Molecular Therapy
Nucleic Acids
Brief report

Broadly cross-reactive mRNA COVID-19 vaccine encoding trimeric RBDs and NSP12 mitigates immune imprinting

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The continuous evolution of SARS-CoV-2 variants, driven by mutations in the spike protein undermines viral recognition by antibodies elicited through prior infection or vaccination with the ancestral Wuhan strain. Original antigenic sin of SARS-CoV-2 ancestral virus or vaccine led to a weakened neutralizing antibody response against successive variants upon administration of an updated vaccine. On the contrary, T cells retain cross-reactivity thanks to the high density of conserved epitopes. We designed mRNA vaccines encoding single-chain heterotrimers of the receptor-binding domain (RBD) natural variants of interest (VOI), (RBD-VOI) and of phylogenetically informed consensus representing the major variant lineages RBD-consensus (RBD-Cons). We demonstrate a broad neutralizing activity against omicron subvariants and mitigated immune imprinting when RBD-Cons was used as a booster after conventional Wuhan spike priming. To enhance cellular immunity, we designed a second mRNA vaccine component encoding the viral polymerase NSP12 able to induce a cross-reactive T cell response to be combined with the heterotrimeric RBD vaccine. Our results offer a rational strategy for next-generation, imprinting-resistant vaccines.

INTRODUCTION

Since its emergence in 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a devastating global pandemic. Emergency coronavirus disease 2019 (COVID-19) vaccination significantly reduced deaths and hospitalizations.[1] The SARS-CoV-2 spike (S) glycoprotein, which mediates viral entry via the human ACE2 receptor, is the principal target of neutralizing antibodies. Within S, the receptor-binding domain (RBD) is the actual bait that interacts with human ACE2 and it contains immunodominant epitopes eliciting most of the neutralizing antibody response.[2] Since 2019, SARS-CoV-2 has continued to evolve under immune pressure, giving rise to successive variants of interest (VOI) and variants of concerns (VOCs) carrying mutations in the S protein. By late 2020–2021, the alpha, beta, gamma, and delta lineages were identified as VOCs. The beta and gamma variants, in particular, showed substantial resistance to neutralizing antibodies elicited by prior infection or vaccination with the ancestral Wuhan strain. The omicron variant emerged bearing a large number of mutations in the RBD and rapidly displaced prior VOCs. Its sub-lineages (BA.1, BA.2, BA.4, BA.5, BA.2.75, BQ.1.1, XBB, XBB.1.5, XBB.1.16, EG.5.1, and others) have since accumulated additional mutations conferring progressively enhanced immune escape.[2] A critical concern in this context is the reported immune imprinting, or "antigenic sin"; a phenomenon that arises when an initial antigenic exposure (i.e., Wuhan S) biases subsequent responses limiting recognition of new variant sites.[3] Recent evidence suggests that the immune imprinting constrains SARS-CoV-2 vaccine responses: individuals primed with three doses of the original vaccine exhibit persistent focus on ancestral epitopes, and a bivalent booster (with BA.4/5 plus Wuhan S) reinforces the recall of outdated immunity toward "old" specificities rather than broadening it. Thus, sera from individuals boosted with a bivalent (Wuhan + omicron BA.4/5) vaccines have 10- to 100-fold reductions in neutralizing titers against recent subvariants such as XBB lineages.[4] Therefore, overcoming antigenic imprinting represents a key goal in next-generation vaccine design. Both clinical and preclinical data showed that trimeric or multimeric RBD constructs generally elicited higher

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neutralizing responses than monomers, while maintaining safety profiles. Several recent studies explored multivalent or heterologous RBD immunogens incorporating multiple variant sequences in one construct to broaden the antibody repertoire by simultaneously exposing the immune system to multiple RBD epitopes. In a notable example, Liang et al. developed a single chain heterotrimer protein carrying RBD from the Wuhan, beta, and kappa strains. While this layout elicited higher neutralizing titers against delta and beta compared with a homotrimer containing three Wuhan RBDs, it was limited to pre-omicron variants and was implemented as a protein subunit vaccine. Similarly, in 2022, Zhang et al. described a mosaic trimeric RBD (mos-tri-RBD) incorporating key mutations from omicron and other circulating variants, which induced a potent cross-neutralizing antibodies against omicron and other immune-evasive variants.[5] However, mos-tri-RBD was still developed as recombinant protein, which suggests a laborious effort for a rapid updating with emerging variants compared to genetic vaccines. Nevertheless, none of the above constructs excluded the original Wuhan RBD to minimize the antigenic imprinting or employed a systematically phylogenetic consensus sequence to select variant representatives.

