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

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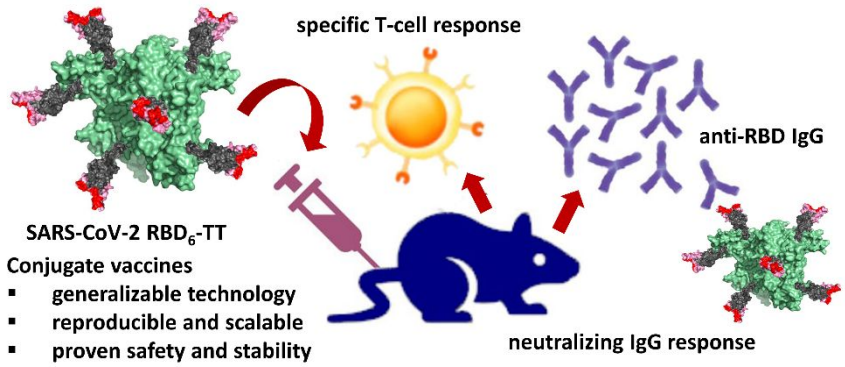
Abstract

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 elicit 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 immunization and correlates well with viral neutralization. Here we show that macromolecular constructs with recombinant RBD conjugated to tetanus toxoid (TT) induce a potent immune response in laboratory animals. Some advantages of the immunization with RBD-TT conjugates include a predominant IgG immune response due to affinity maturation and long-term specific B-memory cells. This result demonstrates the potential of COVID-19 vaccines based on the conjugate vaccine technology and enables the advance to clinical evaluation, paving the way for other antiviral conjugate vaccines.

Graphical Abstract



Introduction

The rapid development of preventive vaccines is crucial for controlling SARS-CoV-2 infection and ending COVID-19 pandemic.[1] 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).[2-5] Most of the 200 COVID-19 vaccines in development[6] aim to block this process.[1] 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 antibodies against the receptor binding motif (RBM), a specific RBD region directly interacting with ACE2.[7] This type of antibodies is not involved in antibody dependent enhancement (ADE).[8]

Key advantages of the well-known recombinant subunit vaccine platform are their safety, stability at 2-8 ^{o}C and facility to scale-up the production.[9] While weak immunogenicity for a small recombinant protein such as the RBD (30 kDa) should be expected, requiring repeated vaccination,[10] it has been found that recombinant RBD in alum is sufficient to induce a neutralizing immune response in laboratory animals,[11] while its simplicity prompted subsequent evaluation in humans.[12] However, the most promising recombinant vaccines relied on large RBD-containing macromolecular constructs as a way to increase the immunogenicity and on the use of potent adjuvants.[13] In addition to the lower immunogenicity, small recombinant RBD exposes to the immune system not only the critical RBM surface but also RBD regions that are well-camouflaged at the virus surface. Antibodies directed to camouflaged RBD regions are not neutralizing, and therefore, an ideal construction should maximize the exposure of the RBM and not of such regions. We hypothesized that the orientation of RBD, when conjugated to a large carrier like tetanus toxoid, exposes better the RBM surface, thereby likely increasing the level of neutralizing antibodies.[14-17]

The SARS-CoV-2 RBD comprises 193 amino acid residues from Thr333 to Pro527, including RBM 438-506 region that interacts directly with ACE2. This domain contains eight cysteines forming four disulfide bridges, three of these stabilizing the RBD core and one within the RBM.[6] Our recombinant RBD 319-541 was obtained in CHO-cells with an 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 spike 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 an unpaired Cys538 intended to be used for chemical conjugation to the highly immunogenic carrier tetanus toxoid (TT). Here we report a promising vaccine candidate based on this high molecular weight conjugate with several copies of recombinant RBD per molecular unit. To our knowledge, neither chemically conjugated constructs nor the immunogenic effect of conjugating viral proteins such as RBD to a protein carrier have been assessed for SARS-CoV-2 or other coronaviruses. Here we demonstrate that RBD-TT conjugates induce a potent immune response in laboratory animals, paving the way for their evaluation in human phase I and II clinical trials.[18]

