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Research paper

Characterisation of tetanus monoclonal antibodies as a first step towards the development of an *in vitro* vaccine potency immunoassay

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A B S T R A C T

Batch release testing for human and veterinary tetanus vaccines still relies heavily on methods that involve animals, particularly for potency testing. The quantity and quality of tetanus antigen present in these products is of utmost importance for product safety and clinical effect. Immunochemical methods that measure consistency of antigen content and quality, potentially as an indicator of potency, could be a better choice and negate the need for an *in vivo* potency test. These immunochemical methods require at least one well characterised monoclonal antibody (mAb) that is specific for the target antigen. In this paper we report the results of the comprehensive characterisation of a panel of mAbs against tetanus with a view to select antibodies that can be used for development of an *in vitro* potency immunoassay. We have assessed binding of the antibodies to native antigen (toxin), detoxified antigen (toxoid), adsorbed antigen and heat-altered antigen. Antibody function was determined using an in-house cell-based neutralisation assay to support prior *in vivo* potency data that was available for some, but not all, of the antibodies. In addition, antibody affinity was measured, and epitope competition analysis was performed to identify pairs of antibodies that could be deployed in a sandwich immunoassay format. Not all characterisation tests provided evidence of "superiority" of one mAb over another, but together the results from all characterisation studies allowed for selection of an antibody pair to be taken forward to assay development.

1. Introduction

This work is part of the VAC2VAC project, which is a public-private consortium of 22 partners funded by the Innovative Medicines Initiative 2 (IMI2). The main objective for VAC2VAC [1] is to develop *in vitro* assays that will support regulatory acceptance of a consistency approach [2] and ultimately reduce the use of animals for batch testing as part of routine vaccine production in the future.

A range of established model vaccines were selected for the VAC2-VAC project based on the large proportion of the animals used for vaccine potency testing, the severity of discomfort in these tests or as models for complex or difficult to assay adjuvants. Established vaccine products are produced by traditional processes of inactivation or attenuation of the micro-organisms or their antigenic components (e.g. vaccines for rabies, pertussis, diphtheria, tetanus and other *Clostridia*) and may historically be less well defined than new generation vaccines, such as recombinant DNA products or sub-unit vaccines, for which consistency testing is already an accepted approach for quality control. Tetanus toxoid (TTxd) was selected as one of the target antigens as it is used widely for the immunisation of both humans and animals. Tetanus vaccine for human use, in combination vaccines containing other antigens, forms part of the routine immunisation schedule for infants in many countries. Around 86% of infants globally received three vaccine doses, containing a tetanus component, in 2018 according to WHO Global Health Observatory data [3]. Tetanus vaccines are also an integral part of preventing disease in highly sensitive animal species. Hence, they are used routinely for immunisation of horses and all small ruminants (sheep, goats, llamas and alpacas), and are also recommended for cattle to prevent bovine tetanus in areas of high risk.

The potency of the tetanus vaccine is determined for each final lot (or final formulated bulk) as part of routine batch release procedures. Although the test methodology varies in different regulatory jurisdictions, all potency tests for human and veterinary tetanus vaccines currently require the use of animals [4,5]. The high number of vaccine

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Table 1
Overview of tetanus mAbs characterised in this study.

Reagent Provider	Species	mAb ID
NIBSC	Rat	TT010
Sanofi Pasteur	Mouse	18E11.3.4
Sanofi Pasteur	Mouse	8E1-1H1.2.1
GSK	Mouse	TT1

batches produced, and the requirement to use animals for quality control testing, means that development of a non-animal potency test for tetanus vaccines could have a significant impact in terms of reducing animal use and enhancing test precision during product manufacturing and batch release.

We have previously developed a capture ELISA method (using a monoclonal antibody for capture and a polyclonal antiserum for detection) for the quantification of tetanus antigen in combination vaccines and showed that this method could be applied to a range of different vaccines licensed for use in humans [6]. As part of the VAC2VAC project, we evaluated this capture antigen ELISA for the characterisation of tetanus vaccines for veterinary use, which have qualitative differences to human vaccines as they contain different adjuvants and additional antigens not licensed for human use [7]. The results suggested that the method could also be appropriate for use in a control strategy for veterinary tetanus vaccines. However, the capture ELISA developed previously uses a polyclonal detection antibody making it more difficult to implement and validate the assay as part of a control strategy because of variability between different production batches and difficulties identifying specific antibodies in a polyclonal population that are targeting functionally relevant epitopes (i.e. epitopes to which neutralising antibodies are directed). Our aim is therefore to improve the assay by using fully characterised monoclonal antibodies for both capture and detection of the target antigen.

In this study we report the results of an extensive characterisation of a panel of four monoclonal antibodies against tetanus with a view to select antibodies that can be used for development of an immunoassay that can potentially serve as an *in vitro* potency assay for tetanus vaccine. To do this we have assessed binding of the antibodies to native antigen (toxin, TTxn), detoxified antigen (toxoid), adsorbed antigen and heat-altered antigen. Where possible, we have used representative antigen samples from two different human and four different veterinary vaccine manufacturers to assess mAb binding. Antibody function was determined using an in-house cell-based neutralisation assay. In addition, antibody affinity was measured, and epitope competition analysis was performed (using toxin) to identify pairs of antibodies that could be deployed in a sandwich immunoassay format.

2. Materials and methods

2.1. Monoclonal antibodies

Four tetanus mAb samples were characterised in this study, which were raised in either rat or mouse, from three providers in the VAC2VAC consortium (Table 1). The mAbs were from different providers, so commercial ELISA kits were used to measure the IgG concentration in mouse (Mouse-IgG ELISA, Roche) or rat (Immunoglobulin G Rat SimpleStep ELISA® Kit, abcam) to help standardise antibody concentrations in binding assays and facilitate comparisons (data not shown).

2.2. Antigen samples

2.2.1. Compliant samples

Non-adsorbed tetanus toxoid (TTxd) and bulk adsorbed tetanus toxoid (Ad-TTxd) from two human vaccine manufacturers (coded HuA and HuB) and four different veterinary manufacturers (coded A-D) were used to assess binding of the mAbs to detoxified antigen. These samples were compliant with all quality requirements for manufacturing, and representative of TTxd used to produce batches that are efficacious in clinical or field studies. The TTxd samples from the human manufacturers and veterinary company D were labelled with a concentration in Lf/ml, whereas the concentration was unknown for samples from companies A-C. For these samples an approximate Lf/ml value was obtained using an in-house capture ELISA [6] to allow suitable working dilutions to be calculated. Details of the TTxd samples are shown in Table 2. Two tetanus toxins (TTxn, from List Biologicals; product code #190B, and from HuA) were used to assess mAb binding to native toxin.

Table 2
Non-adsorbed (non-adjuvanted) and adsorbed tetanus toxoid samples used for mAb binding ELISA assays.

Manufacturer of TTxd	Sampledescription	TTxd content (Lf/ml)	Adjuvant	Adjuvant concentration (Al^{3+} mg/ml)
HuA	TTxd non-adsorbed	2500	N/A	N/A
HuB	TTxd non-adsorbed	4600	N/A	N/A
Veterinary A	TTxd non-adjuvanted	10[a]	N/A	N/A
Veterinary B	TTxd non-adjuvanted	160[a]	N/A	N/A
Veterinary C	TTxd non-adjuvanted	1400[a]	N/A	N/A
Veterinary D	TTxd non-adjuvanted	425	N/A	N/A
HuA	TTxd adsorbed	300	Aluminium phosphate	1.38
HuB	TTxd adsorbed	27	Aluminium hydroxide	1.00

2.2.2. Altered samples

Non-adsorbed TTxd samples from the human manufacturers were deliberately altered by exposure to elevated temperature to determine the impact on mAb binding. Samples were diluted in 0.9% NaCl to a concentration representative of the final vaccine products (20 Lf/ml) and then incubated for 8 weeks at elevated temperatures of +37 °C and +45 °C, with control samples held at the normal storage temperature of +4 °C.

