

# **SARS-CoV-2 RBD-Tetanus toxoid conjugate vaccine induces a strong neutralizing immunity in preclinical studies**

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## **Abstract**

Controlling the global COVID-19 pandemic depends, among other measures, on developing preventive vaccines at an unprecedented pace. Vaccines approved for use and those in development intend to use neutralizing antibodies to block viral sites binding to the host's cellular receptors. Virus infection is mediated by the spike glycoprotein trimer on the virion surface via its receptor binding domain (RBD). Antibody response to this domain is an important outcome of the immunization and correlates well with viral neutralization. Here we show that macromolecular constructs with recombinant RBD conjugated to tetanus toxoid induce a potent immune response in laboratory animals. Some advantages of the immunization with the viral antigen coupled to tetanus toxoid have become evident such as predominant IgG immune response due to affinity maturation and long-term specific B-memory cells. This paper demonstrates that subunit conjugate vaccines can be an alternative for COVID-19, paving the way for other viral conjugate vaccines based on the use of small viral proteins involved in the infection process.

## **Introduction**

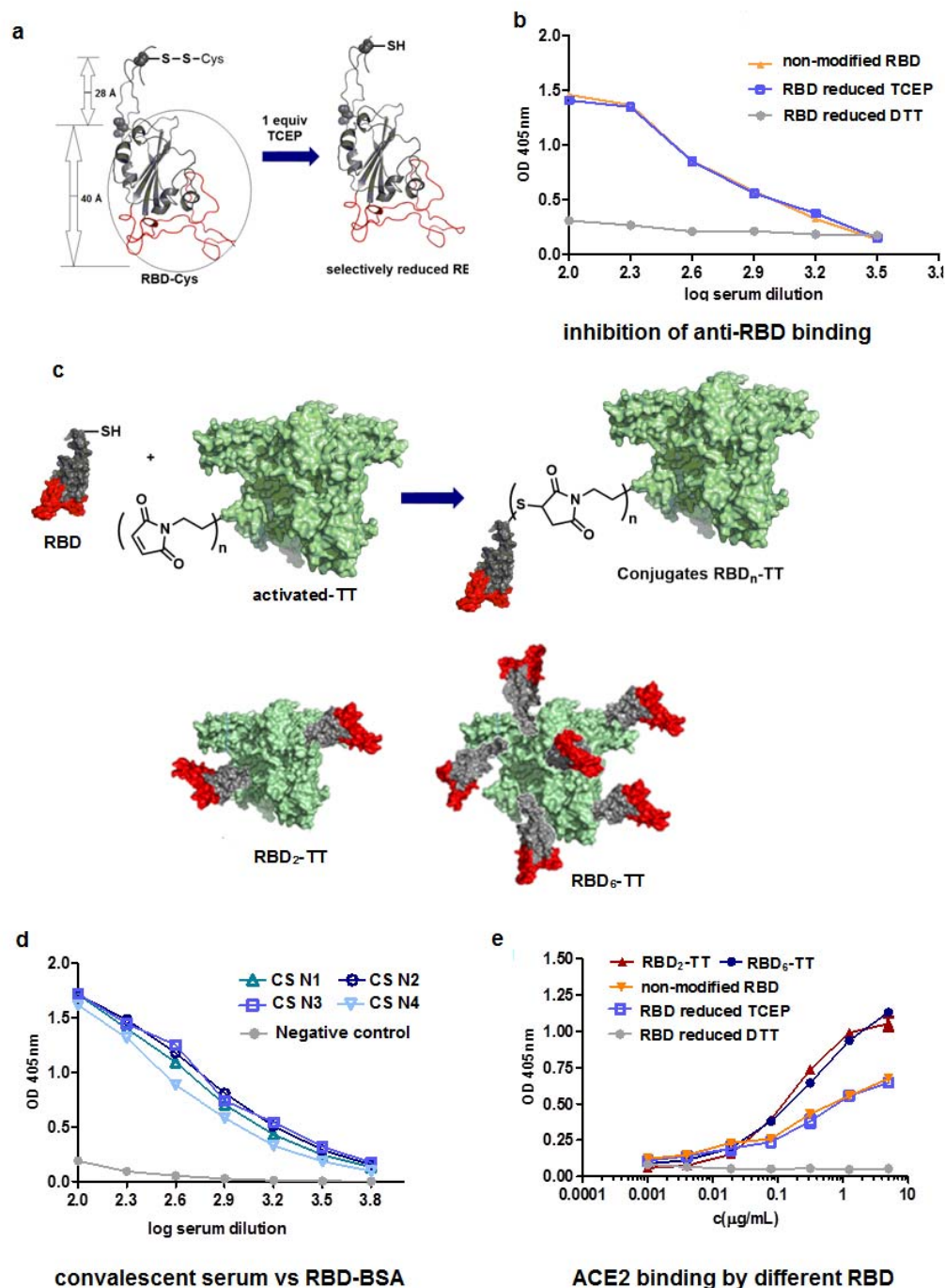
Control of SARS-CoV-2 infection focuses on development of preventive vaccines.<sup>1</sup> Viral particles' initial binding is mediated by the receptor binding domain (RBD) of the spike (S)-glycoprotein trimer to the host's cell surface receptor, the angiotensin-converting enzyme 2 (ACE2).<sup>2-6</sup> Most of the 200 COVID-19 vaccines in development aim to block this process.<sup>7</sup> By focusing on the whole S-protein or its RBD as antigen, the primary goal is induction of anti-RBD antibodies that interfere with RBD-ACE2 interaction, blocking the first step of infection. Virus neutralization is mainly associated with the receptor binding motif (RBM), a specific RBD region directly interacting with ACE2.<sup>8</sup> These types of antibodies are not involved in antibody dependent enhancement (ADE).<sup>9</sup>

