domain of TeNT has been released inside the cytoplasm of target
306 neurons, the toxin cannot be neutralized any longer by anti-TeNT antibodies. However, worsening

307 of the symptoms can be prevented by neutralization of circulating TeNT, and this is the reason
308 supporting the general practice of injecting TIG into hospitalized patients showing symptomatic
309 tetanus. Thus, TIG and TT104 or TT110 were compared in a therapeutic setting for their capacity to
310 interfere with tetanus at different time points after TeNT challenge. We opted to use the Fab
311 derivatives in consideration of their possible use by intrathecal rather than peripheral
312 administration. To properly compare the Fabs and TIG, we estimated the concentration of anti-
313 tetanus specific IgG present in the standard dose of 500 IU (in a putative patient of 70 kg) based on
314 previous quantifications on the serum of hyper-immunized human donors (57). As shown in Figure
315 7E, TeNT inoculation (4 ng/kg) was followed by injection of either TIG or the corresponding amount
316 of TT104-Fab and TT110-Fab in combination after different time periods (1.5, 3, 6 and 12 hours).
317 Table 1 shows that the Fab combination completely protected mice, as TIG did, up to 6 hours after
318 injection of TeNT. However, both forms of serotherapy did not block the toxin completely when
319 injected 12 h after TeNT challenge, in agreement with what is known on the kinetics of TeNT
320 internalization into neurons (5). Notably, in this case, mice developed tetanus symptoms with a
321 similar time course (Figure 7F), further indicating that the Fab combination and TIG are comparably
322 effective in neutralizing the activity of the toxin present in body fluids.

323

324 **DISCUSSION**

325 Here we report the structural and functional characterization of two human monoclonal antibodies
326 that prevent tetanus by neutralizing TeNT at nearly stoichiometric antibody:toxin ratios, a property
327 maintained by their Fabs. TT104 interacts strongly with the HC-C domain that mediates TeNT
328 binding to neurons, whilst TT110 binds the HN domain that mediates the translocation of the
329 metalloprotease domain into the neuronal cytosol.

330 The structure of the [TeNT]-[TT104-Fab]-[TT110-Fab] trimeric immunocomplex has been resolved
331 by cryo-EM and the two TeNT epitopes were identified. This information explains why these
332 antibodies are so potent in preventing tetanus in mice, also after being administered up to 15 days
333 before TeNT. These results are even more relevant considering that the lifetime of human IgGs in
334 mice is expected to be lower than that in humans (58, 59), leading to the prediction that the duration
335 of the great protection exerted by TT104 and TT110 will be even longer in humans. In addition, it
336 should be considered that these antibodies could be engineered to achieve extended half-life and
337 bio-distribution (60). Remarkably, TeNT neutralization was effective in the ng/kg range, a dose

338 orders of magnitude lower than the mg/kg range of TIG, offering the possibility of increasing the
339 dose, if necessary.

340 To the best of our knowledge, this is the first report showing such a potent and long-lasting
341 prophylactic effectiveness of anti-TeNT human monoclonal antibodies, which fully meets the needs
342 of patients entering hospital emergency rooms with necrotic wound(s) possibly contaminated by *C.*
343 *tetani* spores. TT104 and TT110 are expected to be very effective also in preventing maternal and/or
344 neonatal tetanus in the case of delivery from a mother not immunized against tetanus, a frequent
345 condition in several parts of the world (28).

346 The current serotherapy with IgG isolated from the blood of hyperimmune donors, is affected by a
347 number of drawbacks: i) variation of neutralizing power from lot to lot; ii) possible risk of
348 contamination with unknown viruses or with blood proteins, iii) need of injecting relatively large
349 amounts of proteins (typically 16 – 20 % w/v), which may elicit adverse reactions such as
350 angioedema or anaphylaxis, iv) high risk of anaphylactic reactions or serum sickness in the case of
351 horse antisera, v) in addition, patients with IgA deficit could mount an immune response to the small
352 amount of IgA present in TIG. Pain at the site of injection or fever, dizziness and other side effects
353 are not uncommon. All these problems could be overcome by the injection of small amounts of well
354 characterized monoclonal antibodies, such as those identified in this study, or even a single one as
355 exemplified by TT104. Our findings also pave the way to the use of Fab fragments via the intrathecal
356 route. This procedure was found to provide better results than peripheral injections when the toxin
357 has already undergone retroaxonal transport and has been released into the spinal cord but is
358 strictly limited by the amount of antibody protein that can be safely injected intrathecally (30, 31).

359 The isolation of anti-TeNT humAbs from human B memory cells was recently reported (39-42). TeNT
360 neutralization in mice could be achieved only by preincubation of the toxin with a combination of 3
361 antibodies and/or using high molar excess of humAbs with respect to TeNT. As in the present study,
362 the highest neutralizing effect was provided by antibodies specific for the HC binding domain of
363 TeNT. This notion agrees with the numerous reports about the activity of monoclonal antibodies
364 specific for the HC portion of TeNT isolated from mice, or prepared after humanization procedures,
365 or by scFv antibody phage display library (61, 62). Together with our findings with TT104, these data
366 fit with the rather general rule that neutralizing antibodies interfere with pathogen binding to its
367 receptor. However, it is interesting to note that the strong neutralizing activity of TT110 indicates
368 that antibodies can also effectively neutralize TeNT by blocking its membrane translocation.

369 Nine humAbs identified in the present study recognize the HC-N domain, 3 humAbs bind the HC-C
370 domain and 3 bind the HN domain. These results suggest that the most immunogenic domain of
371 TeNT is HC-N, but this possibility calls for more extensive studies. In addition, the fact that HC-N
372 specific humAbs have negligible neutralization activity suggests a structural role for the HC-N
373 domain. This interpretation is in line with the result of Masuyer et al., who showed a direct
374 interaction at low pH of the L and HC-C domains, but not with the HC-N (8).

375 In addition to the biomedical properties of the two anti-TeNT humAbs, the structure of the
376 immunocomplex formed by the toxin with the two Fab derivatives brought novel information on
377 two key steps of the mechanism of nerve terminal intoxication by TeNT. The first insight is about
378 the sequential events leading to a productive binding of TeNT to the presynaptic membrane at the
379 NMJ. TeNT binds both a PSG molecule and the protein nidogen-1/2, but the sequence of binding
380 events is not known. Nidogens are particularly enriched in the NMJ basal lamina which enwraps
381 perisynaptic Schwann cells and the nerve terminal secluding them from the muscle fiber. Nidogens
382 are in a strategic position to capture TeNT molecules entering the NMJ, because they are exposed
383 to the perineural extracellular fluids, yet they are engaged in multiple protein-protein interactions
384 with other basal lamina components, which may limit their availability for TeNT fixation. PSG are
385 glycolipids located on the outer leaflet of the presynaptic membrane, which are endowed with long
386 and flexible headgroups projecting above the plasma membrane. Our data show that TT104 fully
387 disrupts TeNT interaction with PSG but only partially with nidogens, yet this is sufficient to abrogate
388 TeNT binding and internalization at nerve terminals and to prevent tetanus. This strongly suggests
389 that the interaction with PSG is a prerequisite for TeNT toxicity and it is the first step of TeNT binding.
390 Nidogens might then drive the TeNT-PSG complex towards the specific endocytic organelle leading
391 to the formation of signaling endosomes (63).

