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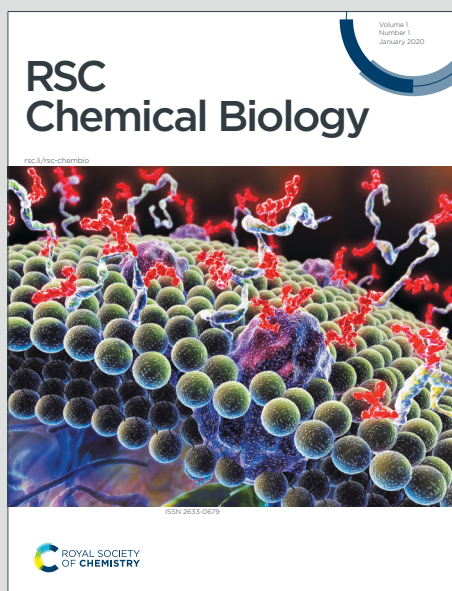
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A COVID-19 vaccine candidate composed of SARS-CoV-2 RBD dimer and *Neisseria meningitidis* outer membrane vesicles

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Dedicated to the memory of Prof. José Luis García Cuevas

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Abstract

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SARS-CoV-2 infection is mediated by the interaction of the spike glycoprotein trimer via its receptor-binding domain (RBD) with the host's cellular receptor. Vaccines seek to block this interaction by eliciting neutralizing antibodies, most of which are directed toward the RBD. Many protein subunit vaccines require powerful adjuvants to generate a potent antibody response. Here we report on the use of a SARS-CoV-2 dimeric recombinant RBD combined with *Neisseria meningitidis* outer membrane vesicles (OMVs) and adsorbed on alum as a promising COVID-19 vaccine candidate. This formulation induces a potent and neutralizing immune response in laboratory animals, which is higher than that of the dimeric RBD alone adsorbed in alum. Sera of people vaccinated with this vaccine candidate, named Soberana01, show a high inhibition level of the RBD-ACE2 interaction using RBD mutants corresponding to SARS-CoV-2 variants of concern and wild-type expressed using the phage display technology. To our knowledge, this is the first time that the immunostimulation effect of *N. meningitidis* OMVs is evaluated in vaccine candidates against SARS-CoV-2.

Introduction

COVID-19 vaccines in use or clinical evaluation seek to induce a protective immunity mainly by eliciting neutralizing antibodies (NAbs)^{1,2,3,4} that block the interaction^{5,6,7,8} between the SARS-CoV-2 receptor binding domain (RBD) of the spike (S)-glycoprotein and the host's cell surface receptor, the angiotensin-converting enzyme 2 (ACE2). Despite the rapid emergence of new platform technologies such as mRNA vaccines delivered by lipid nanoparticles and viral vector vaccines,^{1-4,9} recombinant subunit vaccines remain among the most effective ones to fight SARS-CoV-2 due to their proven safety, clinical efficacy and ease of distribution.^{1-4,10,11} Two main types of COVID-19 subunit vaccines have advanced to clinical evaluation,¹⁻⁴ those based on the full-length spike (S)-glycoprotein ectodomain^{11,12} and those using the RBD as antigen.^{11,13} Whereas the full S-glycoprotein is more immunogenic, the RBD is easier to produce and it also elicits a high-quality



neutralizing response.¹³ The RBD can be obtained as a recombinant protein in a variety of expression systems, including insect cells,^{8,14} yeast (*P. pastoris*)^{15,16,17} and mammalian cells (CHO and HEK293).^{5,16,18}

Different strategies have been implemented to increase the immunogenicity of RBD-based immunogens, including the use of potent adjuvants and multivalent antigen display in larger macromolecular constructs.¹³ Recombinant RBD has been conjugated to large protein carriers as tetanus toxoid¹⁹ and incorporated into self-assembled nanoparticles,^{13,20} leading to highly immunogenic multivalent constructs. Another approach has been the use of dimeric RBD immunogens, which have been produced in the form of fusion RBD-Fc chimeras^{21,22} or as an RBD dimer with a tandem-repeat single chain (sc-dimer) connecting the two RBD units.²³ This RBD-sc-dimer – called vaccine ZF2001 – has completed phase I and II clinical trials²⁴ with a three-dose schedule, proving to be safe and producing very high seroconversion of NABs and a moderate cellular immune response.