2.3. Binding to non-adsorbed antigen (toxin and toxoid)

Direct ELISAs were performed using plates coated overnight at +4 °C with 100 μl/well of TTxn diluted to 30 μg/ml, or TTxd diluted to approximately 2 Lf/ml (based on labelled values of Lf/ml for TTxds from human manufacturers and veterinary company D, and measured values for veterinary companies A-C) in carbonate buffer, except for the TTxd from company A. This had an unusually low measured Lf/ml value and was diluted instead by a magnitude that was comparable to the other veterinary manufacturers (to give an estimated coating concentration of ~0.1 Lf/ml). Following coating, plates were washed (3x) by immersion in phosphate buffered saline containing 0.05% (vol/vol) Tween 20 (PBST), then blocked with 150 μl/well of PBST containing 2.5% (wt/vol) dried skimmed milk powder (PBSTM) for 1 h at +37 °C. Following a second wash in PBST, serial three-fold dilutions of the mAb samples in PBSTM were prepared in the plate (final volume 100 μl) from a starting concentration of 1–10 μg/ml, and the plates were incubated at +37 °C for 2 h. After further washing, bound mAb was detected using 100 μl/well of the relevant HRP-conjugated IgG diluted 1/2000 in PBSTM (rabbit anti-rat for mAb TT010 and rabbit anti-mouse for the other mAbs). After a further incubation of 1 h at +37 °C and a final wash, 100 μl/well of substrate solution containing 0.5 mg/ml ABTS and 0.008% hydrogen peroxide in 0.05 M citric acid buffer was added. The reaction was allowed to develop at room temperature for up to 30 min and the optical density was then measured at 405 nm (Molecular Devices,

Wokingham, UK).

2.4. Binding to adsorbed antigen

A modified version of the Direct Alhydrogel Formulation Immunoassay (DAFIA), established by Zhu et al. and Westdijk et al., was performed to assess binding of the mAbs to Ad-TTxd [8,9]. The assay was performed as described previously using a colorimetric readout with an HRP-labelled secondary antibody, instead of a fluorometric readout. Briefly, Ad-TTxd was diluted to 1 Lf/ml in PBST containing 5% BSA (sample buffer, SB) and titrated using two-fold dilutions in the wells of a 96 well plate (final volume 100 µl). The concentration of aluminium was kept constant by performing the titration in the related aluminium adjuvant diluted in SB. An adjuvant-only control was also included in the plate. Plates were centrifuged at 1000 g for 4 min and the supernatant was gently removed using a pipette. Plates were washed (3x) by adding 200 µl/well of PBST containing 0.2% BSA, centrifuging and removing the supernatant as before. Following washing plates were blocked with 200 µl/well SB at room temperature with agitation for 1.5 h. Plates were centrifuged and washed as described previously and mAb diluted to 1 µg/ml in SB (100 µl/well) was added for a further 1 h at room temperature with agitation. Following another centrifuge and wash step, bound mAb was detected using 100 µl/well of the relevant HRP-conjugated IgG diluted 1/2000 in SB (rabbit anti-rat for mAb TT010 and rabbit anti-mouse for the other mAbs). After a further incubation of 1 h at room temperature with agitation and a final wash step, 100 µl/well of TMB substrate was added and the plates were incubated in the dark for 5 min for the colour to develop. The reaction was stopped by the addition of an equal volume of 1 M H_2SO_4. To read the plates, 100 µl of the supernatant from each well was transferred to a clean flat bottom plate and the absorbance was measured at 450 nm.

2.5. Biosensor analysis

2.5.1. Calibration free concentration analysis (CFCA)

The active concentration of the mAbs for affinity measurements was determined on the Biacore system (Biacore T200, GE Healthcare, US). Briefly, a goat anti-mouse IgG Fc-specific antibody (Thermo Scientific) or a goat anti-rat antibody (Southern Biotech) was immobilised onto a CM5-sensorchip with a target level of 10,000 RU by primary amine coupling. A second flow cell which had been treated chemically without any anti-serum was used as the reference cell for determining non-specific binding. The mAbs were diluted to 0.5–2 µg/ml (based on IgG measurements) in HBS-P buffer, containing 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20 (GE Healthcare, US) and injected during 36s at two different flow rates (5 and 100 µl/min). The sensor chip was regenerated with 10 mM glycine-HCl, pH 1.5. The active concentration was calculated from the slope of the binding curve (assuming a 1:1 interaction model and using the molecular mass of 150 kDa for the antibody).

2.5.2. Affinity measurements

Affinity was determined using a protein G sensorchip. The mAbs were injected over the active flow cell resulting in a specific response of