392 Experiments with TT110 provided new insights on the mechanism of TeNT entry into nerve
393 terminals. TT110-Fab was found to prevent the low pH-driven conformational change of HN by
394 binding to an epitope at the center of an important structural-functional module, the BoNT-switch,
395 previously identified in BoNT/A1, which was suggested to be the pH sensor triggering the initial
396 event of HN membrane insertion (49). The present work indicates that such a structural switch also
397 occurs in TeNT. In addition, the TeNT epitope recognized by TT110 identifies a group of carboxylate
398 residues that are candidates for future studies aimed at determining their role in the TeNT low pH-
399 triggered switch for membrane insertion.

400 The results described here qualify the humAbs TT104, TT110 and their Fabs derivatives as novel
401 therapeutics highly superior to the presently used anti-tetanus immunoglobulins. These humAbs
402 are ready, after appropriate formulation, to replace the immunoglobulins purified from human or
403 equine blood in clinical practice. Their Fab derivatives open new avenues to the treatment of
404 tetanus patients because large doses could be injected intrathecally, thus maximizing the ability of
405 these novel therapeutics to neutralize TeNT in the very area of its action, i.e. the spinal cord.

406

407 **METHODS**

408 ***Human monoclonal antibodies and Fab fragments***

409 30-50 x 10⁶ peripheral blood mononuclear cells (PBMCs) were isolated from adult donors who had
410 received a boost dose of tetanus toxoid vaccine at least one year before sampling. Memory B cells
411 were isolated from PMBCs using magnetic cell sorting with 0.5 µg/ml anti-CD19-PECy7 antibodies
412 (BD, 341113) and mouse anti-PE microbeads (Miltenyi Biotec, 130-048-081) followed by FACS
413 sorting using 3.75 µg/ml Alexa Fluor 647–conjugated goat anti-human IgG (Jackson
414 ImmunoResearch, 109-606-170), 5 µg/ml Alexa Fluor 647–conjugated goat anti-human IgM
415 (Invitrogen, A21215) and PE–labeled anti-human IgD (used at 1:40 dilution; BD Biosciences,
416 555779). Sorted B cells were immortalized with Epstein–Barr virus (EBV) and plated in single-cell
417 cultures in the presence of CpG-DNA (2.5 µg/ml) and irradiated PBMC-feeder cells, as previously
418 described (35). Two weeks post-immortalization, the culture supernatants were tested (at a 2:5
419 dilution) for binding to TeNT by ELISA. Briefly, ELISA plates were coated with 1 µg/ml of TeNT. Plates
420 were blocked with 1% BSA and incubated with titrated antibodies, followed by 1/500 alkaline
421 phosphatase (AP)-conjugated goat anti–human IgG (Southern Biotech, 2040-04). Plates were then
422 washed, substrate (para-nitrophenyl phosphate (p-NPP, Sigma) was added and plates were read at
423 405 nm.

424 Recombinant Fab fragments were produced in HEK-293 cells by introducing a stop codon at the end
425 of C_H1 and purified by affinity chromatography on AKTA Xpress Mab System (Cytiva) with UNICORN
426 5.11 software version (Build 407) using CaptureSelect C_H1-XL MiniChrom columns (ThermoFisher
427 Scientific), buffer exchanged to PBS using a HiPrep 26/10 desalting columns (Cytiva). Purified Fabs
428 were concentrated by Amicon Ultra filter units (Millipore), sterilized through a 0.22 µm filter and
429 stored at -80°C after rapid freezing in liquid N₂.

430

431 ***Cryo-EM data collection and processing***

432 A first screening of the sample and its analysis with Cryo-EF (64) suggested a strong particle's
433 orientation (data not shown). To overcome this problem, data were acquired on a planar orientation
434 and 30° tilted one. For the planar acquisition, 3 ml of the sample at 0.5 mg/ml concentration, were
435 applied to glow-discharged C-flat 2/1 -3 Au holey grid and vitrified in a Mark IV Vitrobot (FEI),
436 whereas, for the tilted acquisition, 3 µl of the sample at 0.35 mg/ml concentration were applied to
437 glow-discharged Au/trafoil 1.2/1.3 grid. Both grids were imaged in a Titan Krios microscope (Thermo
438 Fisher Scientific) at 300 keV with a K2 direct electron camera at 0.827 Å per pixel. The planar dataset
439 is composed by 402 movies (40 frames each and a dose of 1.2 e⁻/Å²) and was combined with the
440 tilted dataset containing 2970 movies (50 frames each with 0.68 e⁻/Å²) after beam-induced motion
441 correction with Motioncor2 (65) and Contrast transfer function (CTF) estimated with Gctf (66). The
442 selected micrographs were picked and analyzed in RELION-3 (67). An initial model generated with
443 EMAN2 (68) was used for the 3D classification and refinement. An analysis of the 2D classes and of
444 the obtained 3D model suggests the presence of some mobility of the domains of the toxin. To
445 overcome this problem, a multibody refinement procedure was performed (69). This refinement
446 gives twelve motion eigenvalues with the first three that explain the 85% of the variance in the data.
447 These eigenvalues allow one to align and subtract the signal of the two domains of the protein and
448 to perform a local reconstruction of the two main flexible regions (70, 71) (see workflow in Figure
449 S5). This protocol yielded a 3.9 Å resolution map that matches to the density of the HC domain and
450 the TT104-Fab formed by 98170 particles, and a 8 Å low-resolution map of the LC-HN domains
451 bound to TT110-Fab.

452

453 ***HumAbs subdomain specificity***

454 TeNT (0.5 µg), reduced TeNT (0.5 µg in 15 mM of dithiothreitol), TeNT-HC (1 µg) or the individual
455 subdomains (HC-C and HC-N) were separated by SDS-PAGE and transferred on nitrocellulose
456 membranes. After saturating for 1 hour with 5% BSA in PBS containing 0.5% of Tween (PBS-T),
457 membranes were incubated (overnight at 4°C) with HumAbs diluted in PBS-T (1 µg/ml) used as
458 primary antibodies. After washing, membranes were incubated with anti-human secondary
459 antibodies conjugated to HRP for 1 hour and then revealed with an Uvitec gel documentation
460 system (Cleaver Scientific) using Luminata Crescendo (Millipore) as substrate.