Here, we designed a next-generation mRNA vaccine that leverages a phylogenetically informed RBD-consensus (RBD-Cons) heterotrimers. By analyzing the evolutionary tree of SARS-CoV-2 lineages, we derived synthetic consensus RBD sequences representing distinct VOCs lineages, such as the delta versus the omicron branch, while deliberately omitting the ancestral Wuhan sequence. These consensus RBDs were assembled into a heterotrimeric immunogen (RBD-Cons), expressed as linked monomers or trimeric fusions, and encoded in mRNA-liponanoparticles (LNPs). This next-generation vaccine was systematically compared with conventional full length S vaccine and with homo- and hetero-RBD trimers demonstrating superior activity. This approach aims to focus immune responses on epitopes shared among multiple VOCs and on new immune-escape mutations, thereby maximizing cross-variant breadth while circumventing original antigenic sin. The use of mRNA delivery further enables rapid adaptation to future variant evolution.

Beyond neutralizing antibody responses, effective protection against coronaviruses also relies on cellular immunity. Many studies have found that many healthy people harbor pre-existing T cells reactive to SARS-CoV-2, likely due to prior exposure to endemic human coronaviruses.[6] Lung tissue from pre-pandemic donors contained $CD4^+$ and $CD8^+$ tissue-resident T cells recognizing SARS-CoV-2 epitopes, and these individuals exhibited stronger immunity to seasonal coronaviruses. Likewise, circulating $CD4^+$ T cells targeting SARS-CoV-2 S peptides are common in uninfected individuals and expand rapidly following infection or mRNA vaccination. These pre-existing cross-reactive memory T cells appear to "jump-start" the immune response upon SARS-CoV-2 exposure, leading to faster antibody production and often an asymptomatic or milder disease outcome.[7] We formulated two mRNA vaccine components: one, expressing a trimeric SARS-CoV-2 RBD (in different layouts) to elicit broadly neutralizing antibodies, and a second, encoding the viral RNA polymerase—NSP12, a component of the replicase polyprotein highly conserved across SARS-CoV-2 variants and even among other beta coronaviruses, thereby resulting as an ideal T cell antigen. This combination vaccine design aims to achieve on one hand a broadly neutralizing antibody response and on the other hand a cross-reactive T cell response, with the objective to elicit a broad immune responses against current and future coronavirus variants.

RESULTS

Antigen design: Clustering of RBD sequences and NSP12 conservation

As a first step, to identify the most relevant genetic drifts, we examined the evolutionary conservation of the S RBD from VOI and VOC using a phylogenetic framework. All RBD sequences available up to the end of 2023 were retrieved, aligned, and clustered to assess patterns of divergence and the emergence of distinct evolutionary lineages. Eight major branches were identified, each defined by a consensus sequence (Figure 1A). Clusters were labeled according to the predominant variant, with the rightmost super-branch composed exclusively of omicron clades. The recurrence of the antigenic epitope in the SARS-CoV-2 sub variants was retrieved from the Immune Epitope Database (IEDB; https://www.iedb.org/) and was depicted in Figure 1B showing the 95% confidence interval (CI) of response frequency (RF) for each position averaged over all epitopes mapped to that position. In Figure 1C, we depicted the number of positive and negative assays along the length of S's RBD. Given the physiological trimeric quaternary structure of S protein, the immunological relevance or receptor binding domain of S protein and mRNA vaccine platform constraints, a trimeric design based on head-to-tail trimeric RBD was selected. Based on previous reports and identification of natural linker sequences bystander canonical RBD, the residue 331–532 amino acids (aa) were selected from full-length S. We validated the stability and expression of monomer (Figures S2A–S2C) and we designed a first version of trimeric RBD based on head-to-tail linkage of prototype Wuhan, and original delta (B.1.617.2) and omicron (BA.1) variants (Figure S2D). This construct is hereinafter referred to as RBD-VOI.

In parallel, given the essential enzymatic activity of RNA-dependent RNA polymerase (RdRp), the viral non-structural proteins, NSP12 was identified as potential conserved antigen shared by all SARS-CoV-2 variants. We validated this conservation in SARS-CoV-2 variants (Figure S1), and also among NSP12 of many human and non-human *Coronaviridae*. In order to align the aa sequence of the NSP12 protein, we extracted its sequence from the polyprotein encoded within open reading frame 1 of the indicated viruses. We reported an identity ranging from 84% to 50%. This level of sequence identity is markedly high (>71%) for human pandemic coronaviruses such as SARS-CoV-1 (84%) and Middle East respiratory syndrome-related coronavirus (MERS, 72%) (Figure S1). However, it is also highly relevant for non-human viruses with documented or potential spillover capacity from bats (e.g., HKU4, HKU5, and HKU9). Moreover, the identity percentage remains elevated (>60%) for