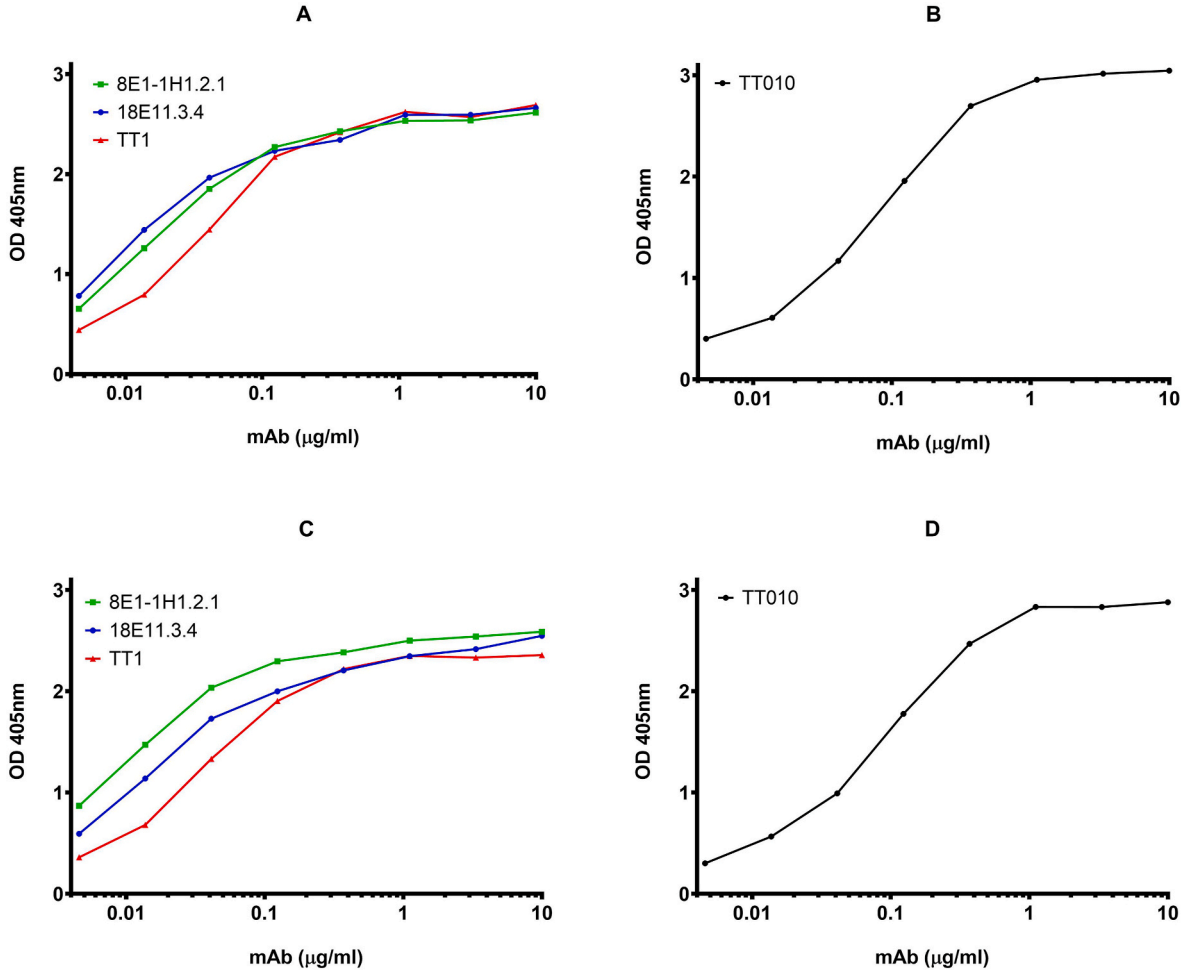

Fig. 1. mAb binding to tetanus toxin (TTxn). Data shows representative results from one of two independent assays for (A) Mouse mAbs binding to HuA TTxn; (B) Rat mAb binding to HuA TTxn; (C) Mouse mAbs binding to LIST TTxn; (D) Rat mAb binding LIST TTxn.

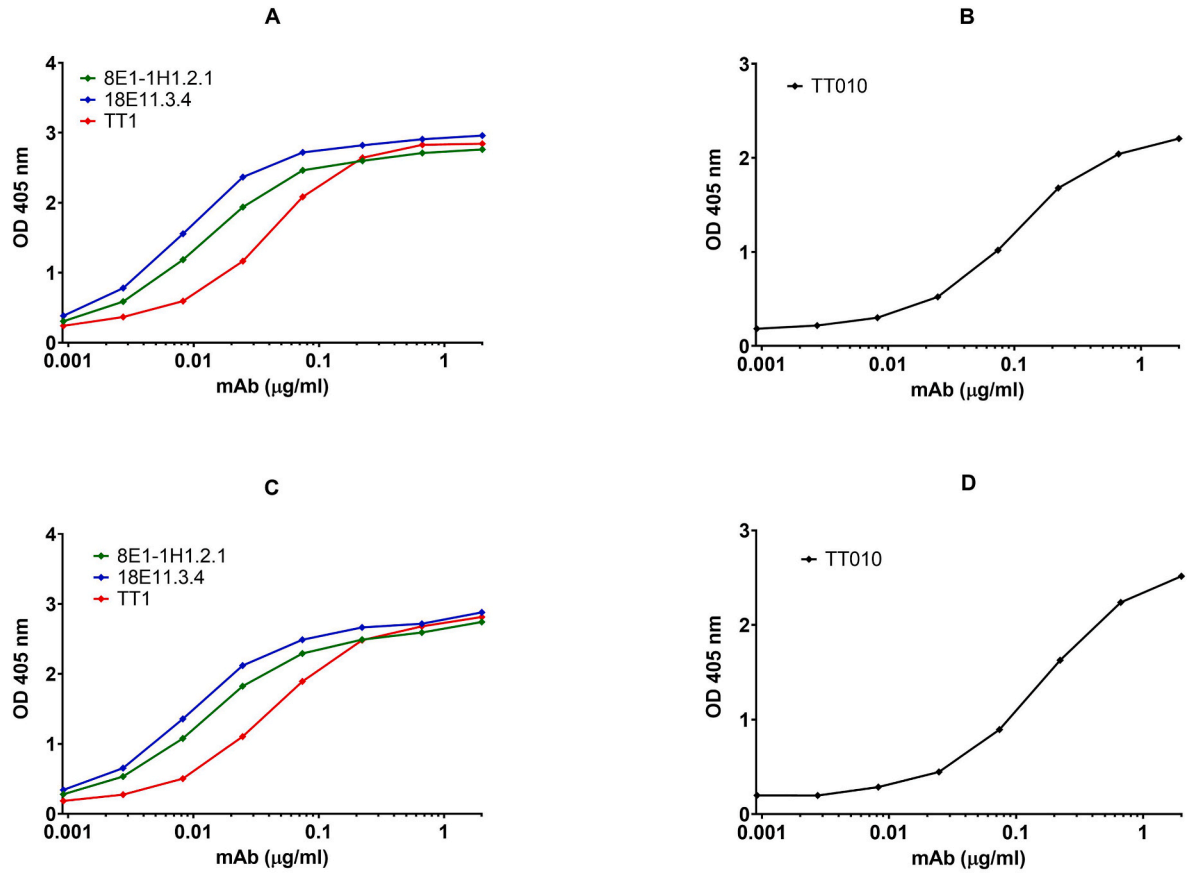

Fig. 2. mAb binding to non-adsorbed detoxified antigen (TTxd) from human vaccine manufacturers HuA and HuB. Data shows representative results from one of two independent assays for (A) Mouse mAbs binding to TTxd from HuA; (B) Rat mAb binding to TTxd from HuA; (C) Mouse mAbs binding to TTxd from HuB; (D) Rat mAb binding to TTxd from HuB.

10–20 RU. TTxn (produced by Intravacc) was diluted to concentrations of 0.6, 1.3, 2.5, 5.0 and 10 µg/ml and injected in a single cycle for 3 min per concentration (flow rate 30 µl/min). The dissociation time was 30 min after the injection of the highest concentration of TTxn. The kinetics were determined by direct curve fitting of the sensorgram to a 1:1 model interaction.

2.5.3. *Epitope competition analysis*

Epitope competition was performed using biosensor analysis with a CM5-sensorchip coupled to the relevant anti-mouse or anti-rat Fc-specific antibody (target level of 3500 RU) as described above. Subsequently, one of the mAbs was captured by the relevant immobilised antibody resulting in a response of 100–400 RU. Blocking was performed using a non-specific monoclonal antibody (ImmunoPure Mouse IgG, Whole Molecule, Thermo Fisher Scientific, US). TTxn (50 µg/mL) was then injected until a plateau level was obtained (typically in 2 min at a flow rate of 30 µl/min). The binding of a second monoclonal antibody was analysed after 2 min (flow rate 10 µl/min).