Results and Discussion

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, the resulting macromolecular construct is endowed with the multivalent RBD display required for a potent B-cell activation. At the same time, the RBM would be well exposed (Fig. 1, represented in red) and likely more available for immune recognition than other immunodominant epitopes present in the RBD core. To achieve this, a site-selective conjugation

by a RBD's residue spatially distant from the RBM is required, a process that should be reproducible and efficient even at large scale to allow a cost-effective vaccine production.

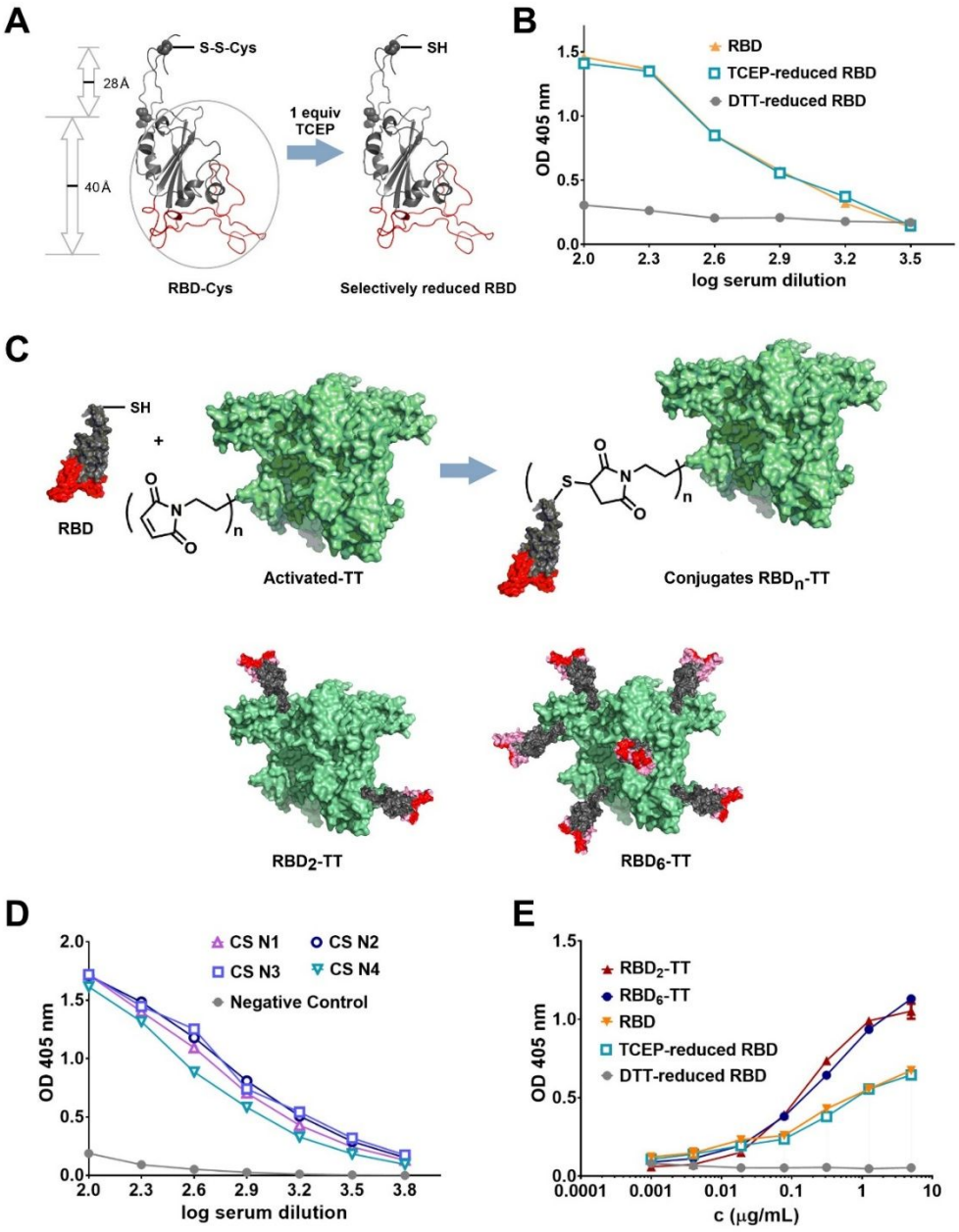


Fig. 1. Synthesis of RBD-TT conjugates. (**A**) Selective reduction of the intermolecular disulfide bond at RBD Cys538 using TCEP. (**B**) Recognition of recombinant RBD and reduced-RBDs by a convalescent serum. (**C**) Conjugation of TCEP-reduced RBD to TT and representation of RBD_2-TT and RBD_6-TT conjugates. (**D**) Recognition of a RBD-BSA conjugate by four convalescent sera (CS), n=1-4. (**E**). Binding to ACE2 of RBD_2-TT and RBD_6-TT conjugates.

We expressed in CHO cells the recombinant RBD(Arg319-Phe541-$(His)_6$) bearing a flexible C-terminal fragment that includes unpaired Cys538, a residue that is very amenable for conjugation and placed far away from the RBM. However, inclusion of an additional free Cys538 in the extended RBD structure could jeopardize RBD folding due to potential disulfide (S-S) rearrangement with the other 8 cysteines (scrambling). Nevertheless, we found that during fermentation and purification, Cys538 is spontaneously protected through an intermolecular S-S bond with