461

462 ***TeNT neutralization assay on primary neuronal cultures***

463 Primary CGNs were prepared from 4- to 6-day-old rats as previously described (44). CGNs at 6–8
464 days in vitro (DIV) were treated with TeNT (50 pM) alone or pre-incubated for 1 hour at RT at the
465 indicated molar ratios of TeNT:humAbs or TeNT:Fabs in complete BME. After 12 hours, CGNs were
466 lysed with Laemmli sample buffer containing protease inhibitors (Roche) or fixed for 10 min with
467 4% (w/v) paraformaldehyde in PBS. Neutralization was determined by western blotting monitoring
468 the cleavage of intact VAMP-2 (Synaptic System, 104 211) and SNAP-25 (Biolegend, SMI81) as
469 loading control (44).

470 Fixed CGNs were analyzed by immunofluorescence staining with primary antibodies specific for
471 intact VAMP-2 (Synaptic System 104 211) detected with a fluorescent secondary antibody (44). DAPI
472 stained the nuclei. Coverslips were mounted using Fluorescent Mounting Medium (Dako) and
473 examined with a Leica SP5 confocal microscope (Wetzlar, Germany).

474

475 ***Internalization of fluorescent HC derivatives.***

476 BoNT/A-HC-CpV (50 nM) and TeNT-HC-555 (50 nM) were mixed for 1 hour at 37°C in complete BME
477 with or without TT104-Fab (30 nM), and then added onto CGNs 6-8 DIV seeded on glass coverslips.
478 At indicated time points, cells were gently rinsed with medium and incubation stopped with 4%
479 paraformaldehyde. Coverslips were extensively washed, mounted and fluorescence examined with
480 a Leica SP5 confocal microscope.

481 In mice, 1 µg of TeNT-HC-555 and BoNT-HC-CpV were mixed in “bioassay solution” (0.9 % NaCl, 0.2
482 % gelatin). Then the solution was split and incubated for 1 hour at 37°C with either TT104-Fab (5
483 molar excess) or the corresponding volume of bioassay solution. 20 µl of either mixtures were
484 injected below the skin at the level of the *Levator Auris Longus* (LAL) of anesthetized CD1 mice. After
485 2 hours, the LALs were dissected, fixed with 4% paraformaldehyde and directly mounted in
486 mounting medium for microscopy analysis with a Leica SP5 confocal microscope.

487

488 ***Binding assay to polysialoganglioside GT1b and nidogens***

489 96-well-plates (Sarstedt, Germany) were coated with either 1 µg of GT1b (Santa Cruz Biotechnology,
490 USA) dissolved in methanol, or 250 ng of nidogen-1/2 (Bio-Techne, USA) diluted in PBS, or their
491 combinations and let to dry overnight at room temperature. Wells were washed with PBS-T, blocked
492 with 1% BSA in PBS for 1 h at RT, and the indicated concentrations of TeNT diluted in PBS, with or
493 without pre-incubation with TT104-Fab (1 hour at RT) were added for 2 hours at RT. Wells were then
494 extensively washed with PBS-T and incubated with a rabbit TeNT antiserum (Istituto Superiore di

495 Sanità, Rome, Italy) diluted in 1% BSA in PBS for 1 hour. After washing with PBST, wells were
496 incubated with appropriate secondary antibody conjugated with HRP. Wells were extensively
497 washed and 100 µL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Absorbance was read
498 at 450 nm with a microplate reader (Tecan).

499

500 ***Low pH membrane translocation of TeNT in CGNs***

501 CGNs were plated into 24-well plates at 4×10^5 density and incubated in ice-cooled complete BME
502 with either 10 nM TeNT or 10 nM TeNT preincubated with 20 nM TT110-Fab. This assay was then
503 performed as described in (51) and the activity of the TeNT L domain into the cytosol was evaluated
504 by western blotting following its VAMP-2 specific proteolytic activity as described above.

505

506 ***Fluorescence assay of ANS binding to clostridial neurotoxins as a function of pH***

507 TeNT alone or TeNT preincubated with tenfold TT110-Fab were diluted to the final concentration of
508 0.8 µM in 100 mM TRIS-citrate buffer, 100 mM NaCl, pH 7.0 in the presence of liposomes (final
509 concentration of 0.4 mM) and 50 µM ANS. The assay was then performed as previously described
510 (56).

511

512 ***Mouse bioassay***

513 Swiss-Webster adult CD1 mice (Charles River) were housed under controlled light/dark conditions,
514 and food and water were provided ad libitum.

515 In pre-incubation assays, TeNT was diluted at a concentration of 8 pg/µl in bioassay solution, split
516 into aliquots and supplemented either with an equivolume of bioassay solution (positive control) or
517 an equivolume of bioassay solution supplemented with the indicated amounts of humAbs or Fabs
518 under a gentle agitation for 1 hour at RT. Female mice (24–26 grams) were randomly injected intra
519 peritoneum with 1 µl per gram of body weight of either toxin alone or toxin-humAbs/Fabs solutions.
520 The final TeNT dose was 4 ng/kg, roughly corresponding to a fivefold lethal dose of our toxin
521 preparation.

522 For the prophylactic activity, TIG (Igantet, Istituto Grifols Poligono Levante S.A., Italy) or humAbs
523 (TT104 and TT110 alone or their combination) were diluted in bioassay solution and
524 intraperitoneally injected with the indicated dose. After 7 or 15 days, TeNT (4 ng/kg) was injected
525 intraperitoneally and mice were monitored for 200 hours, when the experiment was terminated. A
526 human endpoint was set as to when mice showed moderate tetanus symptoms (hunched back and

527 paralysis of rear limbs or disappearance of the righting reflex for 30 s), after which the animal was
528 euthanized by cervical dislocation and considered positive for tetanus. TIG was used at a dose of 7
529 IU/kg, roughly corresponding to the canonic prophylactic injection of 500 IU in a human of 70 kg.
530 To test the post-exposure effect of Fabs, TeNT (4 ng/kg) was i.p. injected. At the indicated time
531 points, either TT104-Fab in combination with TT110-Fab (1.2 µg/kg) or TIG (7 IU/kg) were i.p.
532 injected and mice monitored up to 200 hours. TIG dose was chosen to correspond to the suggested
533 treatment of 500 IU in human of 70 kg. Fab doses were calculated accordingly, estimating the
534 concentration of TeNT-specific IgGs in the serum of hyper-immunized subject equal to 40 µg/ml as
535 averaged from values reported in (57).
536 Data were plotted as Kaplan-Meier survival curves and statistic measured with the Log-rank test.
537 Each curve is representative of at least 6 animals. At each experiment at least 3 mice were treated
538 with TeNT alone. The curve for survival of TeNT alone in Figure 2D and Figure 7B, 7C, 7D and 7F
539 derive from all the data plotted together.