2.6. *Neutralising ability*

The ability of the mAbs to neutralise TTxn was determined using an in-house cell-based assay. This assay is not yet published and is undergoing qualification in our laboratory, and is based on an assay developed for detection of Botulinum Toxin type B [10]. For this assay, a SiMa cell line that had been engineered to stably express luciferase-linked VAMP2 protein (the target of TTxn) is used (NanoLuc-VAMP2 SiMa cells, engineered by University of Sheffield). The assay is based on the principle that tetanus toxin exercises its proteolytic effect on the luciferase-VAMP-2 protein, leaving an exposed epitope that is recognised by a specific cleavage site antibody. The degree of proteolytic cleavage of VAMP-2 is a measure of the activity of tetanus toxin. The cleavage site antibody can be used to capture the cleaved VAMP-2 protein with detection based on the fluorescent luciferase reporter protein. If a tetanus monoclonal antibody has neutralising activity, the amount of VAMP-2 cleavage will be reduced and the fluorescent signal will be reduced. NanoLuc VAMP2 cells were cultured in RPMI medium containing 10% foetal calf serum (FCS) and 5% penicillin/streptomycin (all cell culture reagents obtained from Life Technologies, unless otherwise stated) and passaged once weekly. Cells were differentiated in Neurobasal A Medium containing 1% Glutamax, 2% B27, 1% HEPES, 1% MEM non-essential amino acids and 10 µM retinoic acid (R2625, Sigma), for 72 h. Cells were seeded at a density of 1×10^5 cells/well in 48-well plates precoated with 10 µg/ml laminin (Sigma). Cells were treated for 72 h with 500 µl/well TTxn (190B, List Biologicals) alone or with TTxn that had been pre-incubated for 1 h with the mAbs diluted 1/1000 (final concentration of TTxn 1.2 nM). TTxn that had been pre-incubated with a polyclonal tetanus antitoxin (NIBSC 66/021) diluted to 0.01 IU/ml was included as a positive control. Cells were detached using cell scrapers in 100 µl/well PBS containing 0.5% Triton-X100 and 1x SigmaFast protease inhibitor (Sigma). Cell lysates were incubated for 20 min on ice with frequent vortexing, followed by centrifugation at 14,000 rpm at +4 °C for 15 min. The supernatants were removed from storage at −20 °C prior to the luciferase assay. Lysates were tested for cleaved VAMP-2 using a one-step luciferase assay. Briefly; protein A 96-well plates (Thermo) were coated overnight at +4 °C with a VAMP2 cleavage site-specific antibody (rabbit polyclonal anti-CQ8, in-house) diluted 1:100 in PBS (50 µl/well). Plates were washed with PBST for 5 min on a plate shaker at 600 rpm (3x), and then blocked with PBS containing 1% (wt/vol) BSA for 1 h at room

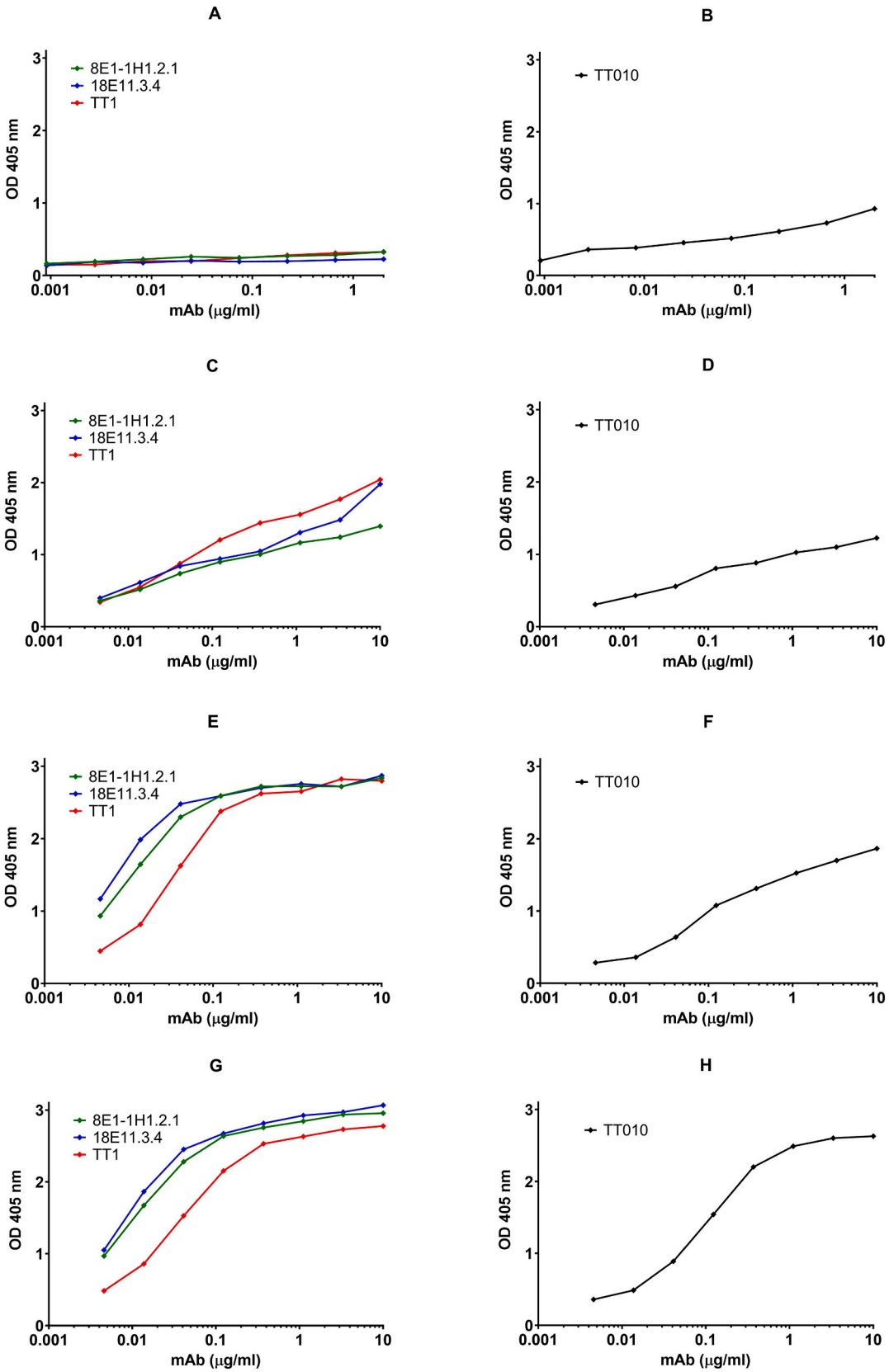

Fig. 3. mAb binding to non-adjuvanted detoxified antigen (TTxd) from veterinary vaccine manufacturers A, B, C and D. Data shows representative results from one of two independent assays for (A) Mouse mAbs binding to TTxd from veterinary company A; (B) Rat mAb binding to TTxd from veterinary company A; (C) Mouse mAbs binding to TTxd from veterinary company B; (D) Rat mAb binding to TTxd from veterinary company B; (E) Mouse mAbs binding to TTxd from veterinary company C; (F) Rat mAb binding to TTxd from veterinary company C; (G) Mouse mAbs binding to TTxd from veterinary company D; (H) Rat mAb binding to TTxd from veterinary company D.