A key advantage of the well-known recombinant subunit vaccine platforms is their safety stability at 2-8°C and scale-up the production.<sup>10</sup> While we should expect weak immunogenicity for such a small recombinant RBD protein (30 kDa), requiring repeated vaccination, it was found that recombinant RBD in alum is sufficient to induce a neutralizing immune response in laboratory animals,<sup>11</sup> and their simplicity prompted subsequent evaluation in humans.<sup>12</sup> However, most vaccines have developed strategies to increase the immunogenicity of this small recombinant RBD protein by incorporating it, for example, into larger molecular constructs and/or by using potent adjuvants.<sup>13</sup> In addition to the lower immunogenicity, small recombinant RBD exposes to the immune system not only the critical RBM surface but also well-camouflaged RBD region at the virus surface. Antibodies directed to camouflaged RBD region are not neutralizing. We hypothesize that the orientation of RBD when conjugate to tetanus toxoid exposes better the RBM surface increasing the level of neutralizing antibodies.<sup>14-17</sup>

The SARS-CoV-2 RBD comprises 193 amino acid residues from Thr333 to Pro527, including RBM 438-506 that directly interacts with ACE2. It contains eight cysteines forming four disulfide bridges, three of these stabilizing the RBD core and one within the RBM.<sup>6</sup> Our extended recombinant RBD 319-541 was obtained in CHO-cells with intentionally extended sequence adding S-glycoprotein residues 527 through 541, in order to include an additional Cys538. This cysteine is usually connected to Cys590 in the S-glycoprotein. The extended sequence includes two N-glycosylation sites at residues Asn331 and Asn343 and two O-glycosylation sites at Thr323 and Ser325. The selected sequence results in a free Cys538 intended to be used for chemical conjugation to the highly immunogenic carrier tetanus toxoid (TT). Here we find a promising vaccine candidate based on this high molecular weight conjugate with several copies of recombinant RBD per molecular unit. To our knowledge, chemically conjugated constructs and the immunogenic effect of conjugating viral proteins such as RBD to a protein carrier have not been assessed for SARS-CoV-2 or other coronaviruses. Here we demonstrate that the RBD-TT conjugate induces a potent immune response in laboratory animals, paving the way for their evaluation in human phase I and II clinical trials.<sup>18</sup>

### **Construction of RBD-TT conjugates**

Our design is based on the hypothesis that by conjugating several copies of the extended RBD to a large carrier protein we can obtain a macromolecular construct displaying multivalent RBD. At the same time, the RBM will be well exposed (Figure 1, represented in red) and better available for immune recognition.