free cysteine present in the culture media, while part of the RBD forms a dimer that is separated from the monomer. ESI-MS showed the presence of the four native S-S bonds, indicating a correctly folded RBD (see the Supporting Information). A key step to achieve a site-selective conjugation is the selective reduction of the intermolecular S-S bond to let Cys538 thiol free without affecting the other four intramolecular bridges that maintain the native RBD conformation. After an extensive derivatization study of reaction time and stoichiometry of various reducing agents, we found that cysteinylated Cys538 could be selectively reduced to free thiol upon treatment with 1 equiv. of tris-(2-carboxyethyl)phosphine (TCEP) for 10 minutes without affecting the native conformation, as proven by recognition with a convalescent serum (Fig. 1B) and with ACE2 (Fig. 1E). In contrast, RBD reduction with dithiothreitol (DTT) led to complete loss of its recognition capacity, suggesting loss of the antigen's 3D structure (Fig. 1B). To prove that the derivatization takes place selectively at Cys538, we carried out the controlled reduction with 1 equiv. of TCEP followed by incubation with 5 mM of *N*-ethyl maleimide (NEM) for 16 h at 4 °C (typical conjugation conditions) to evaluate the integrity of the intramolecular disulfide bonds by ESI-MS. After deglycosylation with PNGase F, the derivatized RBD was digested with trypsin using an in-solution buffer-free digestion protocol.[19] Analysis of the ESI-MS spectra of the tryptic peptides corroborates NEM addition at Cys538, while the other four disulfide bonds were not noticeably affected (see the Supporting Information).