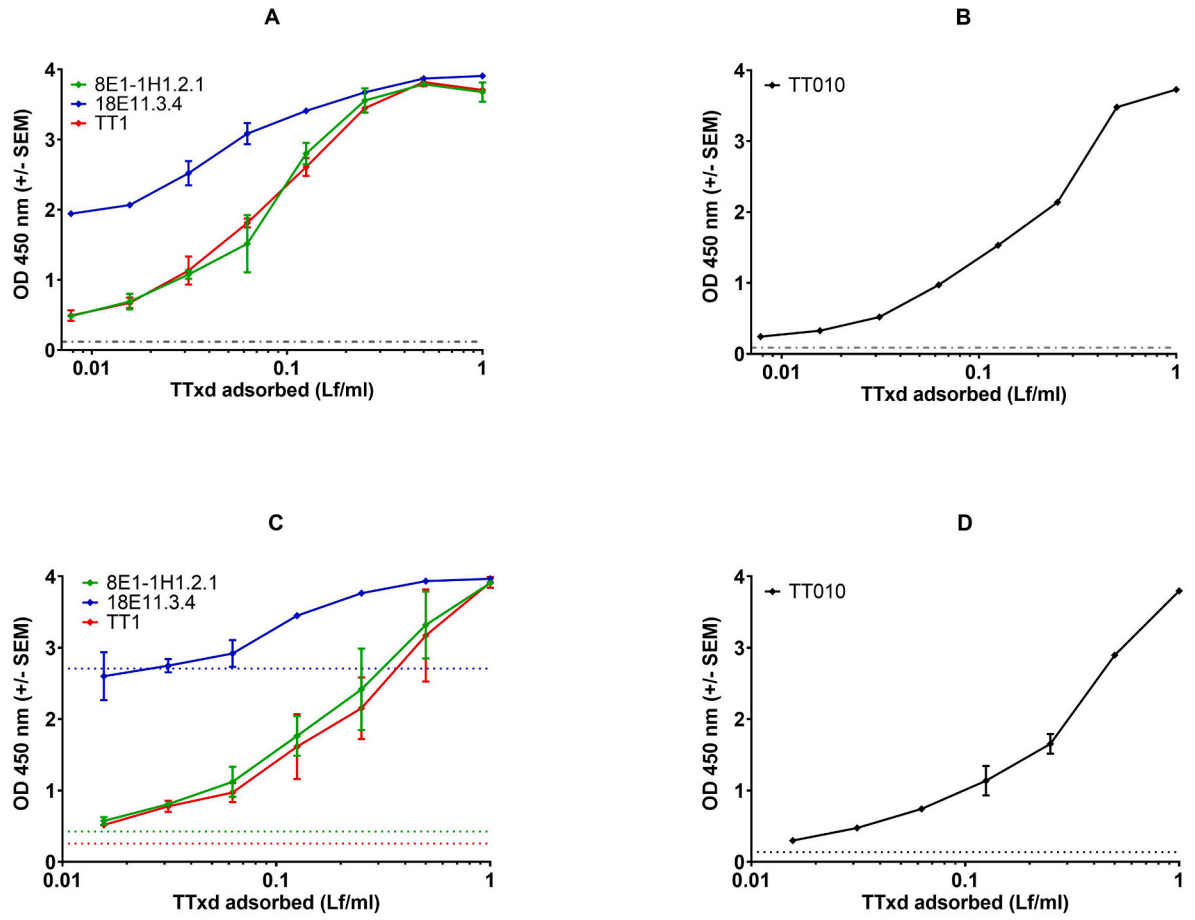

Fig. 4. mAb binding to adsorbed TTxd (Ad-TTxd) from human vaccine manufacturers HuA and HuB. Data shows representative results from one of two independent assays for (A) Mouse mAbs binding to Ad-TTxd from HuA; (B) Rat mAb binding to Ad-TTxd from HuA; (C) Mouse mAbs binding to Ad-TTxd from HuB; (D) Rat mAb binding to Ad-TTxd from HuB. Adsorbed antigen samples were titrated in a fixed adjuvant concentration and detected using a single concentration of mAb. Data are the average OD values from duplicate wells (±SEM). In (A) and (B), the OD value from control wells containing adjuvant, conjugate and substrate only ($n = 4$) is shown by a dashed grey line. In (C) and (D), the control well OD value for each mAb containing no Ad-TTxd ($n = 2$) is shown by a dotted line (green = mAb 8E1-1H1.2.1; blue = mAb 18E11.3.4; red = mAb TT1 and black = mAb TT010). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

temperature with shaking. Following a second wash, cell lysates diluted 1/4 in PBS were added (50 μl/well) and plates were incubated for a further 90 min at room temperature with shaking. Following a final wash, NanoGlo luciferase substrate (Promega) was diluted to 4% in PBS and added to wells (50 μl/well) for 5 min in the dark. Luminescence was measured using a SpectraMax M5 plate reader (Molecular Devices).

3. Results

3.1. Binding to TTxn and non-adsorbed TTxd

At a coating concentration of 30 μg/ml of TTxn, all of the mAbs produced good binding curves, as shown in Fig. 1. The dose response curve for mAb TT010 is shown separately to the other mAbs because a different conjugate antibody (anti-rat) had to be used for detection of this sample.

Binding was observed for all mAbs to TTxd from the two human manufacturers (Fig. 2) and to TTxd from three of the four veterinary manufacturers B, C and D, although binding to toxoid from manufacturer B was notably lower compared to other antigen samples (Fig. 3). None of the mouse mAbs were able to bind TTxd from manufacturer A and low binding was observed for the rat monoclonal antibody against this toxoid sample.

3.2. Binding to adsorbed TTxd

A direct alhydrogel method was used to assess binding of mAbs to Ad-TTxd samples in the presence of aluminium adjuvant. Representative vaccine samples from the two human manufacturers were used. All of the mAbs were able to bind to adsorbed TTxd from both manufacturers in the range of 0.015–1 Lf/ml but we observed high background and non-specific binding with mAb 18E11.3.4 (Fig. 4).

3.3. Binding to heat altered TTxd

The mAbs were tested to determine whether they could detect antigenic changes in non-adsorbed toxoid that had been altered by heat treatment. Representative toxoid samples from the human vaccine manufacturers were used for this analysis. The mAb binding curves to each of the samples at the 8-week time-point is shown in Fig. 5 (HuA TTxd) and Fig. 6 (HuB TTxd). There was no difference in mAb binding to TTxd diluted and incubated +4 °C compared to the same toxoid that had been diluted fresh on the day of the assay (for both HuA and HuB TTxds). Temperature induced changes in the TTxd from HuB were detected to the greatest extent by mAb 8E1-1H1.2.1 (as indicated by a downwards shift of the curve with samples incubated at +37 and + 45), followed by 18E11.3.4 and TT1. The changes were detected the least by mAb TT010. The changes in TTxd from HuA were not as pronounced, and only small