**Fig 1|Synthesis of RBD-TT conjugates.** **a.** Reduction by use of TCEP **b.** Inhibition of anti-RBD binding by reduced RBD, using CS. **c.** Conjugation of RBD with TT and representation of RBD<sub>2</sub>-TT and RBD<sub>6</sub>-TT. **d.** Recognition of RBD-BSA conjugates by convalescent serum (CS), n=1-4. **e.** Binding to ACE2 of conjugated RBD from RBD<sub>2</sub>-TT and RBD<sub>6</sub>-TT.

Inclusion of an additional free Cys538 in our extended RBD, while potentially useful for conjugation, could jeopardize extended-RBD folding, due to potential S-S rearrangement with

the other 8 cysteines (scrambling). Nevertheless, we found that during fermentation and purification, Cys538 is spontaneously protected through an S-S Cys adduct with free cysteine present in the culture media. ESI-MS showed presence of the four S-S bonds, indicating a correctly folded extended RBD (extended Figure 1). Cysteinylation of Cys538 was selectively reduced to free thiol with tris (2-carboxyethyl) phosphine (TCEP)<sup>18</sup> without affecting ACE2 recognition, while—for example—dithiothreitol (DTT) led to complete loss of its ACE2 binding capacity, suggesting loss of the antigen's 3D structure (Figure 1b).

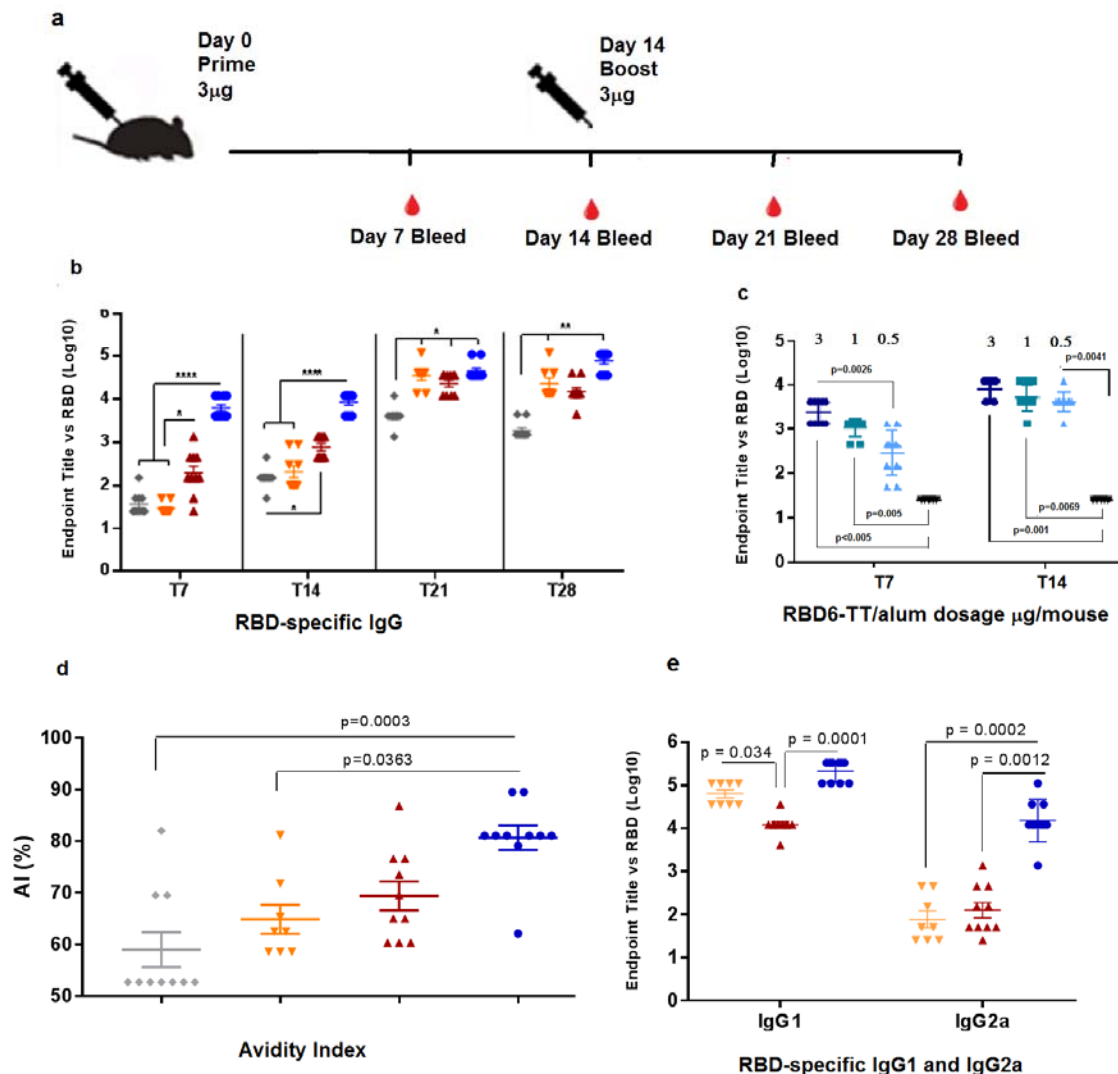
To our knowledge, the immunogenic effect of TT as a carrier has not been assessed previously for SARS-CoV-2 or any other coronavirus. We have successfully used TT as a carrier protein for antibacterial carbohydrate-protein conjugate vaccines.<sup>19,20</sup> The presence of multiple T- and B-cell epitopes of this highly immunogenic carrier<sup>21</sup> might potentiate cellular immunity when compared to use of RBD alone. In addition, multimeric RBD-TT can simultaneously activate several B-cell receptors<sup>22</sup>.