With the site-selective RBD derivatization method in hand, we turned to obtain RBD-TT conjugates for immunogenicity evaluation. 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.[20,21] The presence of multiple T- and B-cell epitopes of this highly immunogenic carrier[22] can potentiate cellular immunity when compared to the use of RBD alone. In addition, multimeric RBD-TT could simultaneously activate several B-cell receptors, thus enhancing B cell response.[23] Considering the urgency to obtain at large scale a safe and effective, but also affordable COVID-19 vaccine, we relied on a conjugation technology that has previously proven success in clinically validated conjugate vaccines.[20] To this end, TT was activated with approximately 20–30 maleimide groups per mol of TT by treatment with *N*-succinimidyl 3-maleimidopropionate (SMP), followed by reactions with 2.5 and 10 equivalents of TCEP-reduced RBD to produce conjugates bearing an average of 2 and 6 mol, respectively, of RBD per mol of TT (Fig. 1C). The amount of RBD conjugated to TT in each reaction was assessed by size-exclusion HPLC (SE-HPLC) quantitation in the crude reaction prior to purification step used to remove unreacted RBD. The RBD_2-TT and RBD_6-TT conjugates were obtained in 72% and 64% yield, respectively, and characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), SE-HPLC and ACE2 recognition assay. Several lots of these conjugates were produced next under good manufacturing practices (GMP) at variable scales, with similar conjugation yield, notable reproducibility and stability proven, at least, over 6 months at 2-8 °C. As shown in Fig. 1E, both conjugates recognize ACE2 slightly better than the original RBD, confirming the preservation of the RBD structure and the suitable exposition of RBM. As convalescent serum usually contains TT antibodies, we also prepared a RBD-bovine serum albumin (BSA) conjugate incorporating 6 RBD units per mol of BSA (RBD_6-BSA), which was recognized remarkably well by convalescent sera (Fig. 1D). Collectively, these results

demonstrate the conservation of the RBD antigenic properties after site-selective carrier conjugation at Cys538 and the synthetic suitability of fine-tuning the number of RBD copies that can be incorporated to achieve the desired multivalent RBD display.

Animal Immunogenicity

Immunization of BALB/c mice with the four different immunogens (Fig. 2) induces a strong IgG RBD–specific immune response as proven by ELISA. RBD_6-TT/alum and RBD_2-TT/alum were compared with RBD/alum and with RBD_2-TT without alum. After the first dose (T7 and T14, Fig. 2C) RBD_6-TT/alum induced the highest level of anti-RBD antibodies. After the second dose, all immunogens adsorbed in alum elicited better anti-RBD IgG levels than without alum (T21 and 28, Fig. 2C). The high and homogeneous early response for RBD_6-TT/alum could be an important attribute for a vaccine in pandemic times. We explored early response to different dosage of RBD_6-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, Fig. 2C).

To evaluate possible immunological advantages of the RBD-TT conjugates, we studied affinity maturation of antibodies elicited by RBD_6-TT compared with the rest of the immunogens. There was an increase in the avidity index (AI),[24] with the highest value of 81% for antibodies induced by RBD_6-TT being consistent with a more pronounced affinity maturation (Fig. 2D). The Th1/Th2 balance can be modulated by vaccination and was also evaluated (Fig. 2E). A biased Th2 immune response was observed for RBD_2-TT/alum (IgG2a/IgG1 ratio 0.54) and RBD/alum (IgG2a/IgG1 ratio 0.40), while RBD_6-TT/alum displayed more balanced Th1/Th2 immunity (IgG2a/IgG1 ratio 0.81).