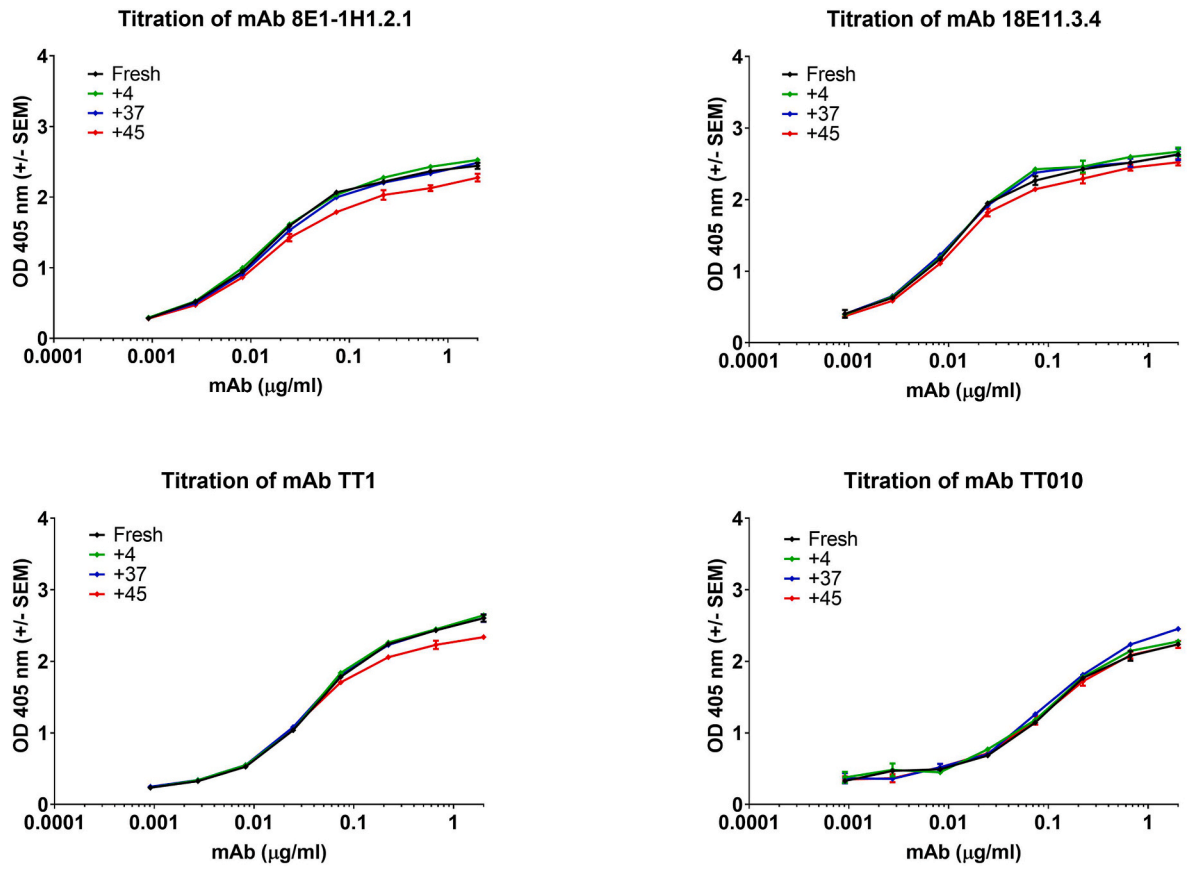

Fig. 5. mAb binding to non-adsorbed antigen (TTxd, HuA) exposed to elevated temperature for 8 weeks. Freshly diluted TTxd and TTxd samples that were incubated at +4, +37 or +45 °C for 8 weeks were titrated in the same assay. Data shown is the average OD value from duplicate wells (±SEM).

differences were observed with mAbs 8E1-1H1.2.1, 18E11.3.4 and TT1, and only for the TTxd sample that had been incubated at the highest temperature.

3.4. Affinity measurements and epitope competition

First the active concentration was determined to select a suitable antibody dilution for the kinetic assay (not shown). Kinetic analysis was done using single cycle analysis, whereby each TTxn concentration was injected in one cycle and the protein G sensorchip was regenerated at the end. The sensorgrams obtained with each mAb are shown in Fig. 7. Results from the subsequent kinetic analysis are shown in Table 3, arranged in order of decreasing affinity for TTxn (the lower the K_D value, the higher the affinity of the antibody). Both TT010 and 8E1-1H1.2.1 have dissociation constants (K_D) in the low nanomolar to picomolar range suggesting that both these mAbs have very high affinity for TTxn.

For the epitope competition, TTxn was captured by a first mAb, and binding of a second mAb was then measured. Additional binding is expected to be observed on the sensorgram if the second mAb binds to a different epitope. Results are summarised in Table 4 and suggest that the four mAbs are all directed against different epitopes.

3.5. Tetanus toxin neutralisation

An in-house cell-based toxin neutralisation test was used to provide an indication of mAb function. The mAb samples, and a polyclonal positive control antitoxin, were pre-incubated with TTxn for 1 h before adding to cells. The polyclonal tetanus antitoxin (66/021) showed complete neutralisation of TTxn activity (as evidenced by a complete reversal of toxin-induced luminescence signal). Strong neutralising activity was observed for mAb TT010 and partial neutralisation of toxin activity was seen with mAbs TT1 and 8E1-1H1.2.1. One of the mAbs, 18E11.3.4, did not show any evidence of toxin neutralisation (Fig. 8).

4. Discussion

Immunoassays are used routinely in the quality control of many vaccines and in some cases have been specifically developed as alternatives to *in vivo* potency tests [4,11–18].

Immunity to tetanus is antibody mediated [19] so an approach to potency testing of tetanus vaccines that relies on the use of specific, functional antibodies targeting protective epitopes (i.e. those epitopes inducing formation of toxin-neutralising antibodies) is clinically relevant. The use of hyperimmune serum to prevent the disease if the patient is not vaccinated also highlights the relevance of using an immunoassay with a neutralising mAb for a tetanus containing vaccine.

Immunochemical methods that measure consistency of tetanus antigen content and quality in vaccine products could potentially negate the need for an *in vivo* potency test, while at the same time increasing precision. Well characterised antibodies are essential for the development of such assays, and where the assay is intended to serve as a potency test, the antibody used should be of high affinity and should target an epitope(s) that is relevant for protection and is stability indicating [20–22]. We report here the thorough characterisation of a panel of existing tetanus mAbs with a view to selection of a pair of antibodies that can be used for development of a replacement *in vitro* tetanus vaccine potency assay.

There was very little to distinguish the mAbs in terms of binding to native antigen (TTxn), or to non-adsorbed detoxified antigen (TTxd). This was the case whether binding to antigen was good (as was the case with most toxoid samples) or poor (as was the case with one toxoid in particular). The reason for poor binding to non-adsorbed toxoid from