TT was activated with an average of 20–30 maleimide groups per mol of TT by reaction with *N*-succinimidyl 3-maleimidopropionate (SMP) followed by reaction with 2.5 or, alternatively, 10 equivalents of TCEP-reduced extended RBD, to produce conjugates bearing 2 or 6 mol, respectively, of RBD per mol of TT (Fig. 1c). The RBD<sub>2</sub>-TT and RBD<sub>6</sub>-TT conjugates were produced under good manufacturing practices (GMP) in 72% and 64% yield, respectively, and characterized by SE-HPLC and MS. Both conjugates recognize ACE2 slightly better than the original RBD, (Figure 1e), confirming preservation of their structure and, probably, a better exposition of RBM. As convalescent serum usually contains TT antibodies, we prepared a RBD-bovine serum albumin (BSA) conjugate incorporating 6 RBD units per mol of BSA (RBD<sub>6</sub>-BSA), which was recognized well by various convalescent sera, proving conservation of the RBD antigenic properties after conjugation (Figure 1d).

## Animal Immunogenicity

Immunization of BALB/c mice with the four different immunogens (Figure 2) induces a strong IgG RBD-specific immune response detected by enzyme-linked immunosorbent assay (ELISA). RBD<sub>6</sub>-TT/alum and RBD<sub>2</sub>-TT/alum were compared with RBD alone and with RBD<sub>2</sub>-TT without alum. After the first dose (T7 and T14, Figure 2c) RBD<sub>6</sub>-TT/alum induces the highest level of anti-RBD antibodies. After the second dose all immunogens adsorbed in alum elicit better anti-RBD IgG levels than without alum (T21 and 28, Figure 2c). The high and homogeneous early response for RBD<sub>6</sub>-TT/alum could be an important attribute for a vaccine

in pandemic times. We explored early response to different dosage of RBD<sub>6</sub>-TT/alum, finding a dose-dependent response at day 7. At day 14, before the second dose, the response was very high even for the lowest dosage (T14, Figure 2c).