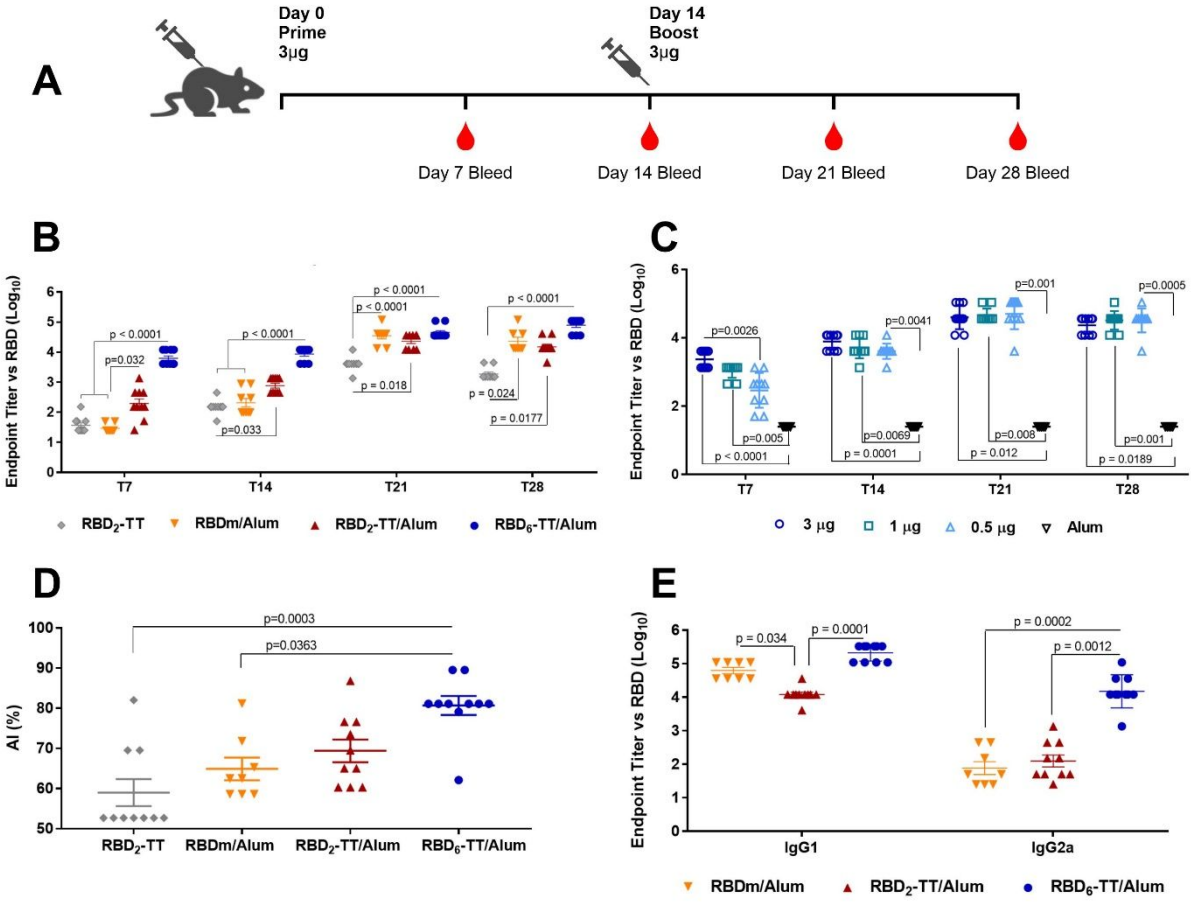


Fig. 2. Immunogenicity Evaluation. Immunization of BALB/c mice with RBD_2-TT/alum and RBD_6-TT/alum compared to RBD/alum and RBD_2-TT**.** The serum of individual mice is represented by ▼ (RBD/alum), ▲ (RBD_2-TT/alum), ◆ (RBD_2-TT) and ● (RBD_6-TT/alum). (**A**) Representation of the immunization protocol. (**B**) anti-RBD specific IgG at days 7, 14, 21, and 28. (**C**) Dose response to RBD_6-TT/alum on days 7, 14, 21 and 28. (**D**) Avidity index of antibodies elicited on day 28. (**E**) RBD-specific IgG1 and IgG2a.

Fig. 3 shows the induction of memory antigen-specific B and T-cells, an important property of conjugate vaccines. In this experiment, splenocytes from mice immunized with two doses of conjugate RBD_6-TT/alum were taken at day 28, purified and intravenously transferred to naïve mice that were then boosted by a single dose of 3 µg RBD/alum. In parallel, mice not previously

receiving splenocytes from immunized mice were also primed with a dose of 3 μg RBD/alum for comparative purpose. As shown in Fig. 3B, mice receiving splenocytes from RBD_6-TT/alum-immunized animals responded with a strong secondary RBD–specific IgG response (titers 10^3-10^4) after 7 days, while those only receiving the RBD/alum prime showed a typically poor primary response to this antigen. This finding demonstrated the presence of RBD-specific memory B cells in the transferred splenocytes, which were able to be activated after a stimulus with RBD/alum (here considered as alternative to SARS-CoV-2 virus).