540

541 **Statistics**

542 Statistical analyses were carried out with GraphPad Prism. Statistical significance in ELISA assays was
543 calculated using a two-tailed unpaired t tests. The curves of survival were compared using the
544 Mantel-Cox log-rank test. In any case, results were considered significant when the *P* value was
545 lower than 0.05.

546

547 **Study Approval**

548 Our studies were carried out in strict accordance with the European Community Council Directive n°
549 2010/63/UE and approved by the local (University of Padova) and national ethic committees of the
550 Italian Ministry of Health.

551

552 **Data deposition**

553 Atomic coordinates and density maps have been deposited for immediate release as PDB ID 7OH0
554 and EMD-12890 for the HC-TT104-Fab region and as PDB ID 7OH1 and EMD-12891 for the L-HN-
555 TT110-Fab region.

556

557 **Author Contribution**

558 Conceptualization, C.M., A.L. and G.Z.; Investigation M.P., A.G., O.L., F.V., M.T. S.B, D.C., C. SF., E.K.,
559 L.O.; Data Curation, A.G. and G.Z., Resources, G.S., S.B., D.C., C. SF., E.K., L.O.; Supervision, C.M., A.L.
560 and G.Z. Writing Original Draft, C.M., A.L. G.Z., G.S. and M.P.; Writing, Reviewing and Editing, all
561 authors.

562

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570

571 **Declaration of interests**

572 A.L., L.P., C.S. and D.C. are employees of VIR Biotechnology and may hold shares in Vir
573 Biotechnology.

574

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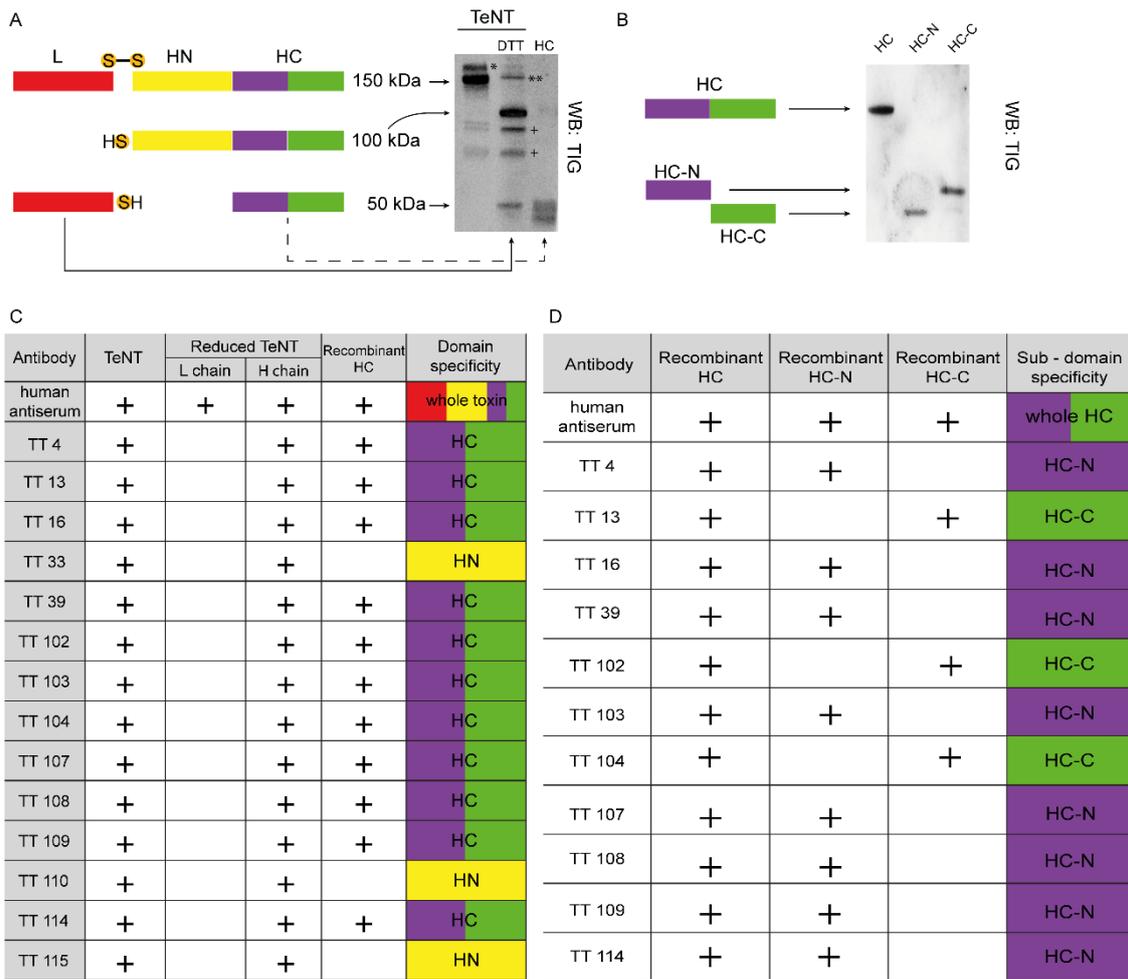
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756 **Figures and legends**