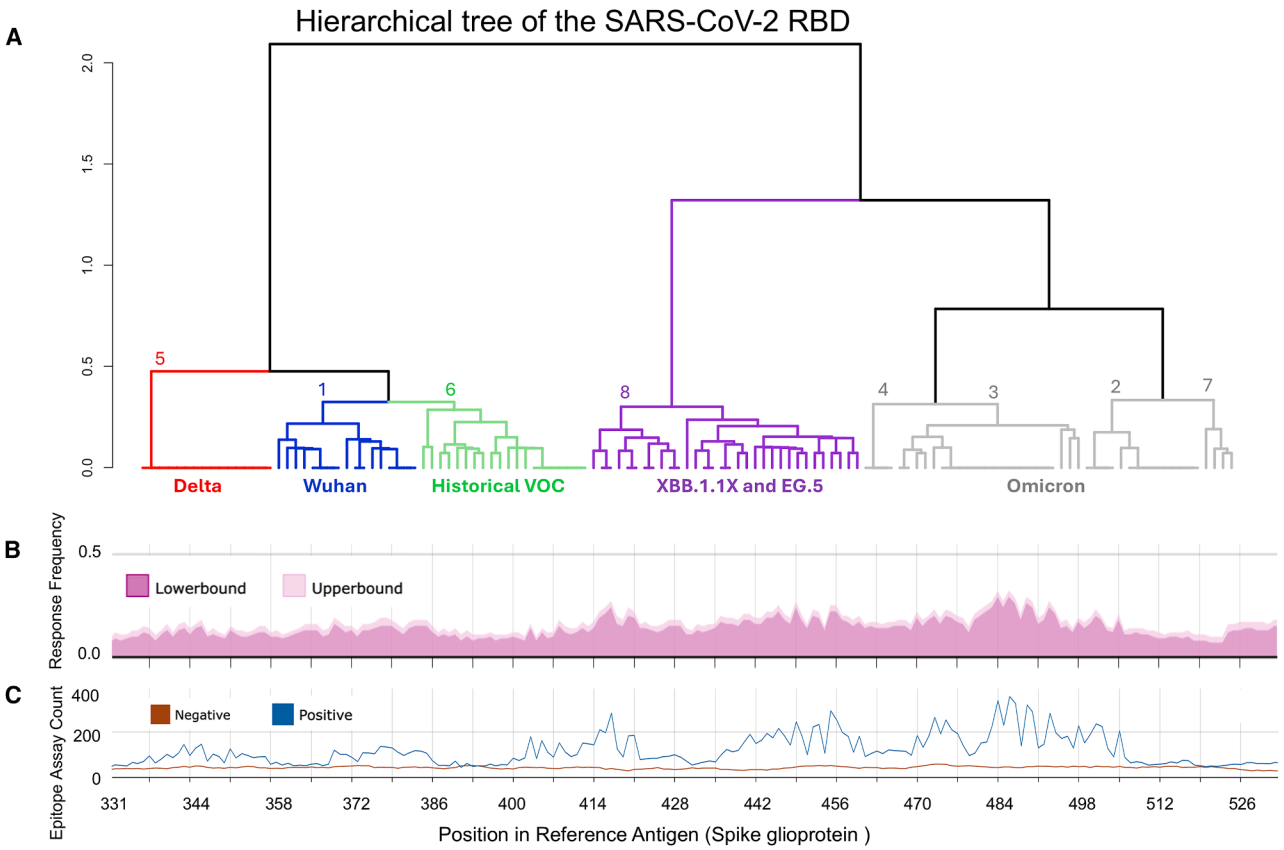


Figure 1. Bioinformatic analysis of spike protein

(A) Hierarchical tree of spike RBD: Ward's clustering of the identity distance of the sequences. Labels report the most representative variant for each branch. Branch 2, 3, 4, and 7 are all slightly different omicron variants. (B and C) epitopes along the length of the RBD of spike tested in B cell immune assays. (B) Plot representing the 95% confidence interval (CI) of response frequency (RF) for each position averaged over all epitopes mapped to that position. Upper and lower bound in light and dark color, respectively. (C) Plot representing the total number of assays (positive and negative, in blue and red, respectively) along the length of RBD of spike.

endemic human coronaviruses, including the alpha coronaviruses HCoV-229 E and HCoV-NL63, and the beta coronaviruses HCoV-OC43 and HCoV-HKU1 (Figure S1). Notably, such conservation is maintained even in coronaviruses that are phylogenetically distant from both human strains and SARS-CoV-2 (e.g., night heron HKU19, as well as various avian and porcine coronaviruses), likely reflecting the essential and conserved nature of their enzymatic functions (Figure S1). Moreover, as for the RBD of S, we exploited the IEDB looking for NSP12 antigenic epitopes of SARS-CoV-2 but we selected only the ones supported by T cell assays in at least 10 subjects. With the goal to assert in how many different species each epitope was conserved, we blasted each one individually against *Coronaviridae* (taxid:11118) database (percent identity > 90%). The positions of the epitopes on the NSP12 and their abundance in *Coronaviridae* family were depicted as a lollipop plot in Figure S1B.