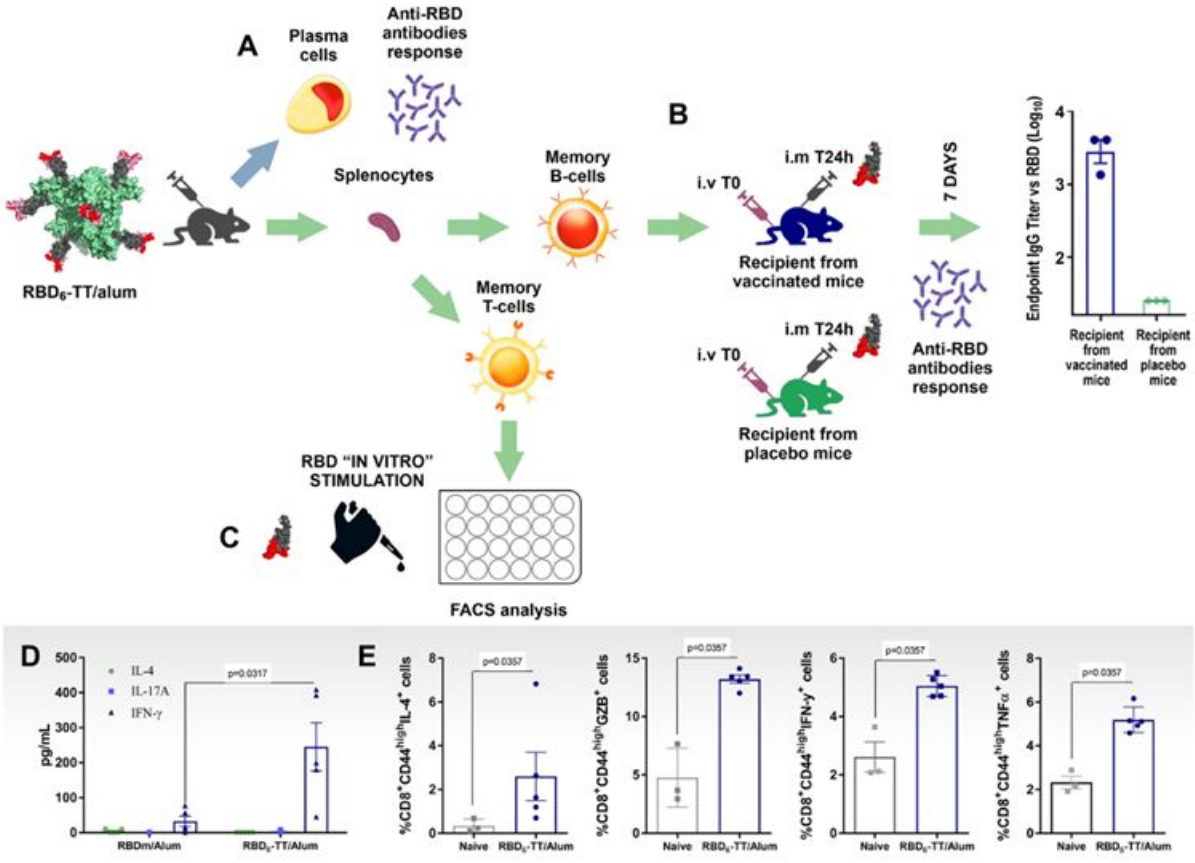


Fig. 3. Memory B and T cells induced by RBD_6-TT. (**A**) Representation of the primary immune response to RBD_6-TT/alum (blue arrow). (**B**) Classical passive transfer of splenocytes from RBD_6-TT/alum BALB/c and stimulated with RBD/alum (strong secondary response after day 7). (**C**) T-cell stimulation with RBD. (**D**) Cytokine secretion after *in vitro* RBD stimulation. (**E**) % RBD-

specific memory T $CD_8^+CD_{44}^{high}IL\text{-}4^+$; % RBD-specific memory T $CD_8^+CD_{44}^{high}Granzyme^+$; % RBD-specific memory T $CD_8^+CD_{44}^{high}IFN\gamma^+$; % RBD-specific memory T $CD_8^+CD_{44}^{high}TNF_\alpha^+$.