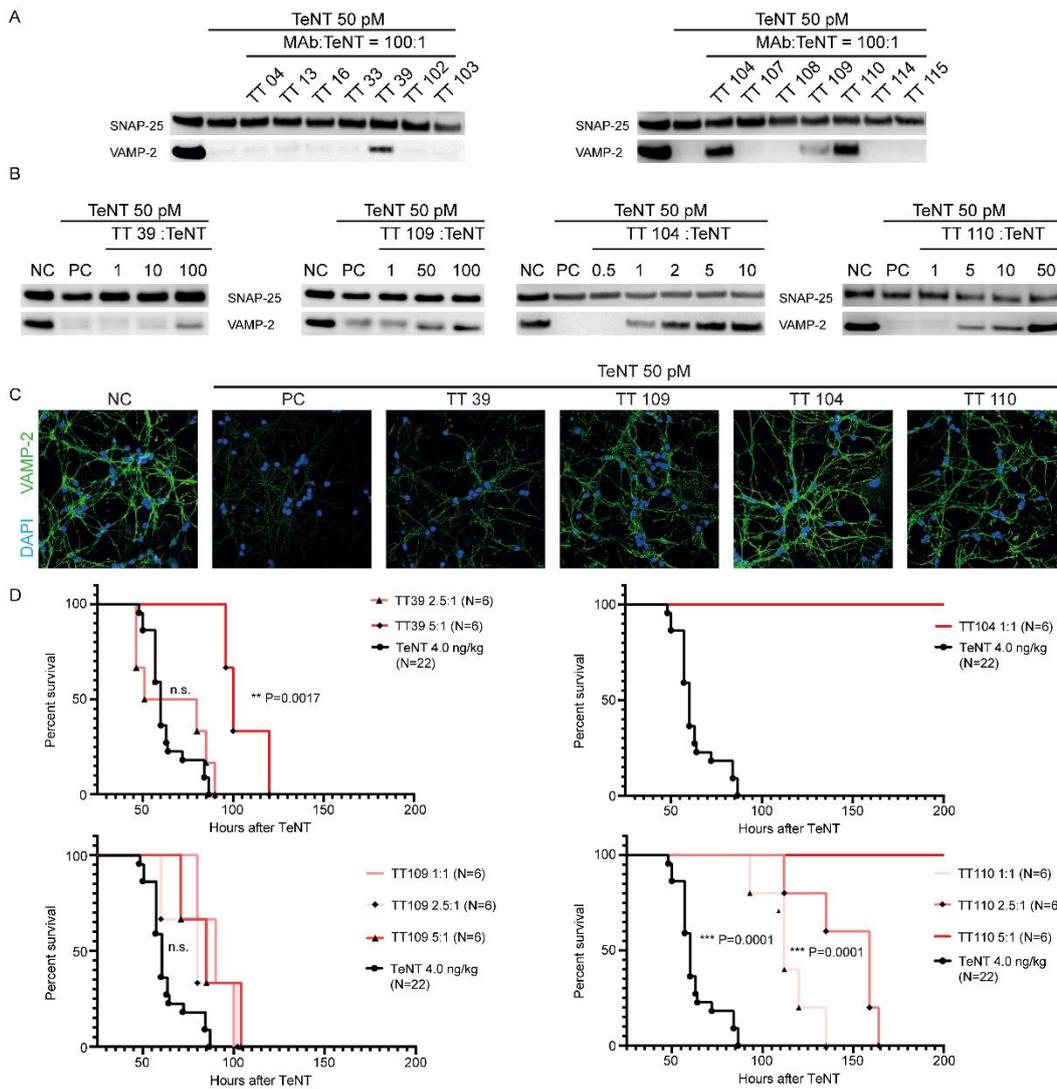


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758 **Figure 1. TeNT polypeptide chains and domain-specific recognition by TT-humAbs.** A) Schematic
 759 structure of TeNT (top), TeNT-HC (middle). At the bottom the L chain (red) and HC subdomains HC-
 760 N (violet) and HC-C (green) and as they appear in western blotting (right panel) using TIG as primary
 761 antibody. Whole TeNT has a molecular weight of 150 kDa corresponding to the L chain + H chain
 762 (left line). Reduction with dithiothreitol (DTT) generates two bands corresponding to HC (HN+HC,
 763 100 kDa) and L (50 kDa). Recombinant HC has a molecular weight of ~50 kDa. The L chain has weak
 764 signal possibly due to a low immunoreactivity of L-specific IgGs in TIG. * indicates a redox isomer of
 765 TeNT; ** indicates single-chain TeNT; + indicates degradation fragments. B) Schematic structure of
 766 HC (top), HC-N (middle) and HC-C (bottom) and how they appear in western blotting stained with
 767 TIG. C-D) Summary tables of TeNT domains and subdomains recognition by TT-humAbs as detected
 768 by western blotting. The specificity of TT-humAbs was determined with at least three independent
 769 trial per antibody.

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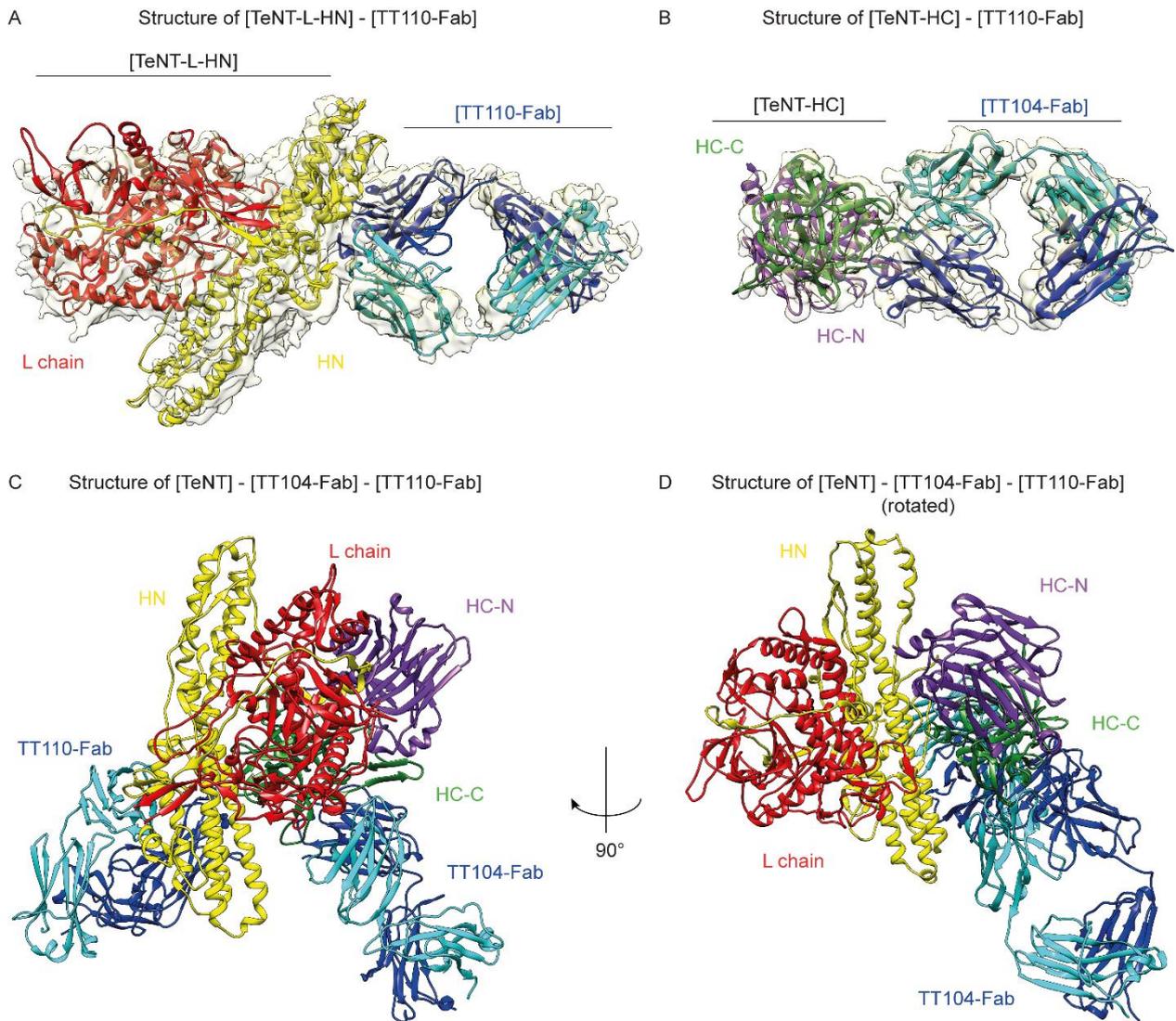
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Figure 2. Preliminary screening for TeNT neutralization by TT-humAbs assayed *in vitro* and *in vivo*.