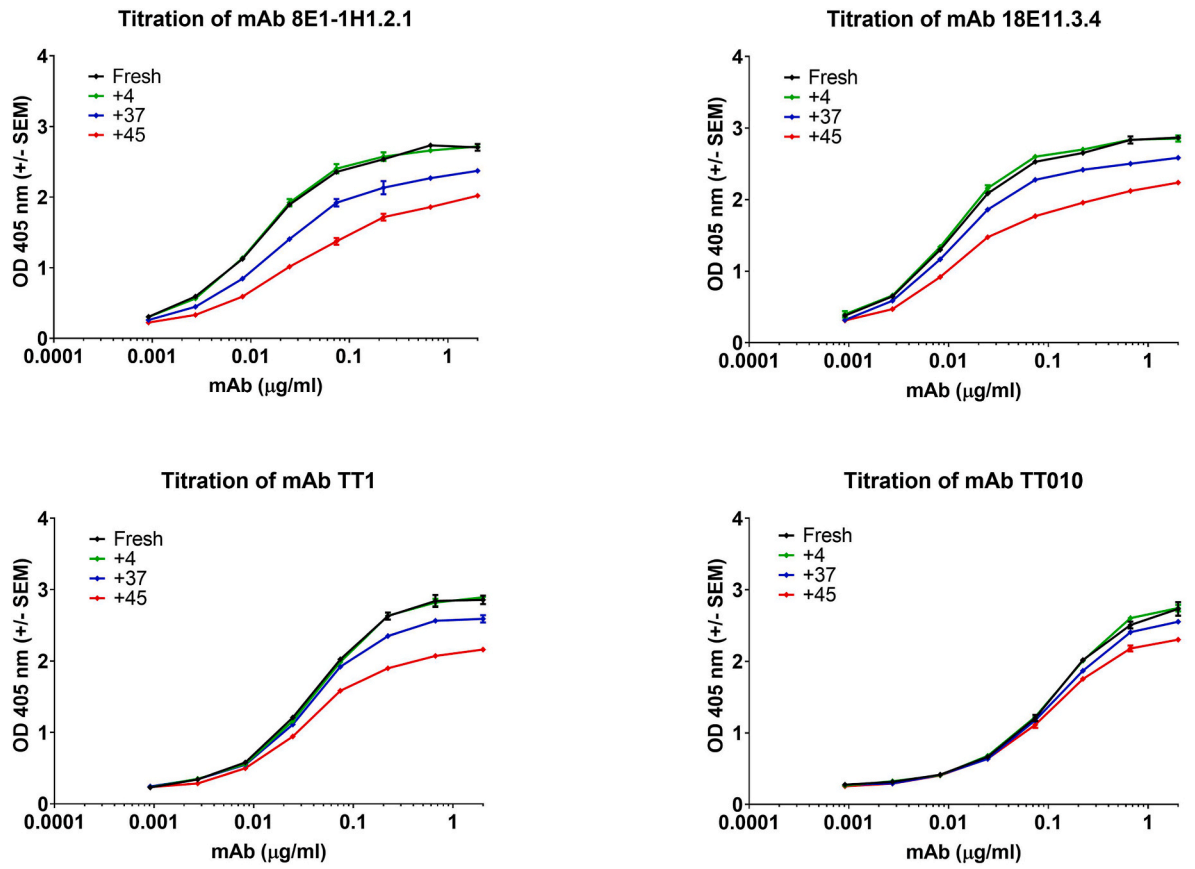

Fig. 6. mAb binding to non-adsorbed antigen (TTxd, HuB) exposed to elevated temperature for 8 weeks. Freshly diluted TTxd and TTxd samples that were incubated at +4, +37 or +45 °C for 8 weeks were titrated in the same assay. Data shown is the average OD value from duplicate wells (±SEM).

veterinary manufacturer A is unclear but may be related to the direct ELISA format used for the binding experiments. The lower coating concentration used for this toxoid can also not be ruled out, however we have shown previously that one of the mAbs (a poor binder in this ELISA format) can function as a capture antibody for the tetanus toxoid from this manufacturer when the sample is tested in a sandwich ELISA format at the same concentration used for coating plates [7]. Preliminary data from sandwich ELISA assays using two of the four tetanus mAbs from this current study also show that the tetanus toxoid from this manufacturer can be reliably detected (unpublished observations). This suggests that it is the direct ELISA format causing the poor binding observed for TTxd from manufacturer A. Direct coating of protein onto an ELISA plate may induce conformational changes that could reduce epitope availability [23–25], however this is an unlikely explanation for the results we observe here since all four mAbs, which target different epitopes, are poor binders. More likely is binding of non-toxin proteins in the toxoid sample that reduce the amount of target antigen bound to the plate and therefore reduce sensitivity. The ultimate aim is to develop a capture ELISA assay which will overcome any issues related to purity of toxoid or vaccine samples.

For development of an *in vitro* potency assay, the test needs to be suitable for use with the drug product, i.e. the final vaccine. Many tetanus vaccines, whether for human or veterinary use, are formulated using an aluminium adjuvant, the presence of which may interfere with binding of antibody to the target antigen [7,8,26–28]. To assess binding of mAbs in the presence of adjuvant, we used a modified version of the Direct Alhydrogel Formulation Immunoassay (DAFIA) [8,9] where adsorbed antigen samples are titrated in a fixed concentration of adjuvant. The principle of the assay is similar to an ELISA except that TTxd adsorbed to aluminium adjuvant is pelleted by centrifugation in wells of an ELISA plate (rather than being directly bound to the surface of the plate as in a traditional ELISA). The assay response was quite variable, mainly due to the difficulties in removing all of the reagent/wash buffer consistently from the adjuvant pellet during the wash steps but allowed us to identify low or high binders to the adsorbed antigen. All of the mAbs were able to bind to Ad-TTxd, however a high background was obtained with mAb 18E11.3.4 caused by non-specific binding of the mAb to the adjuvant. Although we only assessed non-specific binding of the antibodies to one of the human manufacturer vaccines containing an aluminium adjuvant, the significant non-specific binding observed makes mAb 18E11.3.4 unsuitable for taking forward into development of an immunoassay for measuring adsorbed tetanus antigen.

To provide an indication of whether one or more of these antibodies is sensitive to changes in the antigen that are relevant for quality assessment, we deliberately altered non-adsorbed TTxd samples by exposure to heat. Two of the antibodies (8E1-1H1.2.1 and TT1) were sensitive to heat-induced changes in TTxd from HuA, but only at the highest storage temperature. More pronounced changes were observed with the TTxd from HuB and mAb 8E1-1H1.2.1 was most able to detect heat induced antigenic changes, with mAb TT010 being least sensitive. The altered sample study was performed using temperatures no higher than +45 °C with the aim of producing changes in the toxoid that would allow discrimination between the monoclonal antibodies in terms of their ability to recognise any alteration in the toxoid. The extent to which binding of each antibody was affected by heat-exposure of the toxoid may be related to the nature of the epitope to which they are directed, although we have not yet determined whether these antibodies recognise linear or conformational epitopes. Epitope mapping is the subject of ongoing work using cross-linking mass spectrometry.

In vivo data on neutralising activity of the tetanus mAbs was available for some, but not all antibodies prior to initiating this work. Therefore, we assessed antibody function using an in-house cell-based