Our endeavor on the use of recombinant RBD as antigen focuses on exploring different alternatives to improve its immunogenicity. RBD(Arg319-Phe541-(His)₆), which intentionally includes unpaired Cys538,¹⁹ was expressed in CHO cells as a mixture of RBD monomer and dimer. RBD monomer (RBD-m) was site-selectively conjugated to tetanus toxoid (TT) for producing an RBD-TT conjugate vaccine candidate,¹⁹ which recently completed phase III clinical evaluation under the name SOBERANA02 and received emergency approval in Cuba.²⁵ Here we report on the development of a new vaccine candidate, SOBERANA01, containing the recombinant RBD dimer (RBD-d) and *Neisseria meningitidis* outer membrane vesicles (OMVs) adsorbed in alum. We recently communicated the successful use of the RBD-d adsorbed in alum as booster vaccine for COVID-19 convalescents.²⁶ Here, we describe the preparation and characterization of the RBD-d and compare the immunogenicity evaluation of formulations either containing or not *N. meningitidis* OMVs. Assessment of the RBD-ACE2 interaction inhibition of sera from vaccinated people using phage-



displayed RBD mutants presents in variants of concern (VOC) is also described, which sheds light into effect of the adjuvant in the neutralizing response not only in mice but also in humans.

Results and Discussion

Vaccine design

N. meningitidis OMVs, at 50 μg per dose, are a component of antimeningococcal vaccine VAMENGOC-BC[®] that is produced on large scale in Cuba and from which nearly 70 million doses have been applied in humans.²⁷ *N. meningitidis* OMVs are not only protective against meningitis, but also have well-known adjuvant properties associated with stimulation of neutrophils,²⁸ bone marrow-derived dendritic cells²⁹ and macrophages,³⁰ which are key players of the innate immune system recognizing pathogen-associated molecular patterns through toll-like receptors (TLR). Stimulation of dendritic cells and macrophages induces the production of two proinflammatory cytokines IL-1 β and IL18, serving as a bridge with the adaptive immune system.^{31,32} These properties prompted us to explore the capacity of *N. meningitidis* OMVs for improving RBD-d immunogenicity initially in mice, and in case of satisfactory results, extend the study to evaluation in humans.

Preparation and characterization of the RBD dimer

The formation of stable disulfide-linked dimeric RBD has been observed for MERS-CoV RBD (Glu367-Tyr606)²³ expressed in CHO cells and for SARS-CoV-2 RBD(Arg319-Phe541) expressed using the Bac-to-Bac baculovirus system.⁸ We focused on the production in CHO cells of the SARS-CoV-2 RBD with the sequence from residue 319 to 541. This system proved to efficiently produce RBD-d bearing an intermolecular Cys538-Cys538 disulfide bond (Fig. 1A), along with a monomeric RBD-Cys adduct due to cysteinylolation of Cys538 with free cysteine present in the culture medium.¹⁹ The RBD-d was separated from the monomer using size exclusion chromatography (SE-HPLC) in a Superdex 200 column (Fig. 1B). Consistent with the SE-HPLC results, dynamic light scattering (DLS) indicated the greater hydrodynamic diameter of the RBD dimer compared to the monomer



(Fig. S1, Supplementary Information), The monomeric RBD-Cys adduct can be decysteinyliated¹⁹ and eventually converted into RBD-d under oxidative conditions.