In vivo immunogenicity

mRNA encoding the selected antigens (NSP12 and trimeric RBD-VOI) were formulated into LNPs. Full length S mRNA (Wuhan) recapitulating the clinically approved Spikevax (COVID-19 mRNA-1273) was used as control. BALB/c mice were immunized once with 80 μg/Kg of mRNA encoding S or NSP12 or the same amount of both. For NSP12, as it is naturally part of a polyprotein, we added an ectopic methionine as first aa. Three weeks post-treatment, the S specific or NSP12-specific T cell response was evaluated by IFN-γ ELISpot, confirming that both vaccines were immunogenic (Figure 2A). We then focused on the characterization of the RBD vaccine component, with the aim of generating a highly immunogenic construct capable of eliciting a potent cross-neutralizing response. Specifically, the RBD-VOI trimeric form described above (composed of the Wuhan, delta, and omicron RBDs) was compared to a monomeric Wuhan-derived RBD. As expected, the T cell response against the S1 subunit of S (which includes the RBD) was comparable between mice vaccinated with the monomeric or trimeric RBD (Figure 2B). Total IgG titers at 4 weeks post-immunization were also similar between the monomeric and trimeric groups, although a slight trend toward higher titers was observed with the trimeric construct (Figure 2C). In contrast, the trimeric vaccine induced a markedly superior neutralizing response. This enhanced neutralization of the trimer compared with the monomer