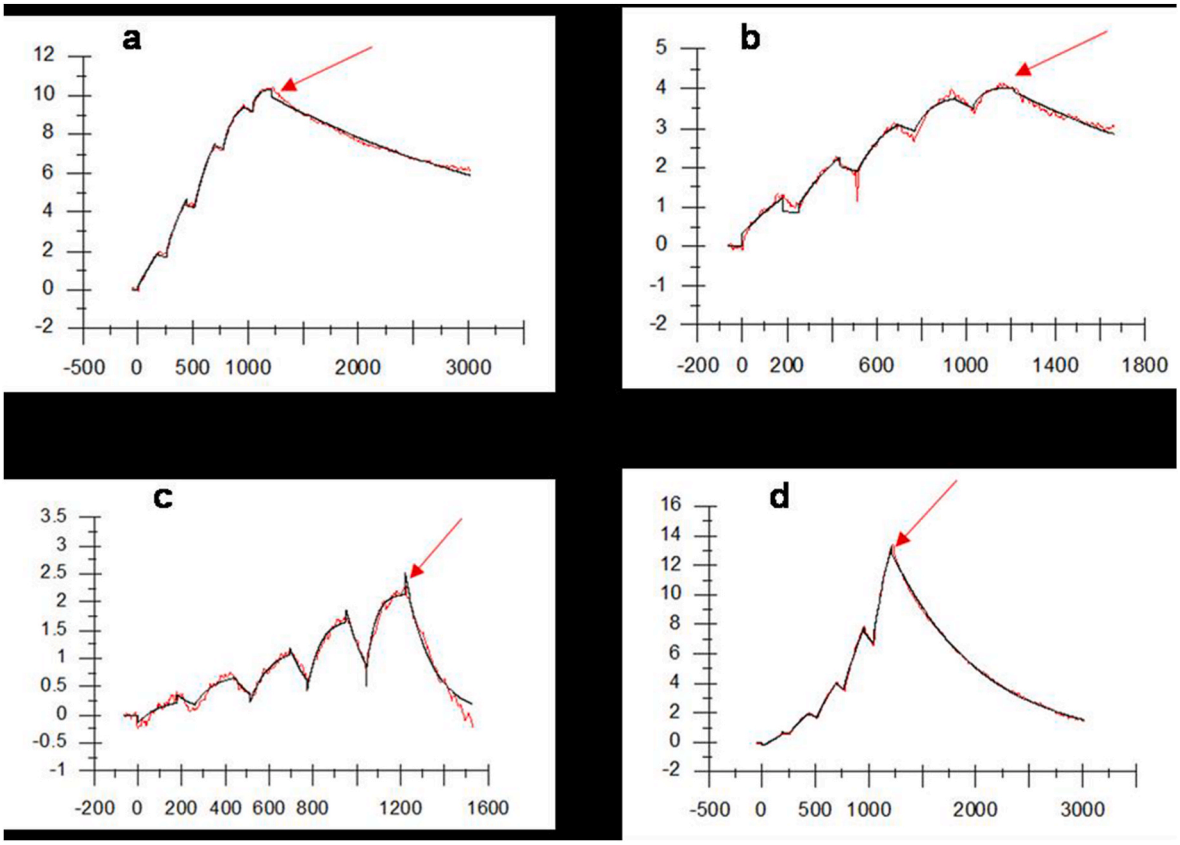

Fig. 7. mAb-antigen interactions using the single cycle kinetic assay. Representative sensorgrams for (A) TT010, (B) 8E1-1H1.2.1, (C) 18E11.3.4 and (D) TT1 are shown. The red arrow indicates where the dissociation starts after binding of TTxn in increasing concentrations. Response units are displayed on the y-axis and seconds are displayed on the x-axis of each graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Affinity of the tetanus mAbs for tetanus toxin.

Ranking	mAb ID	Association rate(K_a, 1/Ms)	Dissociation rate(K_d, 1/s)	Equilibrium dissociation constant (K_D, M)
1	TT010	3.27E+05	3.12E-04	9.53E-10
2	8E1-1H1.2.1	3.63E+05	7.14E-04	1.97E-09
3	18E11.3.4	2.64E+05	8.25E-03	3.13E-08
4	TT1	3.75E+04	1.18E-03	3.16E-08

Table 4
Epitope competition for the tetanus mAbs.

		Second mAb			
First mAb		**TT010**	**8E1-1H1.2.1**	**18E11.3.4**	**TT1**
	TT010	-	+	+	+
	8E1-1H1.2.1	+	-	+	+
	18E11.3.4	+	+	-	+
	TT1	+	+	+	-

assay. This assay allows detection of all steps of tetanus toxin action (binding to the cell surface, endocytosis, translocation of the toxin light chain and enzymatic cleavage of VAMP-2) using a luminescent enzymatic reaction. The assay is based on the principle that toxin-induced cleavage of VAMP-2 protein can be detected using a specific neo-epitope antibody. The assay used is based on an assay developed for Botulinum toxin type B, which shares the same intracellular target as tetanus toxin [10]. Three of the four mAbs were able to partially prevent the toxin-mediated cleavage of VAMP-2, with TT010 showing near complete neutralisation – bettered only by the polyclonal antitoxin positive control. Antibody 8E1-1H1.2.1 showed partial neutralisation but mAb 18E11.3.4 did not show any reduction of toxin effect in this assay model. This compared well with available (unpublished) data from mouse bioassays where 18E11.3.4 did not provide any protection and 8E1-1H1.2.1 did protect mice but only at the highest dose used (not shown), and where TT010 has a reported *in vivo* neutralising potency of 13–33 IU/μg IgG [29]. Neutralisation data was not available for TT1 but the results obtained in the cell-based assay suggest that this mAb does have moderate toxin-neutralising ability. Two of the mAbs, TT010 and 8E1-1H1.2.1, had affinities for tetanus toxin in the nano- or sub-nanomolar range and epitope competition studies showed that the mAbs were all directed against different epitopes, and therefore potentially any combination would be suitable for use in the development of a sandwich immunoassay. The recognition of distinct epitopes by these monoclonal antibodies together with the evidence suggesting three of them have neutralising activity is consistent with previous studies that suggest there are multiple distinct epitopes on tetanus toxin to which neutralising antibodies may be directed [30,31].

Based on all these characterisation results, mAbs TT010 and 8E1-1H1.2.1 are the preferred pair to take forward to assay development. TT010 has the best neutralising ability and the highest affinity for TTxn. 8E1-1H1.2.1 has a similar high affinity for TTxn and was also the best at being able to detect heat induced antigenic changes in non-adsorbed TTxd. The two selected mAbs are different species which avoids the need to biotinylate one of the antibodies in a sandwich ELISA. The development of the ELISA, using these two antibodies, is now underway and will assess the ability of this mAb pair to detect antigenic changes that are indicative of vaccine quality and stability for a wide range of

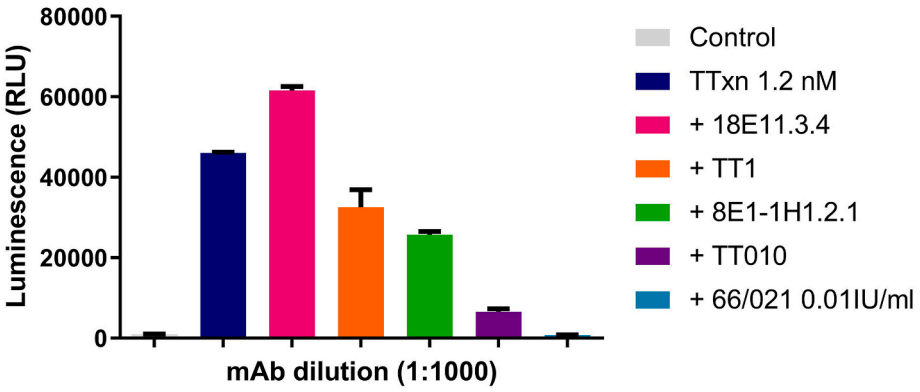

Fig. 8. mAb neutralisation of tetanus toxin activity using a developmental cell-based assay. Cells were treated for 72 h with TTxn or TTxn that had been pre-incubated (for 1 h) with mAb or positive control antiserum (66/021). Cell lysates were then used for detection of VAMP-2 cleavage using a one-step luciferase assay. Data are from a single experiment conducted in duplicate with error bars representing the standard deviation of the average from duplicate wells.

tetanus vaccines from both human and veterinary manufacturers.

The extensive characterisation of the monoclonal antibodies performed in this study, including affinity, functional activity and recognition of heat-altered antigen, provides a platform of evidence to support their use in development of a quantitative immunoassay that, subject to appropriate validation and regulatory approval, can be implemented as part of a control strategy for tetanus vaccines, potentially as a substitute for *in vivo* potency. The antibodies characterised here recognise tetanus toxoid from multiple sources and three of the antibodies appear to be suitable for use with final lot vaccine (i.e. in the presence of adjuvant). An immunoassay developed using these antibodies should therefore have wide applicability across a range of tetanus vaccines. Our aim is to use an antibody pair where, in addition to the recognition of multiple tetanus toxoids, including adjuvanted toxoid, the detection antibody recognises a functional epitope and is sensitive to heat-altered antigen. Further antigen stresses will be explored using an antibody pair to determine the extent to which a monoclonal antibody immunoassay is stability indicating. The possibility to produce recombinant versions of the selected antibodies will also be explored to ensure sustainability of critical reagents.

Author contributions

RRD and LH designed and performed experiments, analysed data and drafted the manuscript. AK and JW participated in the design of the study and performed affinity and competition experiments. AD and AF participated in the design of the study. BD and CD developed and provided cells for the toxin-neutralisation test and SR designed and performed experiments using this in-house cell-based assay. PS conceived the study, contributed to design of experiments and drafted the manuscript.

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Declaration of competing interest

None.

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