Specific CD8+ T cells also play an important role in protection against SARS-CoV-2, as recently demonstrated.[25] To evaluate the specific T-cell response, we compared RBD_6-TT/alum and RBD/alum immunized mice. After *in vitro* RBD stimuli of both groups of animals, splenocytes from mice immunized with RBD_6-TT secreted higher levels of IFNγ compared to those immunized with RBD/alum (Fig. 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-γ, TNF-α and Granzyme B increased significantly in RBD_6-TT immunized mice with respect to control mice (Fig. 3E), as shown by flow cytometry, indicating the relevant activation of cytotoxic T immune memory.

Antibody functionality

We evaluated antibodies' ability to block interaction between the virus and its receptor,[10] using the molecular Virus Neutralization Test ($mVNT_{50}$)[26] and the conventional Virus Neutralization Test ($cVNT_{50}$).[27] $mVNT_{50}$ evaluates the inhibition of interaction between recombinant RBD and ACE2 at the molecular level; while at the cellular level, $cVNT_{50}$ 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_2-TT/alum and RBD_6-TT/alum were compared to antibodies elicited after immunization with RBD/alum. $mVNT_{50}$ showed a high level of inhibition for all sera (Fig. 4A), indicating that all tested antibodies displayed a similar efficacy in interfering with RBD-ACE2 interaction at the molecular level. On the other hand, $cVNT_{50}$ (Fig. 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, while for both conjugates

there was a much higher level of virus neutralization, i.e., 1303 for RBD_2-TT and 2568 for RBD_6-TT. The $mVNT_{50}/cVNT_{50}$ ratio was 0.143, 0.732 and 1.08 for RBD, RBD_2-TT and RBD_6-TT, respectively. As depicted in Fig. 4C, while antibodies neutralizing the virus are mainly directed at the RBM,[7] there are antibodies recognizing soluble RBD not only by the RBM but also by a different lateral region. This type of 'lateral' antibodies could contribute to $mVNT_{50}$ with soluble monomeric RBD (RBDm), but probably will not recognize this RBD region that is camouflaged in the spike protein at the virus surface.

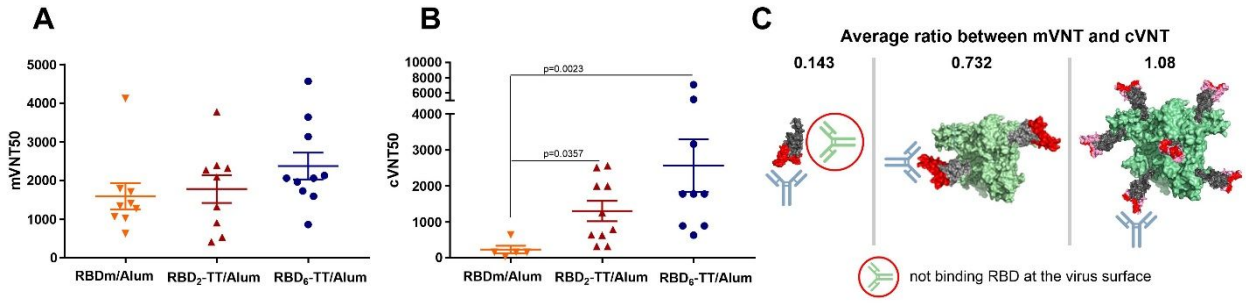


Fig. 4. Virus neutralization by anti-RBD antibodies induced by RBD-TT conjugates. (**A**) $mVNT_{50}$ representing the serum dilution giving 50% inhibition of ACE2-RBD interaction. (**B**) $cVNT_{50}$ measured as serum dilution giving 50% of virus neutralization (**C**) $mVNT_{50}/cVNT_{50}$ ratio and a possible schematic representation of the differences found between RBD and the conjugates.