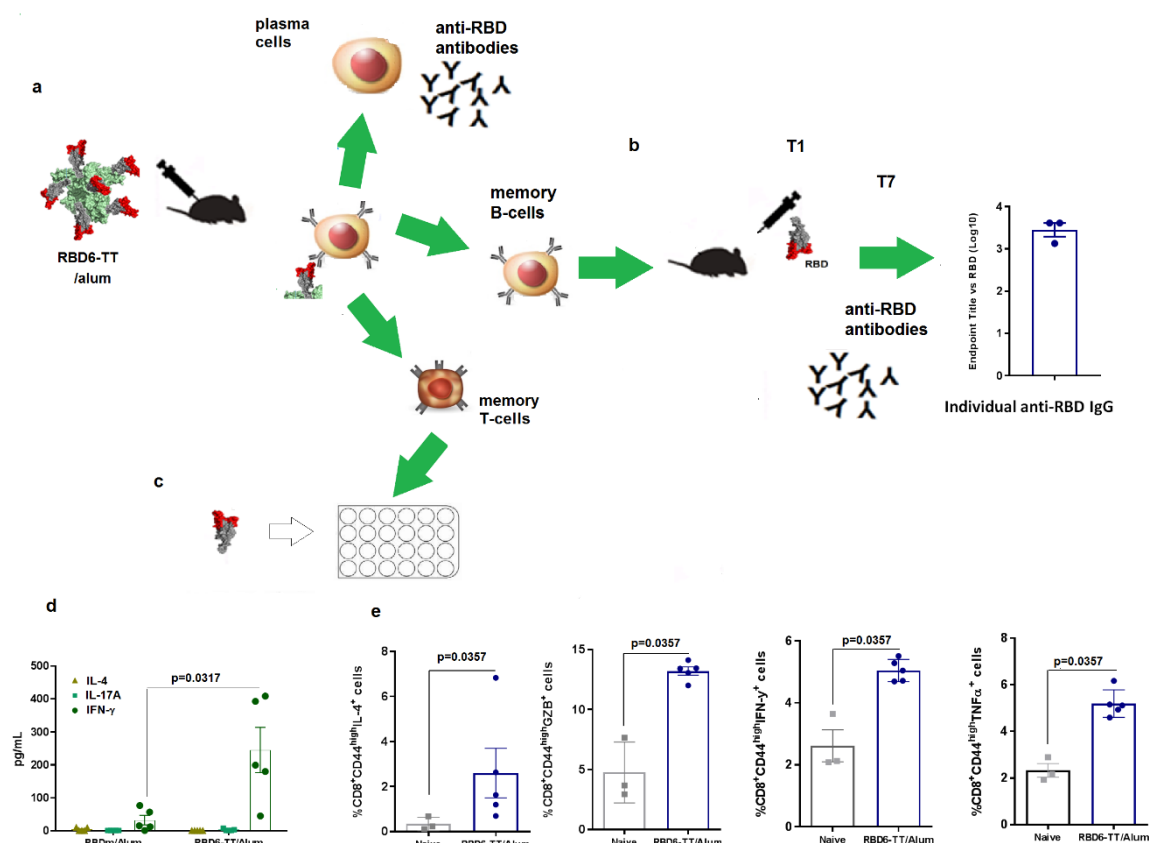
**Figure 2** | Immunization of BALB/c mice with RBD<sub>2</sub>-TT/ alum and RBD<sub>6</sub>-TT/alum compared to RBD and RBD<sub>2</sub>-TT. The serum of individual mice is represented by RBD/alum ▼, RBD<sub>2</sub>-TT/alum ▲, RBD<sub>2</sub>-TT ◆, RBD<sub>6</sub>-TT/alum ● **a.** Immunization protocol **b.** anti-RBD-specific IgG at days 7, 14, 21, and 28. **c.** Dose response to RBD<sub>6</sub>-TT/alum at days 7 and 14. **d.** Avidity index of antibodies elicited at T28. **e.** RBD-specific IgG1 and IgG2a.