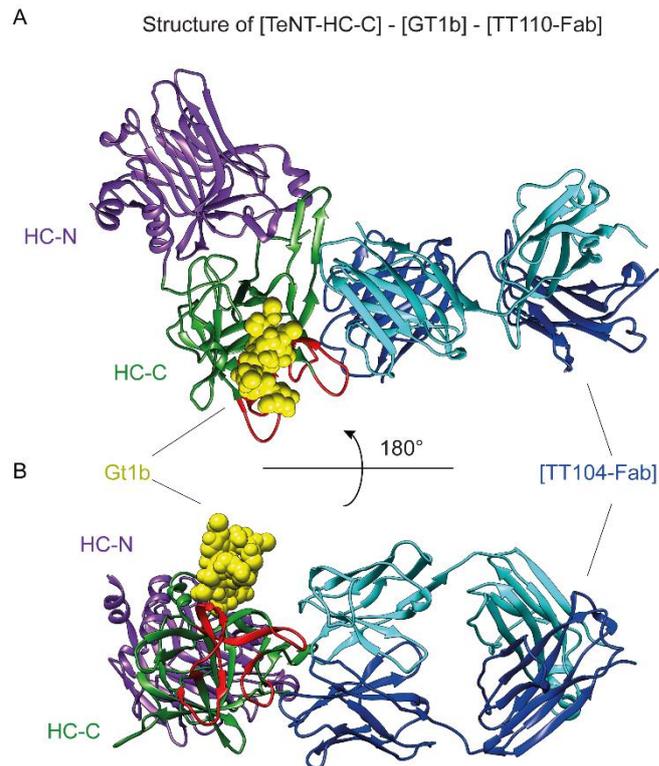
A) TeNT (50 pM) was diluted in complete culture medium alone (PC) or supplemented with a 100X molar excess of the indicated humAb. The mixture was then added to CGNs for 12 hours and TeNT activity evaluated by monitoring the cleavage of VAMP-2 with an antibody recognizes only the intact form. SNAP-25 was used as loading control. NC indicates control CGNs. B) Effect of different TeNT:humAbs ratios for the humAbs displaying toxin neutralizing activity on CGNs. C) Immunofluorescence analysis performed with an antibody specific for intact VAMP-2 (green) to assay for the TeNT neutralizing activity of TT39, TT104, TT109 and TT110 preincubated with TeNT (100:1 molar ratio) and added to the primary culture of CGNs. Control CGNs (NC) are labelled in green whereas neurons treated with TeNT alone (PC) do not display this signal due to the complete cleavage of VAMP-2. CGNs treated with the indicated humAbs display intermediate signals depending on the neutralization activity of humAbs. Images are representative of three independent experiments. D) Mice were injected intraperitoneally with TeNT (4 ng/kg, black trace) alone or pre-incubated with the indicated molar ratios humAb:TeNT and survival plotted as a function of time after toxin injection. Statistical significance was calculated with Mantel-Cox test. Group numerosity is indicated in the panels.



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790 **Figure 3. Cryo-EM structure of [TeNT-HC]-[TT104-Fab]-[TT110-Fab] ternary complex.** A) Structure
 791 of HN (yellow) and L (red) domains in complex with TT110-Fab (light and dark blue for the variable
 792 L and H chain, respectively). B) Structure of the HC-C (green) and HC-N (purple) domains in complex
 793 with TT104-Fab (light and dark blue for the variable L and H chain, respectively). C-D) Overall
 794 structure of the TeNT-Fabs complex colored as in A and B.