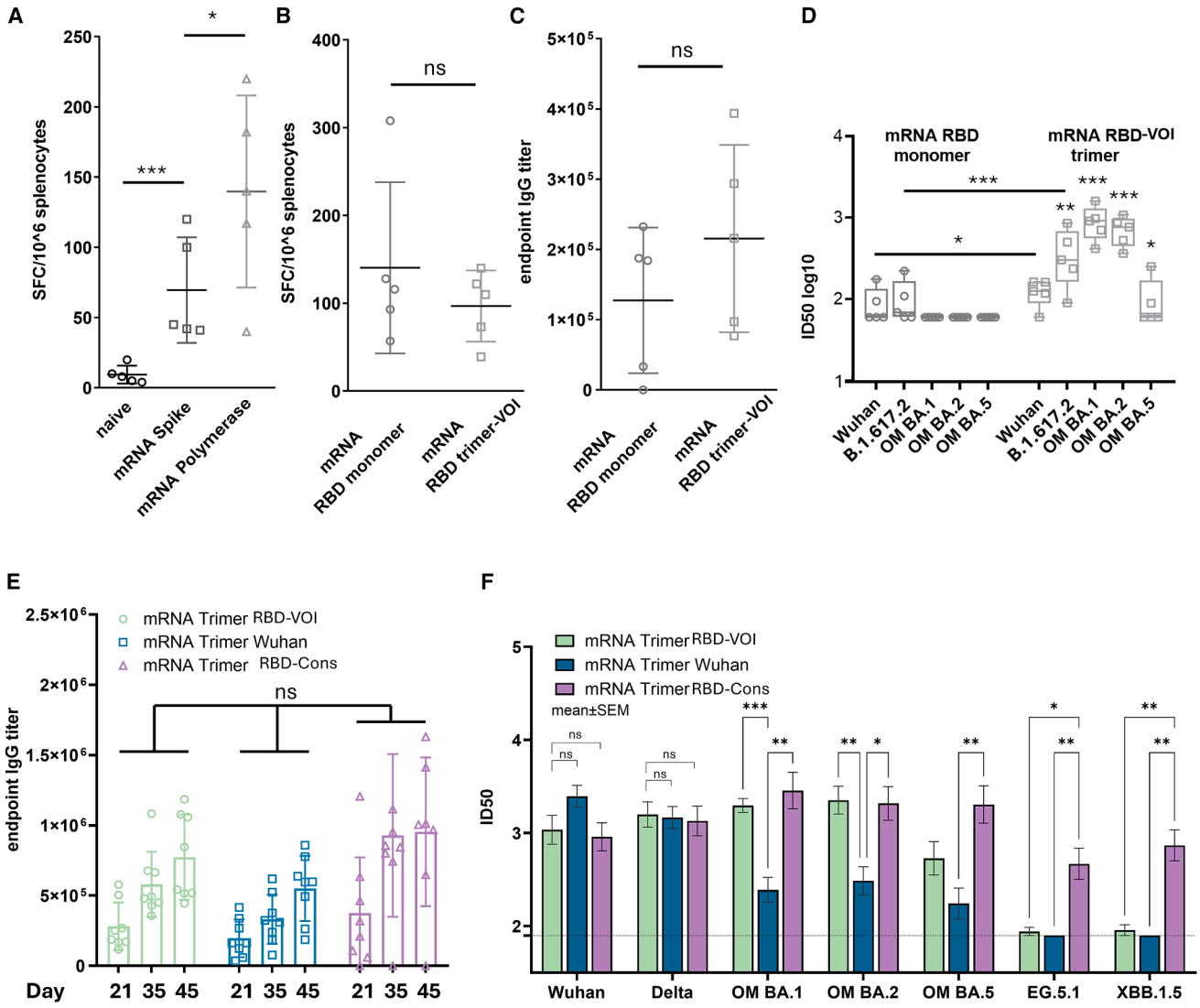
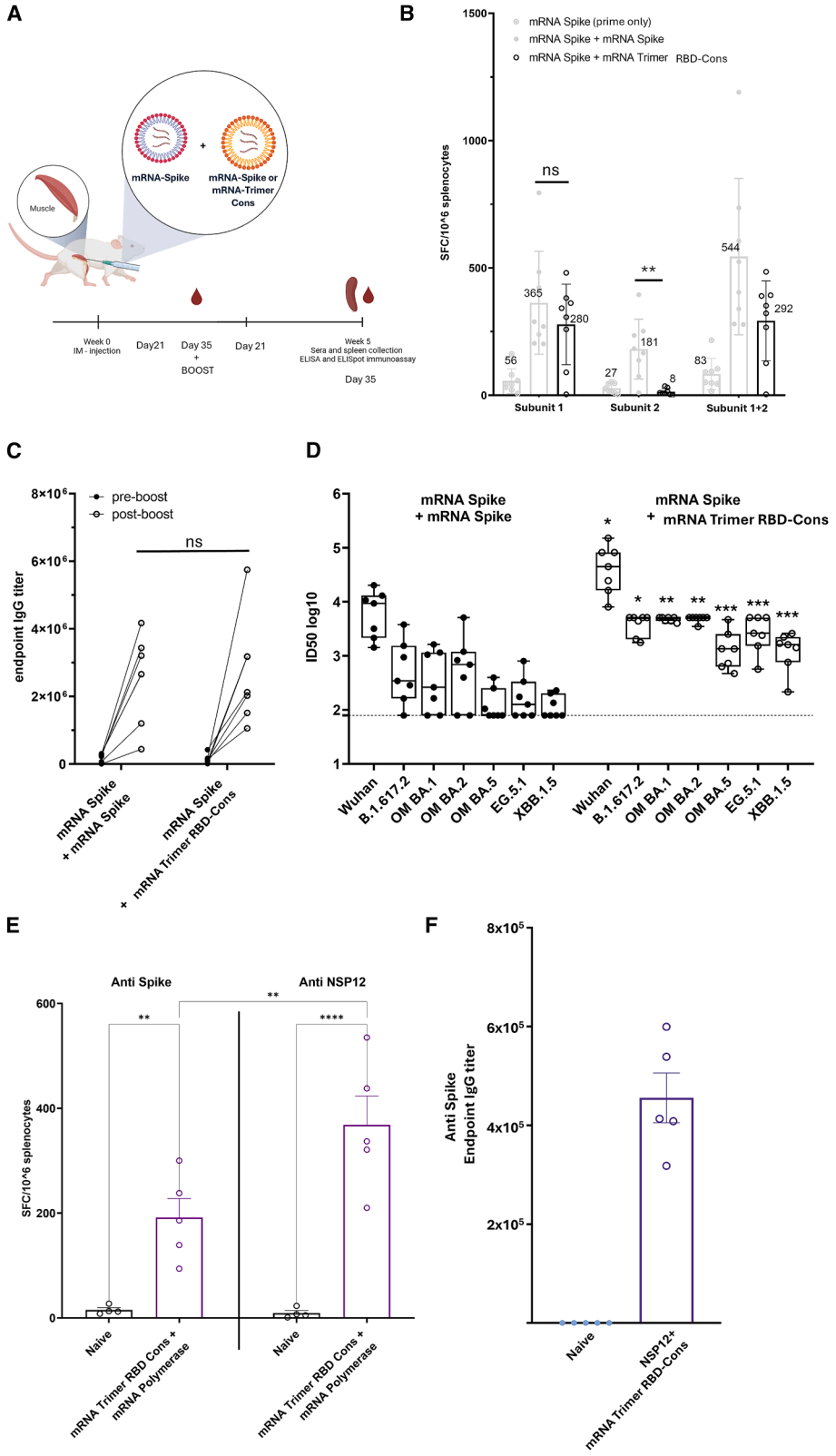


Figure 2. *In vivo* immunogenicity of conserved polymerase and RBD constructs

(A) ELISpot quantification of IFN-γ-secreting splenocytes following stimulation with polymerase-derived peptides in BALB/c mice immunized with mRNA encoding spike, polymerase, or untreated controls (naive) (mice per group $n = 5$). (B) Comparison of T cell responses elicited by the Wuhan RBD monomer and the trimeric RBD construct (RBD-VOI). (C) Endpoint IgG titers measured by ELISA in sera collected 4 weeks after immunization. (D) Neutralization titers (ID_{50}) in mice vaccinated with RBD monomer or trimer mRNA (RBD-VOI). The dotted line indicates the assay cut off (minimum serum dilution tested 1:60 dilution). Asterisks indicate a statistically significant difference for each variant between groups vaccinated with trimer. Asterisks on lines indicate statistical significance between monomer and trimer constructs.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-tailed t test. (E) Endpoint IgG titers in mice immunized with the original RBD-VOI trimer (green), a Wuhan-based trimer (blue), or the RBD-Cons trimer (purple) at days 21, 35, and 45 post-immunizations (mice per group $n = 8$). (F) Neutralization titers (ID_{50}) against Wuhan, delta, and omicron subvariants (BA.1, BA.2, BA.4/5, EG.5.1, and XBB.1.5) measured at 45 days post immunization. The dotted line indicates the assay cut off (minimum serum dilution tested 1:80 dilution). Asterisks indicate a statistically significant difference for each variant between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA