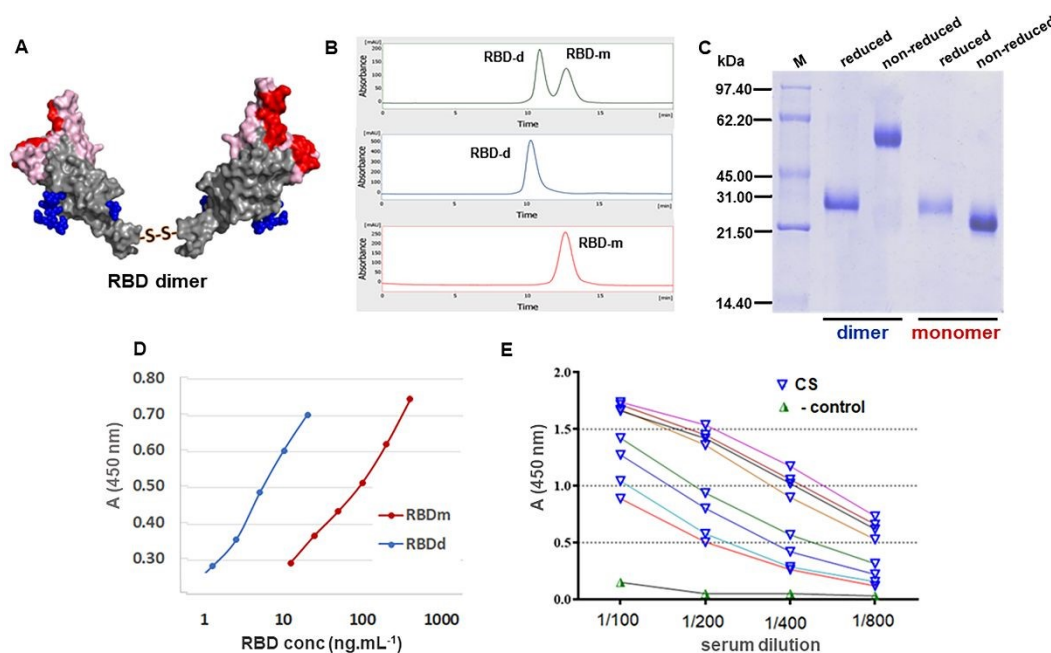


Figure 1. Characterization of the recombinant RBD dimer. **A)** Structure of RBD dimer (red: amino acids contacting ACE2 receptor upon binding, pink: RBM, blue: glycan chains, grey: RBD core). **B)** SE-HPLC chromatograms of the RBD-d/RBD-m mixture and the purified antigens. **C)** RBD-d and RBD-m in reducing and non-reducing SDS-PAGE. **D)** Interaction of RBD-d and RBD-m with the ACE2 receptor expressed in Vero cells. **E)** Recognition of RBD-d by eight convalescent sera from a Cuban panel.

As shown in figure 1 C, reduction of dimer into monomer was verified on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by a band shift from ~60 kDa in non-reducing gel electrophoresis to ~30 kDa under reducing conditions. The ESI-MS spectrum of the PNGase-F deglycosylated RBD-d showed multiply-charged ions due to the heterogeneous nature of *O*-glycans (Fig. S3 in the SI). The experimental molecular masses determined by ESI-MS confirm: a) the dimeric nature of this antigen, b) the presence of the four disulfide bonds at each RBD unit and the nature of the *O*-glycosylations at Thr323/Ser325 residues (Table S1). In addition, after deglycosylation with PNGase F in presence of *N*-ethylmaleimide (NEM), the RBD dimer was digested with trypsin using an in-solution buffer-free



digestion protocol,³³ which allowed a full-sequence coverage including the identification of the intramolecular disulfide bonds. The confirmation of the disulfide bond linking the two RBD units at Cys538 came from the unequivocal identification of the 4+ and 5+ ions of the C-terminal peptide (Cys538-His547)-S-S-(Cys538-His547). The assignment of all proteolytic peptides are listed in Table S2 in the SI.

To confirm the correct exposure of the receptor-binding motif (RBM) in the dimeric antigen, we analyzed by ELISA the interaction of RBD-d with the ACE2 receptor either present at the surface of Vero cells (Fig. 1E) and with recombinant human ACE2 (hACE2-Fc, Fig. S4 in the SI). Compared to RBD-m, the dimeric antigen binds more efficiently to the ACE2 receptor. The RBD-d was also recognized by several convalescent sera from the Cuban panel (Fig. 1F), corroborating the good antigenic properties of this dimer.

Immunogenicity in mice

Previous immunogenicity studies of the RBD-sc-dimer proved that this immunogen induced a significantly higher anti-RBD IgG response compared to the RBD-m.²³ Accordingly, we also anticipated high immunogenicity for our disulfide-linked RBD-d, but at the same time, we sought to evaluate whether the potent innate-immunity stimulation with *N. meningitidis* OMVs can further improve the quality and the magnitude of the anti-RBD immunity. Two different formulations were prepared: a) RBD-d/OMV/alum (10 µg/4 µg/250 µg) and b) RBD-d/alum (10 µg/250 µg). Balb/c mice were immunized following a three-dose schedule (Fig. 2A). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Finlay Vaccine Institute and approved by its Animal Ethics Committee.

Compared to RBD-d/alum, the formulation containing RBD-d/OMV/alum induced a stronger IgG response already on day 14 after the first dose, and there was a significant ($p < 0.05$) increase in IgG titers at all times evaluated (Fig 2B). In addition to the greater anti-RBD antibody response, the avidity index increased from 63.8 for RBD-d/alum to 80.7 for RBD-d/OMV/alum (Fig. 2C).