To evaluate possible immunological advantages of the RBD-TT conjugate, we studied affinity maturation. There was an increase in the avidity index (AI).<sup>23</sup> The highest value of 81% for antibodies induced by RBD<sub>6</sub>-TT is consistent with a more pronounced affinity maturation. (Figure 2d). The Th1/Th2 balance can be modulated by vaccination and was also evaluated



(Figure 2e). A biased Th2 immune response was observed for RBD<sub>2</sub>-TT/alum (IgG2a/IgG1 ratio 0.54) and RBD/alum (IgG2a/IgG1 ratio 0.40), while RBD<sub>6</sub>-TT/alum displayed more balanced Th1/Th2 immunity (IgG2a/IgG1 ratio 0.81).

Figure 3 shows the induction of memory antigen-specific B and T-cells, an important property of conjugate vaccines. Mice immunized with both conjugates RBD<sub>6</sub>-TT/alum was compared to mice receiving RBD/alum. Both groups developed a primary immunity as shown previously (Figure 2b). After two doses at T28, splenocytes purified from both groups were intravenously transferred to naïve mice that were then boosted by a single dose of 3 µg RBD/alum (Figure 3b). Mice receiving splenocytes from RBD<sub>6</sub>-TT/alum responded with a strong secondary RBD-specific IgG response (Title $10^3$ - $10^4$ ), while those receiving splenocytes from RBD/alum did not (Results not shown). This finding demonstrated presence of RBD-specific memory B cells in transferred splenocytes, able to activate in the presence of RBD/alum (alternative SARS-CoV-2 virus) stimuli.



**Figure 3|Memory B and T cells induced by RBD<sub>6</sub>-TT. a.** Primary immune response to RBD<sub>6</sub>-TT/alum (green arrows). **b.** Classical passive transfer of splenocytes from RBD<sub>6</sub>-TT/alum BALB/c and stimulated with RBD/alum (strong secondary response after day 7). **c.** T-cell stimulation with RBD **d.** Cytokine secretion after *in vitro* RBD stimulation **e.** % RBD-specific memory T CD8<sup>+</sup>CD44<sup>high</sup>IFN $\gamma$ <sup>+</sup>; % RBD-specific memory T CD8<sup>+</sup>CD44<sup>high</sup>TNF $\alpha$ <sup>+</sup>;

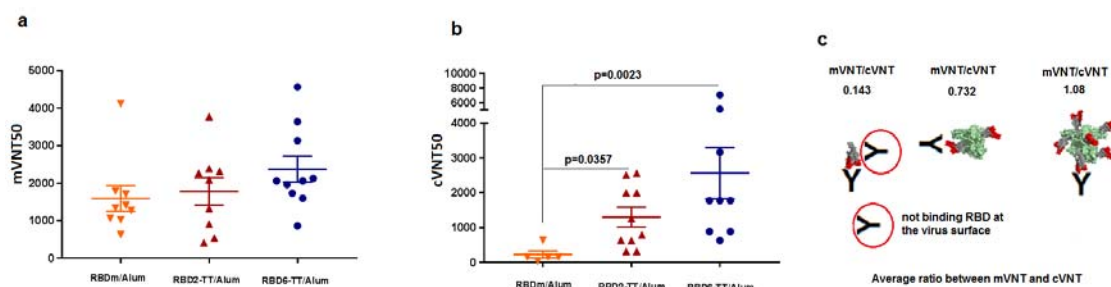
RBD-specific memory T  $CD8^+CD44^{high}Granzyme^+$ ; % RBD-specific memory T  $CD8^+CD44^{high}IL-4^+$

Specific  $CD8^+$  T cells also play an important role in protection as recently demonstrated.<sup>21</sup> To evaluate the specific T-cell response, we compared RBD<sub>6</sub>-TT/alum and RBD/alum. After *in vitro* RBD stimuli, splenocytes from mice immunized with RBD<sub>6</sub>-TT secreted higher levels of IFN $\gamma$  compared to those immunized with RBD/alum (Figure 3d), suggesting a Th1 pattern, while IL-4 (characteristic of Th2 pattern) and IL-17A (characteristic of Th17 pattern) were not detected. Frequency of  $CD8^+CD44^{high}$  memory T-lymphocytes producing IFN- $\gamma$ , TNF- $\alpha$  and Granzyme B increased significantly in RBD<sub>6</sub>-TT immunized mice with respect to control mice (Figure 3e) as shown by flow cytometry, indicating activation of cytotoxic T immune memory. This activation can be elicited by viral infection.