is already detectable when assessing neutralization of a Wuhan pseudovirus. Notably, when evaluating neutralization against additional variants (including delta and omicron) this difference becomes substantial (Figure 2D). The monomeric Wuhan RBD vaccine did not induce neutralizing antibodies against these heterologous variants, whereas the trimeric RBD elicited robust neutralizing responses against Wuhan, delta (B.1.617.2), and multiple early omicron variants (Figure 2D). Despite its superior ability to induce broadly neutralizing antibodies, the RBD-VOI vaccine loses efficacy against BA.5. To determine whether the improvement in neutralizing titers reflected trimeric architecture or sequence diversification, mice were immunized either with the original RBD-VOI (Wuhan-delta-omicron) or with a Wuhan-based RBD trimer (Wuhan-Wuhan-Wuhan).



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While in time course (21–45 days) the total anti-S IgG titers were essentially identical across the RBD-VOI vs. trimeric Wuhan constructs (Figure 2E), their ability to elicit neutralizing antibodies differed markedly. Firstly, the trimeric conformation is per se responsible for improved immunogenicity compared to RBD monomer in the induction of neutralizing or cross-neutralizing antibodies. Indeed, the trimer composed of three repeated Wuhan RBDs was able to neutralize Wuhan and delta strains better than Wuhan monomer (Figure 2D and 2F). However, the heterotrimeric Wuhan-delta-omicron construct exhibited robust neutralizing activity against Wuhan, delta, and omicron variants BA.1 and BA.2, and despite at less extend, BA.5. Given the known agnostic immunostimulatory activity of LNPs, we also vaccinated mice with empty particles, and we stimulated *ex vivo* splenocytes by S or NSP12 peptide demonstrating the absence of non-specific stimulation. As control, Wuhan, delta, and omicron RBD monomers were also tested revealing no substantial differences in antibody titers among monomers (Figure S3B).

To further increase cross-neutralization activity and to overcome resistance to BA.5, we engineered a next generation of phylogenetically informed trimeric RBD antigen based on consensus sequences constructed from a hierarchical tree of the HCov-19 RBD. To this end, we excluded from the vaccine the ancestral Wuhan and early VOC sequences. We selected three representative RBDs based on the degree of divergence from each other. Three clusters were identified as (1) cluster "delta," (2) cluster 8 (XBB.1.1X/EG.5), and (3) cluster 3, omicron-derived cluster (Figure 1A). Majority-rule consensuses based on position-specific frequency were generated and assembled into a trimeric construct, hereinafter referred to as RBD-Cons, for *in vivo* immunogenicity studies in the form of mRNA-LNP vaccine. Mice were immunized in the same experimental setting described above in comparison with RBD-VOI and trimeric Wuhan RBD. After a single dose of RBD-Cons, endpoint titer was almost identical to those induced by RBD-VOI and trimeric Wuhan RBD over a period of 21–45 days. Notably, the next-generation antigen (here defined as Consensus W/o Wuhan) induced significant neutralizing antibodies not only against BA.5, but it is also able to efficiently neutralize EG.5.1 and XBB.1.5 as well as against original Wuhan, delta, and early omicrons (Figure 2F).

Boosting with the Wuhan-free trimer mitigates antigenic sin

Having confirmed the superior performance of the novel RBD-Cons mRNA vaccine in terms of breadth of response against VOI, we sought to evaluate its ability to mitigate the antigenic imprinting resulting from prior exposure to ancestral Wuhan antigens. To mimic a clinically relevant scenario, mice were first vaccinated with full-length Wuhan mRNA and boosted on day 35 with either of a second dose of full-length Wuhan S or, alternatively, the RBD-Cons trimer (Figure 3A).

As expected, the homologous S-S regimen induced a higher number of S-specific T cell response measured by ELISpot (Figure 3B). This was as consequence of the lack of antigenic contribution against subunit 2 in RBD-Cons (RBD is a small fragment of subunit 1 of S protein). However, antibody titers pre-boost and on day 35 post-boost showed comparable levels between groups (Figure 3C). Notably, neutralization assays demonstrated that boosting with the RBD-Cons trimer achieved a markedly higher neutralizing activity against recent omicron variants, including EG.5.1 and XBB.1.5 (Figure 3D). Interestingly, also anti Wuhan response resulted enhanced. These results indicate that the consensus-based trimer broadens the neutralizing spectrum and may overcome the constraints of antigenic sin induced by prior exposure to the ancestral S.