Similarly, both IgG1 (5.05 vs 4.27) and IgG2a (3.89 vs 2.15) titers were superior ($p < 0.0001$) for RBD-d/OMV/alum than for the one lacking the bacterial OMVs (Fig 2D). The increase of IgG2a and a higher IgG2a/IgG1 ratio (Fig. 2E) is consistent with the detected greater production of IFN- γ (Fig. 2F). Altogether, these results indicate a more balanced Th1/Th2^{34,35} response for RBD-d/OMV/alum and, which is favorable for the prevention of COVID-19, as Th1 immune response helps viral clearing while Th2 cells associate to a poor prognosis.³⁶

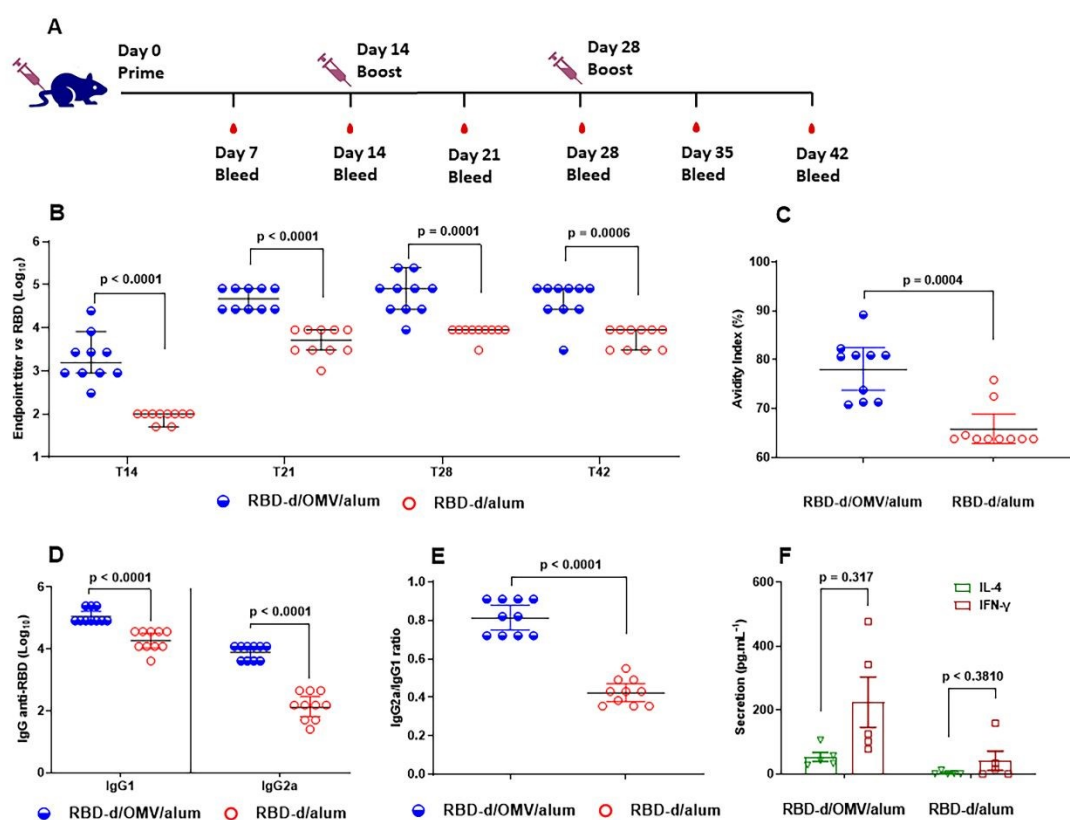


Figure 2. Preclinical immunogenicity evaluation. Immunization of BALB/c mice with RBD-d/OMV/alum (blue) and RBD-d/alum (red). **A)** Immunization protocol. **B)** anti-RBD IgG at days 14, 21, 28 and 42. **C)** Avidity index of anti-RBD IgG elicited on day 42. **D)** anti-RBD IgG1 and IgG2a titers. **E)** anti-RBD IgG2a/IgG1 ratio. **F).** Cytokine secretion (IL-4 and IFN- γ) after *in vitro* stimulation with RBD-d.