Conclusions

We demonstrated that after a site-selective functionalization of the SARS-CoV-2 RBD followed by chemical conjugation to a highly immunogenic carrier protein rendered conjugates with the proper RBD orientation, as shown by recognition studies. This was achieved by employing an extended RBD that incorporates the well-exposed Cys538 at the C-terminal tail placed far away from the key RBM region, so conjugation did not block the neutralizing RBM epitopes by steric hindrance despite the large size of TT. However, expressing the antigen with an unpaired Cys was

not enough, because the reactive thiol side chain turned out to be capped with an additional Cys. Accordingly, the key step of this strategy was the selective reduction of the intermolecular S-S bond at the Cys538 without affecting the four S-S bridges that preserve the antigenic RBD conformation.

Another advantage of the conjugation technology here employed is the possibility to adjust the number of RBD units attached to the carrier. In this case, this proved to be important since RBD_6-TT showed better immune response than RBD_2-TT, especially in the early response. The superior multivalent display achieved in RBD_6-TT, compared to RBD_2-TT, might enable a more suitable cross-linking of B-cell receptors,[23,28] thus helping explain the higher IgG response of the larger conjugate construction. We also proved that conjugation to TT led to a notable enhancement of the neutralizing response, likely due to the better spatial accessibility of the RBM compared to other 'lateral regions', which concentrates the antibody response towards this motif. In contrast, in the soluble monomeric RBD, the RBM is as accessible as other immunodominant epitopes, thus hampering the neutralizing effect that depends on targeting the RBM. Finally, when considering application in humans, a further benefit of RBD-TT conjugates is the carrier-specific pre-existing T helper cells, which can help improve antibody production.

Based on the results presented here, various GMP batches of the conjugates RBD_2-TT and RBD_6-TT were produced and absorbed on alum as final vaccine candidates. A phase I clinical trial[29] was initiated in October 2020, and after preliminary results confirming a better performance of a vaccine based on RBD_6-TT/alum, this candidate advanced on December 2020 to a phase II clinical trial with 910 subjects.[30] Whereas clinical trial results will be reported in due course, the encouraging interim data prompted moving forward to a phase III trial in March 2021. The resulting vaccine composed of a RBD_6-TT conjugate, the first one developed and produced in a Latin-American country, has important advantages to consider it as a promising and available

candidate. These are: a) it induces a strong IgG neutralizing antibody as well as specific T-cell response, b) the well-known safety record of this vaccine platform (which is being confirmed in our clinical trials), c) the storage temperature of 2-8 ^{o}C is the desirable one for vaccines to achieve a rapid distribution worldwide, and finally, d) both the expression and conjugation technologies can be adapted to existing vaccine production capacities available at several countries. Some of these advantages are a 'must' for developing nations, in which not only the distribution chain but also recombinant and synthetic capacities are well prepared for producing a conjugate vaccine like this one, but not others currently licensed. The results shown here open a venue of possibilities for the generalization of the conjugate vaccine technology for SARS-CoV-2 and other coronaviruses. Finally, we sincerely expect that this vaccine completes clinical evaluation and receives approval, so it can contribute to win the battle against the COVID-19 pandemic.

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Author contributions

Y.V.B., D.G.R., and V.V.B. designed and led the study. S.F.is the manager of the project. D.G.R. and D.S.M. designed and directed the conjugation protocols and characterization. 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.P., 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 MS studies of the conjugates G.R., E.R-H., Y.C.I., 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. contributed to the study design and analysis. All authors revised the manuscript and approved submission.

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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