As a final objective, we investigated whether a pan-coronavirus vaccination strategy could be achieved by combining the T cell antigen based on NSP12 with the consensus RBD trimer immunogen within a single immunization regimen.

To this end, mRNAs encoding NSP12 and the consensus trimer were formulated separately as LNPs, subsequently mixed, and administered in a single injection to naive animals. Four weeks after immunization, animals were sacrificed and immune responses were assessed by measuring S-specific T cell responses (directed against the S1 subunit) and NSP12-specific T cell responses, together with anti-S antibody titers. As expected, the inclusion of two different antigens within a single vaccine was not detrimental (Figures 3E and 3F) compared with the efficacy observed with the individual antigens (Figures 2B and 2C). This multi-antigenic vaccine, which is also polyvalent with respect to the RBD, could represent an excellent candidate for rapid clinical translation.

DISCUSSION

The relentless evolution of new variants of S has allowed SARS-CoV-2 to escape antibody recognition induced by vaccination with the ancestral Wuhan S. The original-antigenic-sin phenomenon limits recognition of novel variant. Studies found that bivalent Wuhan/omicron mRNA boosters primarily recalled antibodies against the

Figure 3. Heterologous boosting with the RBD-Cons trimer mitigates antigenic sin

(A) Schematic of immunization and boosting regimen in BALB/c mice. (B) IFN-γ ELISpot responses in splenocytes stimulated with two peptide pools covering conserved spike epitopes from S1 and S2, respectively. Numbers above bars indicate mean SFC/10^6 splenocytes (mice per group $n = 8$). (C) Serum endpoint IgG titers measured pre- and post-boost in homologous and heterologous groups ($n = 7$). (D) Neutralization titers (ID_{50}, log_{10}) measured post-boost showing that boosting with the variant-based trimer enhanced breadth and potency of neutralization compared to homologous spike-spike boosting ($n = 7$). Statistical analysis in 3D refers to neutralization titer against a given variant between spike-spike vs. Spike-RBD_Cons. (E) T cell response measured by IFNg EliSpot against polymerase (NSP12) and subunit 1 of spike protein. (F) Endpoint titer against full length spike protein.

original Wuhan S with modest neutralization of divergent omicron subvariants.[3] Overcoming such imprinting by designing a variant agnostic vaccine is therefore a key goal for next-generation vaccines. To broaden neutralizing antibody responses, in this study we used the receptor binding domain of S based on previous evidence reporting the ability of folding as stand alone. In the recently described muti-tri-RBD vaccine, three variant-derived RBDs were fused into one trimer and elicited much higher neutralization against delta and beta variants than a trimer of Wuhan RBD.[5] In this work, we extended this observation by selecting three divergent RBD sequences via phylogenetic consensus analysis (specifically, representatives of a delta-like branch, an emerging XBB-like branch, and another omicron lineage) and assembling them head-to-tail in an mRNA-encoded vaccine trimer (RBD-Cons). The guiding rationale remained the inclusion of the most antigenically distinct branches to maximize cross-reactivity. By deliberately excluding any original Wuhan RBD, we hypothesized that this "Wuhan-free" consensus trimer would achieve focusing of the immune response on conserved antigens, thereby broadening neutralization activity while sidestepping original-antigenic-sin bias. In an experimental mimicking of clinical condition, boosting Wuhan-primed mice with this fully novel consensus trimer led to strong neutralizing antibody titers even against recent XBB-derived omicrons, suggesting a significant mitigation of imprinting. In summary, the trimeric RBD immunogen dramatically enhanced antibody breadth, eliciting potent neutralization of diverse variants (including omicron sublineages), where monomeric RBD failed. It should be noted that the present work considered the variants circulating at the time the study was conceptualized, namely in 2023. However, the intent of the project is precisely to avoid a race against the emergence of new variants, and instead to generate a broadly protective vaccine strategy that may also retain relevance against future mutations. By including in the vaccine, the coronavirus RdRp (NSP12) as a second antigen, we aimed at increasing T cell immunity. The rationale behind this choice based on observation of cross-reactive memory $CD4^+$ and $CD8^+$ T cells to SARS-CoV-2 (likely derived from past common-cold coronavirus infections). At the same time, a potent T cell responses correlate with milder COVID-19 symptoms. NSP12 is extremely well conserved across SARS-CoV-2 strains and even across diverse coronaviruses, so it makes an ideal pan-coronavirus T cell target. In mice, the NSP12 mRNA vaccine elicited robust IFN-γ T cell responses (even exceeding those induced by full-length S). Because of conserved enzymatic function and aminoacidic primary sequence shared with many different and divergent coronaviruses, such polymerase-specific T cells should recognize essentially any SARS-like coronavirus. This is a powerful feature for future pandemic readiness: vaccines that prime conserved T cell epitopes may confer broad cross-protection against new zoonotic coronaviruses.