RBD-specific memory B cells. There was difference between RBD-d/OMV/alum and RBD-d/alum concerning the induction of RBD-specific memory B cells. As shown in Fig 3A, splenocytes



collected on day 42 from mice immunized with these formulations were transferred intravenously to naive mice that were then boosted with a single dose of 10 μg of RBD-d/alum. There was a secondary RBD-specific IgG response for both formulations, but more intense for RBD-d/OMV/alum, with titers as high as 10^2 to 10^3 on day 7 after immunization.

Inhibition of the RBD-ACE2 interaction. To assess the functionality of the elicited anti-RBD antibodies, we measured the inhibition of the RBD-ACE2 receptor interaction in a molecular virus neutralization tests (mVNT)³⁷ and a conventional virus neutralization test (cVNT₅₀) (Fig. 3B).³⁸ mVNT is based on antibody-mediated blockage of RBD-hACE2 interaction, and it is an *in vitro* surrogate of virus neutralization test, detecting neutralizing antibodies in a short time. It uses recombinant RBD mouse Fc (RBD-mFc) and the host-cell receptor hACE2-Fc as coating antigen. Anti-RBD antibodies recognize the RBD-mFc, blocking its interaction with the ACE2 receptor. RBD-mFc not inhibited by the antibodies can bind to hACE2-Fc, being recognized by a monoclonal antibody anti-gamma murine conjugated to alkaline phosphatase. This inhibition ELISA mimics the virus-host interaction at the molecular level. We initially evaluated the inhibition of the RBD-hACE2 interaction at 1/100 dilution of the sera of immunized mice. Mice immunized with the RBD-d/OMV/alum formulation gave a higher ($p < 0.0007$) and more uniform inhibition than those immunized with RBD-d/alum (Fig. 3B). mVNT₅₀, representing the serum dilution giving 50% inhibition of ACE2-RBD interaction, also resulted in a significant difference ($p < 0.0004$) in the inhibition titers induced by RBD-d/OMV/alum compared to RBD-d/alum (Fig. 3C). The superior neutralizing response ($p = 0.0013$) elicited by RBD-d/OMV/alum was also confirmed in the cVNT₅₀, which evaluates the inhibition of the interaction between the live SARS-CoV-2 virus and Vero E6 cells bearing ACE2 receptors. There was a higher level of neutralization for sera from most mice immunized with RBD-d/OMV/alum, while a lower NAb response was achieved after immunization with RBD-d/alum (Fig. 3D). In summary, *N. meningitidis* OMVs in the RBD-d formulation leads to an important increase in the NAb response in mice compared to formulations containing only alum



as adjuvant. The presence of this bacterial adjuvant also makes the anti-RBD IgG response in mice comparable to that of the vaccine Soberana02, whose immunogen is a RBD-tetanus toxoid conjugate adjuvated in alum.¹⁹

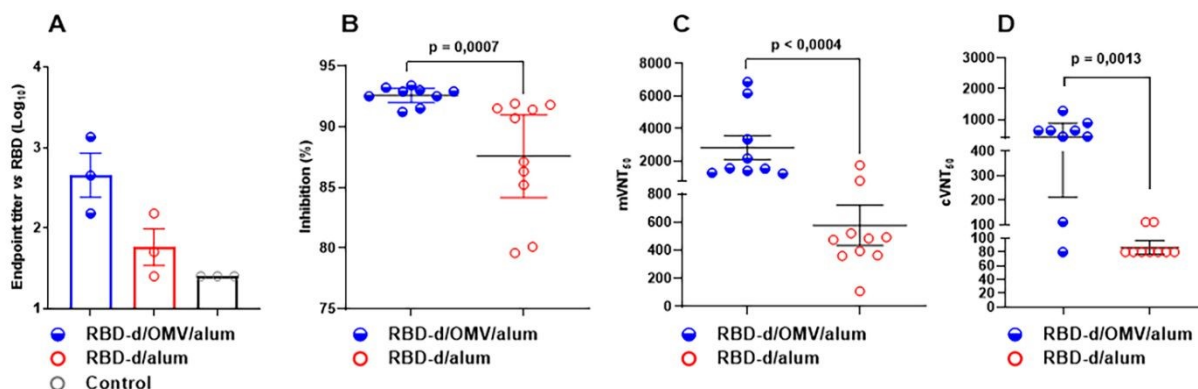


Figure 3. Virus neutralization by anti-RBD antibodies induced by formulations RBD-d/OMV/alum and RBD-d/alum. **A)** Classical passive transfer of splenocytes from BALB/c mice immunized with each vaccine formulation or placebo to naïve recipient mice and stimulated with RBD-d/alum (secondary response on day 7 after immunization). **B)** Percent of inhibition of the RBD-ACE2 interaction at 1/100 serum dilution. **C)** mVNT₅₀ representing the serum dilution giving 50% inhibition of RBD-ACE2 interaction. **D)** cVNT₅₀ measured as serum dilution giving 50% of virus neutralization.