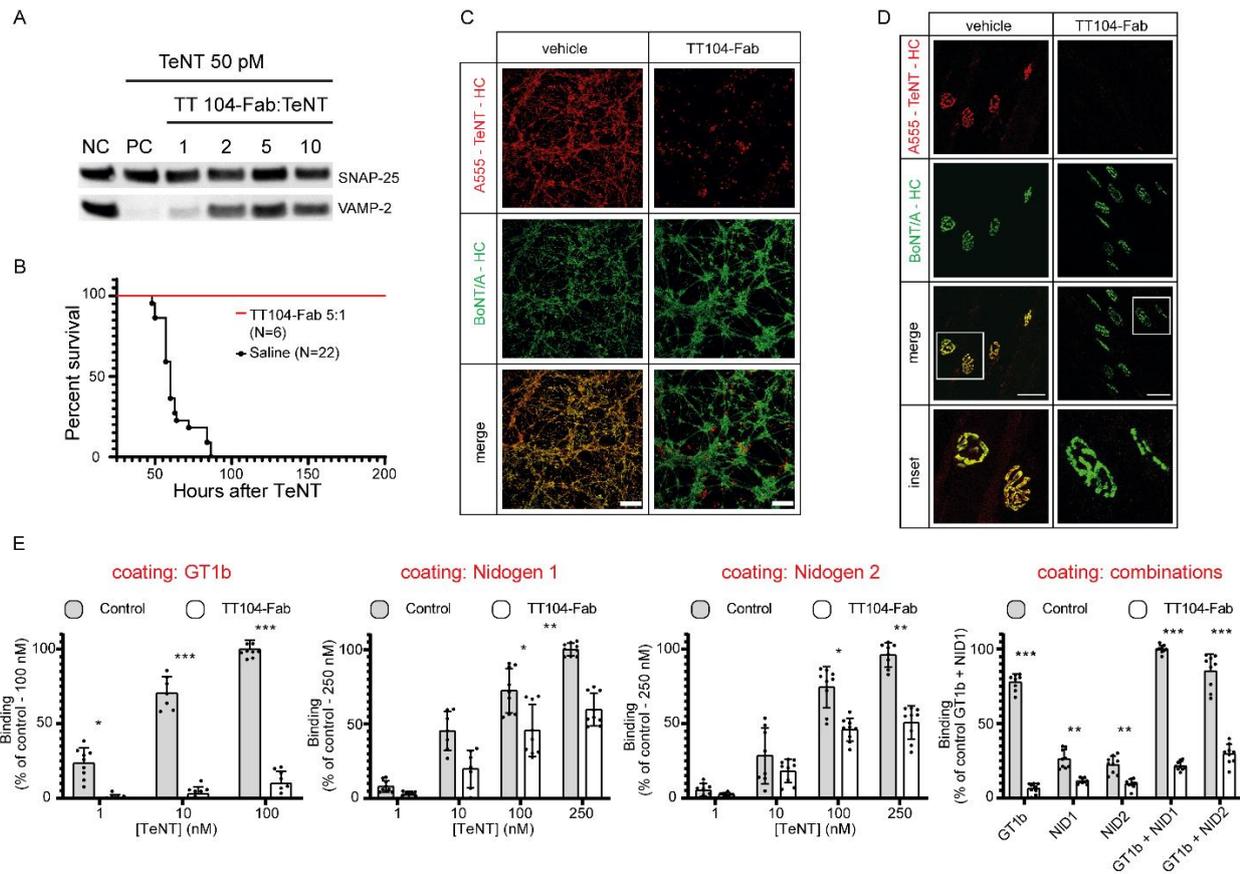
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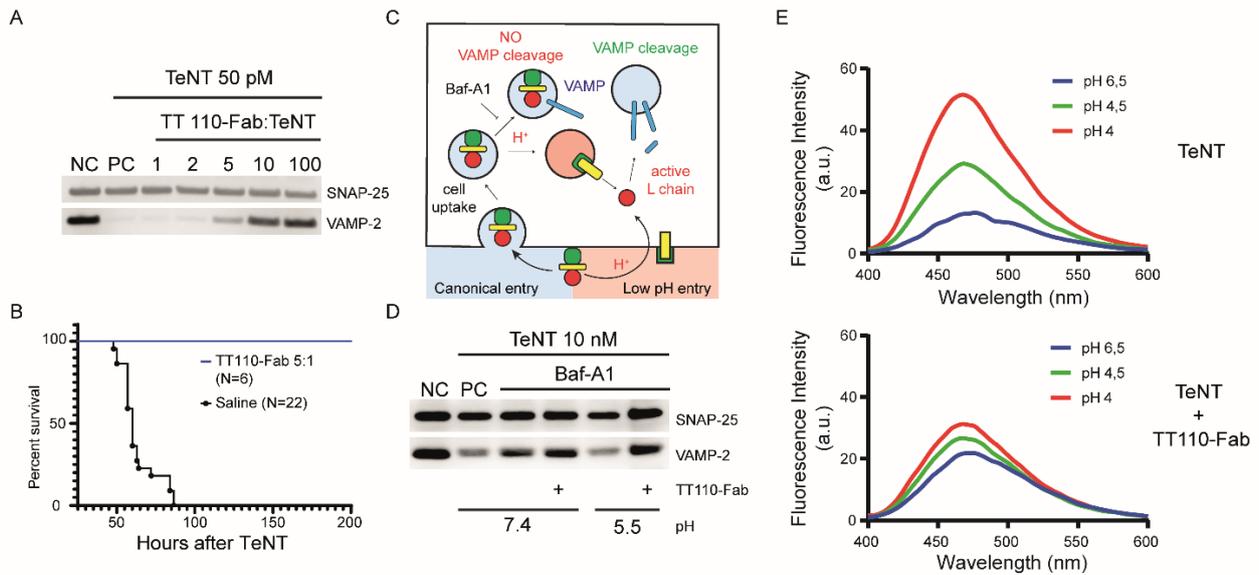
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797 **Figure 4. Model of the [TeNT-HC]-[TT104-Fab] interaction in complex with GT1b.** A) The HC-N
 798 (purple) and HC-C (green) domains of TeNT complexed with TT104-Fab (light and dark blue), and
 799 the oligosaccharide portion of the GT1b colored in yellow (bound as in pdb:1FV3). In red is
 800 highlighted the nitrogen binding region. B) View of the model after a 180° horizontal rotation.

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802
 803 **Figure 5. TT104-Fab prevents TeNT toxicity by interfering with toxin binding to**
 804 **polysialogangliosides and nidogen.** A) Western blotting analysis of CGNs treated with 50 pM TeNT
 805 alone (PC) or preincubated with the indicated TT104-Fab:TeNT molar ratios. After 12 hours, TeNT
 806 activity was evaluated by monitoring the cleavage of VAMP-2 as in Figure 2. NC indicates control
 807 CGNs. Shown is one representative out of three independent experiments. B) Survival of mice
 808 injected intraperitoneally either with TeNT alone (4 ng/kg) or premixed with 5:1 molar ratio TT104-
 809 Fab:TeNT. Group numerosity is indicated within the panel. C) Fluorescence in CGNs treated with a
 810 mixture of 50 nM A555-TeNT-HC (red) or 50 nM CpV-BoNT/A-HC (green) preincubated either with
 811 culture medium or with a 2:1 molar ratio of TT104-Fab:TeNT-HC for 2 hours and observed with a
 812 confocal microscope. Images are representative of one representative out of three independent
 813 experiments. D) Immunofluorescence staining of the *Levatoris Aureus Longus* muscle injected *in*
 814 *vivo* with A555-TeNT-HC (1 μ g) or CpV-BoNT/A-HC (1 μ g) preincubated either with vehicle or with a
 815 2:1 molar ratio of TT104-Fab:TeNT and observed after 2 hours with a confocal microscope. Images
 816 are representative of one representative out of three independent experiments. E) Purified GT1b
 817 (0.5 μ g/well, left panel), recombinant nidogen-1/2 (250 ng/well, middle right and middle left panels)
 818 or their combination (right panel) were adsorbed by overnight incubation on ELISA plates and the
 819 binding of indicated concentrations of either TeNT alone (filled columns) or TeNT preincubated with
 820 TT110-Fab (white columns) was tested as described previously (12). Data are reported as percentage
 821 of the highest value in the graph and averaged from at least three independent experiments (each
 822 dot represent a single well). Graphs show mean \pm S.D. Significance was calculated by a two tailed t
 823 test (*=P<0.05, **=P<0.01; ***P<0.001).