### Antibody functionality

We evaluated antibodies' ability to block interaction between the virus and its receptor, using the molecular Virus Neutralization Test (mVNT50)<sup>24</sup> and the conventional Virus Neutralization Test (cVNT50).<sup>25</sup> mVNT50 evaluates inhibition of interaction between recombinant RBD and ACE2 at the molecular level; at the cellular level, cVNT50 evaluates inhibition of interaction between the live virus and Vero E6 cells bearing ACE2 receptors. Antibodies resulting from immunization of Balb/c mice with two doses of RBD<sub>2</sub>-TT/alum and RBD<sub>6</sub>-TT/alum were compared to antibodies elicited after immunization with low molecular weight RBD/alum. mVNT50 showed a high level of inhibition for all sera (Figure 4a), indicating that all tested antibodies displayed a similar efficacy in interfering with RBD-ACE2 interaction at the molecular level. cVNT50 (Figure 4b) showed sharp differences between sera from animals immunized with RBD/alum and those with both conjugates. For RBD/alum, the neutralization titer was 232; for both conjugates, there was a higher level of virus neutralization: 1303 for RBD<sub>2</sub>-TT and 2568 for RBD<sub>6</sub>-TT. The mVNT50/cVNT50 ratio was 0.143, 0.732 and 1.08 for RBD, RBD<sub>2</sub>-TT and RBD<sub>6</sub>-TT respectively. Antibodies neutralizing the virus are mainly directed at RBM<sup>7</sup>. Presence of antibodies recognizing soluble RBD not only by RBM but also on a different region as shown in Figure 4c. This type of "lateral" antibodies can interfere in mVNT50 with soluble RBD but will probably not recognize this RBD region camouflaged at the virus surface.





**Figure 4| Virus neutralization by RBD antibodies induced by conjugates. a.** mVNT50 **b.** cVNT50 measured as serum dilution giving 50% of virus neutralization representing the serum dilution giving 50% inhibition ACE2-RBD interaction. **c.** mVNT50/cVNT50 ratio and possible explanation of differences.

Based on the results presented here, GMP batches of the conjugates RBD<sub>2</sub>-TT and RBD<sub>6</sub>-TT were obtained and absorbed on alum for final vaccine candidates. A phase I clinical trial<sup>26</sup> was initiated in October 2020, and after preliminary results, the vaccine based on RBD<sub>6</sub>-TT/alum was moved on December 21 to a phase II clinical trial with 910 subjects.<sup>27</sup> The encouraging results of the clinical trial will be published in due course.

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## Author contributions

Y.V.B., D.G.R., and V.V.B. designed and lead the study. S.F. is the manager of the project. D.S.M., D.G.R., L.Q., U.R., J.P.S., Y.M., H.G., and M.G.R. performed chemical conjugation, L.R., B.S.R., R.P., C.A., T.H., G.B., F.Pi., A.V., performed the immunologic assays, M.F. and R.O. led the clinical care of the animals, F. C., R.G., M.L. led the analytical chemistry of the conjugates and vaccines J.E., N.G., and A.S. performed the virologic assays L.A.E., Y.R., and L.J.G. performed mass-spectra studies of the conjugates G.R., E.R-H., Y.C., S-L.L., T.B., E.O., K.L.M., C.F., and G.W.C. led the CHO-cells RBD preparation, Y.C., F.C. and F.P. supported and contributed to the study design and analysis.

## Competing interests

The authors declare no financial conflicts of interest. Y.V.B., D.S.M, S.F., M.R., L.R., U.R., D.G.R., T.B., E.O., D.G.R., D.G.R., and V.V.B. are co-inventors on provisional SARS-CoV-2 vaccine patents (Cu 2020-69).

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