Human immunogenicity

A phase I clinical trial was conducted to compare these and other vaccine candidates, in groups of 20 subjects following a three-dose schedule at intervals of 28 days.³⁹ While the safety, reactogenicity and immunogenicity were reported,⁴⁰ here we describe additional results which enable to further assess the effect of the OMVs in the quality of the antibody response in humans. The analysis was performed with sera from trial participants vaccinated with of RBD-d/OMV/alum (50 µg/20 µg/1250 µg) and RBD-d/alum (50 µg/1250 µg).

Figure 4A shows the anti-RBD IgG titer 28 days after the second (T=56) and third doses (T=84). The number of subjects with IgG concentration >70 AU/mL increased after the third dose with respect to



the second one: for RBD-d/OMV/alum from 13/20 (65%) to 18/20 (90%) and RBD-d/alum from 10/20 (50%) to 13/20 (65%).⁴⁰ Interestingly, while the IgG response at day 28 after the second (T=56) and third doses (T=84) was rather similar,⁴⁰ after the third vaccination the quality of the IgG response was certainly higher for both formulations on day 28, as determined by the inhibition of the RBD-ACE2 interaction. In mVNT₅₀, the percentage of subjects attaining a ID₅₀ > 150 was 90% for the formulation RBD-d/OMV/alum and 55% for that without OMVs (Fig 4B). These results confirm the higher quality humoral response after vaccination with the OMV-containing formulation found in the preclinical evaluation.

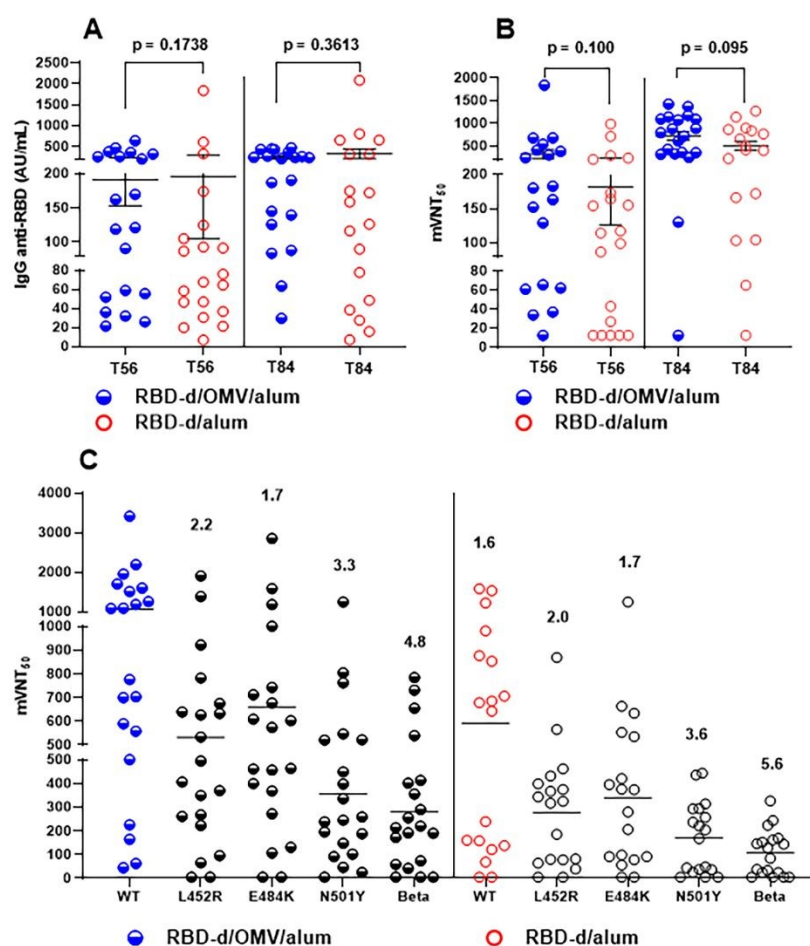


Figure 4. Immunogenicity evaluation from a phase I clinical trial.^{39,40} Subjects immunized with RBD-d/OMV (50 μ g/20 μ g, blue) vs RBD-d (50 μ g, red) adsorbed in alum. **A)** anti-RBD IgG titers. **B)** mVNT₅₀ representing the serum dilution giving 50% inhibition of RBD-ACE2 interaction. **C)** mVNT₅₀ comparing the inhibition of the interaction between phage-displayed RBD mutants and the