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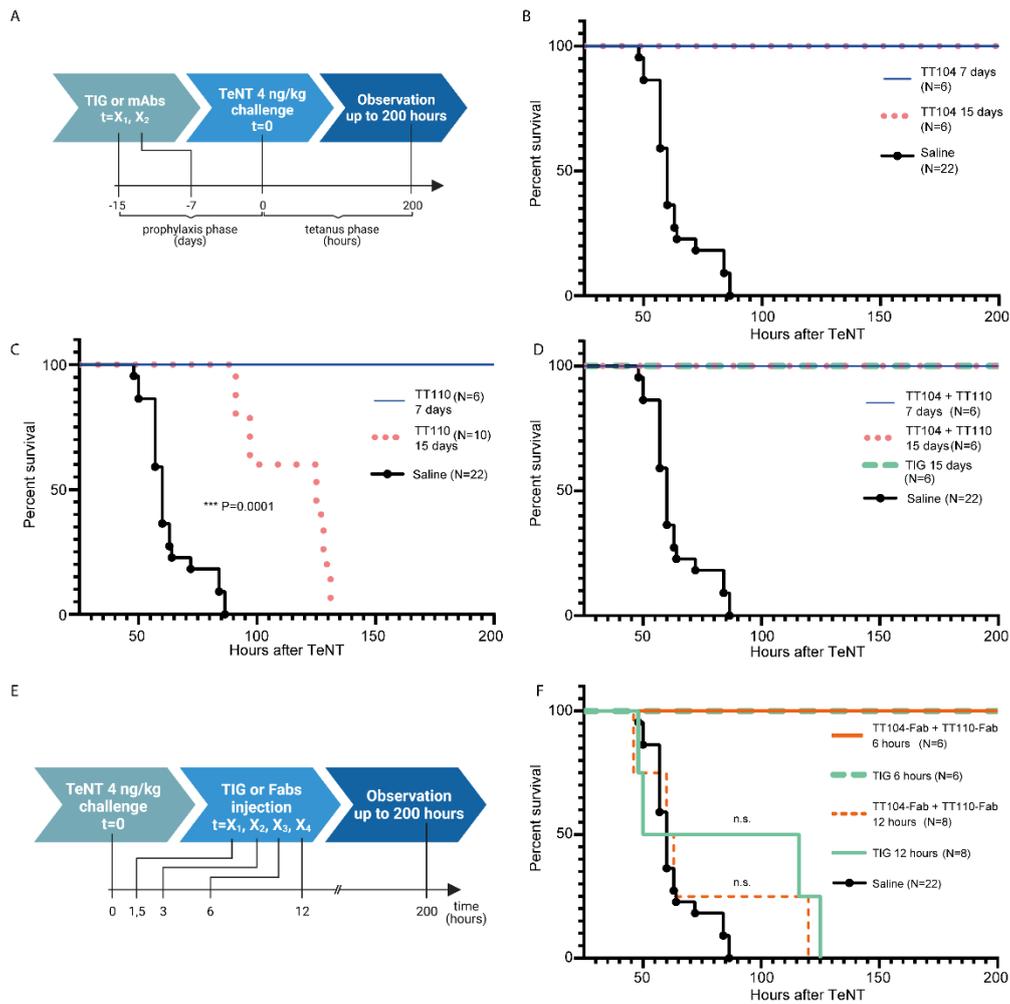
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Figure 6. TT110-Fab neutralizes TeNT toxicity by preventing the translocation of the L chain into the nerve terminal cytosol. A) Western blotting of CGNs treated with 50 pM TeNT alone (PC) or preincubated with the indicated TT104-Fab:TeNT molar ratio. After 12 hours, CGNs were lysed and immunoblotted for VAMP-2 and SNAP-25 as in Figure 2. NC indicates control CGNs. Shown is one representative out of three independent experiments. B) Survival curve of mice injected intraperitoneally with either TeNT alone (4 ng/kg) or premixed with 5X molar excess of TT104-Fab. Group numerosity is indicated within the panel. C) Scheme illustrating the entry of TeNT L chain into the neuronal cytosol via either: the canonical receptor-mediated cell uptake and translocation across the membrane of synaptic vesicles triggered by the acidification of their lumen due to the proton pump activity of the V-ATPase (light blue) or the low-pH translocation across the plasma membrane in the presence of bafilomycin A1 (Baf-A1). D) Western blotting analysis showing the inhibition of TeNT L chain membrane translocation by TT110-Fab. CGNs were incubated at 4°C for 15 minutes with either TeNT (10 nM) or TeNT preincubated with TT110-Fab. The culture medium was then replaced with a 37°C buffer for 10 minutes either at pH 7.4 or pH 5.0. Samples were then incubated for 12 hours with normal medium (PC) or normal medium supplemented with Baf-A1 (100 nM). Membrane translocation was assessed by VAMP-2 cleavage. SNAP-25 served as loading control. NC indicates control CGNs. Shown is one representative out of three independent experiments. E) ANS fluorescence binding experiment showing the pH-induced conformational change of TeNT blocked by TT110-Fab. TeNT (0.35 μM) (top) or TeNT pre-incubated with TT110-Fab (bottom) were incubated at pH 7.0 in the presence of 50 μM ANS and liposomes. The conformational change was triggered by lowering the pH with sequential addition of specific volumes of HCl and evaluated following the ANS fluorescence intensity at 470 nm. Shown is one representative out of two independent experiments.



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852 **Figure 7. TT104 and TT110 HumAbs allow a long-lasting prophylactic protection against TeNT and**
 853 **their Fabs derivatives prevent tetanus development after toxin challenge.** A) Time-course to test
 854 the prophylactic activity of humAbs. Mice were intraperitoneally pre-injected with either TT104 (400
 855 ng/kg) or TT110 (400 ng/kg) or their combination (200 ng/kg+200 ng/kg) or with TIG (3.5 IU/kg
 856 roughly corresponding to 250 IU/70 kg) for 15 or 7 days. TeNT (4 ng/kg) was then inoculated intra-
 857 peritoneally and the animals observed for 200 hours for tetanus symptoms. The prophylactic
 858 profiles for TT104 and TT110 injected alone are shown in (B) and (C), respectively. Panel D shows
 859 the survival curves of TT104+TT110 in combination compared to TIG. E) Time-course to test TeNT
 860 neutralization by Fabs in post-exposure challenge. TeNT (4 ng/kg) was inoculated via intraperitoneal
 861 injection. At indicated times, the combination of TT104 + TT110 Fab derivatives (1.2 μ g/kg) or TIG
 862 (7 IU/kg) were injected intra peritoneum and the animals observed for 200 hours. F) Survival plot of
 863 mice injected with TeNT and treated with either TT104 + TT110 Fab derivatives (1.2 μ g/kg, orange
 864 traces) or TIG (7 IU/kg, cyan traces) after 6 or 12 hours. Statistical significance was calculated with
 865 Mantel-Cox test. Group numerosity is indicated within the panels. Panels 7B, 7C, 7D and 7F display
 866 the same lethality curve for the saline group as they derive from the data of all the mice treated
 867 with TeNT alone plotted together.

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869 **Table 1: TeNT neutralization *in vivo* showing the prophylactic and therapeutic activities of**
 870 **HumAbs and Fabs.** HumAbs, TIG or Fab were injected at the indicated time points with respect to
 871 TeNT inoculation. Survival percentage was calculated based on animals surviving up to 200 hours.

Prophylactic use			
Agent	Dose	Time before TeNT injection (days)	% survival
HumAbs combination	400 ng/kg	7 days	100 %
		15 days	100 %
TIG	3.5 U/kg (corresponding to 0.6 µg/kg of specific anti-TeNT IgGs and to ~ 2.5 mg/kg of total proteins)	15 days	100 %
Therapeutic use			
Agent	Dose	Time after TeNT injection (hours)	% survival
Fab combination	1.2 µg/kg	1.5 h	100 %
		3 h	100 %
		6 h	100 %
TIG	7 U/kg (corresponding to 1.2 µg/kg of specific anti-TeNT IgGs and to ~ 5 mg/kg of total proteins)	1.5 h	100 %
		3 h	100 %
		6 h	100 %

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