ACE2 receptor. Represented are the ratios of geometric mean titers (GMTs) between the RBD wild-type and the RBD mutants for each of the two vaccine formulations and the ratio of GMTs between the two formulations for the RBD wild-type.

Sera of vaccinated people were studied for their neutralizing effect against RBD mutants of SARS-CoV-2 variants of concern (VOC), and compared with that against the RBD wild-type (Arg328-Thr533). The RBD single mutants L452R, E484K and N501Y and the triple mutant K417N, E484K and N501Y, corresponding to the SARS-CoV-2 beta variant (B.1.351) were expressed using the phage display technology and used in a molecular virus neutralization test. As shown in Fig. 4C, the inhibition effect, determined as mVNT₅₀, was higher for sera of people vaccinated with OMV-containing formulation against the phage-displayed RBD wild-type and all mutants. For the RBD wild-type, the ratio of geometric mean titers (GMTs) between subjects vaccinated with RBD-d/OMV/alum and with RBD-d/alum was 1.6. We also measured the GMT ratios between the RBD wild-type and the mutants for both formulations, showing a similar decrease in GMTs for phage-displayed RBD mutants L452R, E484K and N501Y compared to the wild-type. However, for the triple mutant corresponding to the beta variant, the GMTs declined 4.8-fold for sera of people vaccinated with RBD-d/OMV/alum, while the decrease was 5.6-fold for people vaccinated with RBD-d/alum. Taken together, these results suggest that, in humans, the improvement in the quality of the antibody response due to the use of OMVs as adjuvant leads to a better neutralizing effect not only against SARS-CoV-2 wild-type but also its variants.

Conclusions

We have demonstrated the substantial increase in immunogenicity and neutralizing IgG response of recombinant dimeric SARS-CoV-2 RBD (319-541) when formulated together with *N. meningitidis* OMVs in alum. The RBD-d antigen bears an intermolecular S-S bond that connects the two RBD subunits, exposing well their receptor-binding motifs as demonstrated by its good antigenic



properties. The innate-immunity stimulation provided by the OMV adjuvant contributes not only to the higher humoral response and better IgG functionality, but also to a more balanced Th1/Th2 response. The formulation containing the bacterial OMVs elicits a more potent neutralizing effect against RBD mutants presents in VOCs, including the triple RBD mutants of the beta variant. These results, together with those of phase I clinical trial,^{40,40} pave the way to phase II and III clinical trials for the RBD-d/OMV/alum formulation. They also demonstrate the advantages of using *N. meningitidis* OMVs not only in antibacterial vaccines, but as adjuvants in antiviral protein subunit vaccines. This candidate, named Soberana01, adds a new asset to Cuba's research program on COVID-19 vaccines.

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Electronic Supplementary Information: Materials and methods, ESI-MS spectra, DLS and SE-HPLC chromatogram of the RBD dimer.

Competing interests

The authors declare no financial conflicts of interest. Some of the authors are co-inventors on a provisional SARS-CoV-2 vaccine patent (Cu 2020-57) based on these results.

Author contributions:

D.G.-R., Y.V.-B., and V.V.-B. conceived the idea and directed the research. D.S.-M., R.P.-N., Y.C., L.R., B.S.R., G.-W.C., and D.G.R. proposed original ideas and conducted experimental work and data analysis. S.P.-R., M.R., C. L., T.H., M.D., I.O., U.R., R.O., R.G., F.C., M.L., R.M., H.G., T.H., R.O.-A., J.L. P., J.E., N.G., Y.I., L.A.E., Y.R., L.J.G., C.V., A.V.C., B.F., G.R., B.P.-M., Y.T., E.B.,



C.A.P., T.B., E.O., F.C., S.F., F.P., C.F., and G.-W.C. contributed to the experimental work and characterization. D.G.R., D.G.-R., and V.V.-B. wrote the article with contribution of all authors.

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