In conclusion, our dual-antigen mRNA strategy: a multivalent "imprinting-resistant" RBD heterotrimer for broad B cell neutralization and a conserved polymerase antigen for increased T cell immunity, may represent a promising blueprint for next-generation coronavirus vaccines.

MATERIALS AND METHODS

Bioinformatic analysis

From Global Initiative on Sharing All Influenza Data (GISAID; https://gisaid.org/) EpiCoV we retrieved the full-length variant alignment translated file reporting 98 SARS-CoV-2 different sequences at 2022-08-15. To integrate and update this database, we retrieved the circulating SARS-CoV-2 VOIs up to the end of year 2023 from GISAID. To detect the representative sequences of the latest VOIs, we clustered by hierarchical clustering (distance = "identity," method = "ward.D2") the translated sequences for each VOI, grouped similar sequences and labeled them with the VOI's name followed by the cluster number, obtaining 35 representative RBD of S the XBB.1.5, XBB.1.16, and EG.5 VOIs. We finally merged all the representative S's RBD sequences, clustered them by hierarchical clustering (distance = "identity," method = "ward.D2") and grouped all the RBDs into 8 clusters, labeling them according to the representative VOIs as depicted in Figure 1A. To construct the RBD consensus trimer, we excluded the Wuhan's branch, then we selected 3 different branches. To evaluate the immunogenicity of the epitopes found in human from SARS-CoV-2 RBD and nsp12, we then exploited the (IEDB; https://www.iedb.org/) selecting the ones supported by validated B cell and T cell assays, respectively. Regarding RBD, we obtained 1,698 epitopes found on different people and tested by different B cell immune assays (see supplemental file RBD_immunomeBrowser). In Figure 1B, we depicted the 95% CI of RF for each position averaged over all epitopes mapped to that position. In Figure 1C, we depicted the number of positive and negative assays along the length of RBD. For more insight about IEDB immunome browser result (see https://doi.org/10.1093/bioinformatics/bty463). To show the similarities of the SARS-CoV-2 NSP12's sequence among the *Coronaviridae*'s family, we blasted (blastp) such sequence against the *Coronaviridae* (taxid:11118) database with default parameters. We obtained 91 significant (E-value = 0) hits with at least 98% and 50% of coverage and percent identity, respectively. The result, purged of duplicates, was depicted in a slanted cladogram (hierarchical tree) labeling the sequences according to the "taxonomic name." Moreover, among the epitopes of NSP12 in IEDB, we selected the ones supported by T cell assays in at least 10 subjects (see supplemental file NSP12_immunomeBrowser). Subsequently, we blasted each epitope individually against *Coronaviridae* (taxid:11118) database (percent identity > 90%) to obtain in how many different species ("taxonomic name") it was conserved. The positions of the epitopes on the NSP12 and their abundance in *Coronaviridae*'s family were depicted as lollipop plot using the trackViewer package in Figure S1B.

mRNA vector generation and LNP formulation

In vitro transcribed mRNA was performed using the T7 FlashScribe kit (CellScript) with complete substitution of uridine by N1-methylpseudouridine-5′-triphosphate and co-transcriptional capping with 3′ OMe CleanCap AGG (TriLink), as described in previous publication.[8]

mRNA-LNPs were generated using Ignite (Cytiva) by using SM-102:DSPC:cholesterol:DMG-PEG2000 = 50:10:38.5:1.5.

RBD expression and biophysical characterization

The SARS-CoV-2 RBD (residues 331–532, Wuhan-Hu-1) was cloned into pETM11 with an N-terminal His-tag and expressed in *E. coli* BL21 (DE3). After induction with 0.75 μM IPTG for 3 h at 37°C, cells were lysed and inclusion bodies solubilized in 8 M urea. Proteins were purified by Ni-affinity chromatography, refolded by stepwise dialysis, and analyzed by SDS-PAGE. Folding was confirmed by size-exclusion chromatography (Superdex 75 Increase 10/300 GL) and circular dichroism spectroscopy (JASCO J-810).

Animal studies

Six-week-old female BALB/c mice were immunized with 80 μg/Kg (about-1,6-2 μg) of LNP-formulated mRNAs (i.e., Spike, NSP12, Trimers, RBD monomers) either in a prime only or prime-boost schedule (day 0 and day 35). Splenocytes (2.5–5 $\times$ 10^5 cells/well) were stimulated for 18 h at 37°C with overlapping 15-mer peptide pools spanning SARS-CoV-2 S (Wuhan) or NSP12 polymerase (JPT PepMix) as described in previous publication.[9] S-specific IgG were quantified using His-tagged S (Wuhan) immobilized on Ni-NTA plates. Sera were incubated for 2 h at room temperature and detected with alkaline phosphatase-conjugated secondary antibodies as previously reported.[10] Luciferase-expressing lentiviral vectors (LV-Luc) pseudotyped with SARS-CoV-2 S (Wuhan-Hu-1 and VOCs) were produced as described Borghi et al.[11] and Gallinaro et al.[12] Titers were determined on Vero E6 cells by luciferase activity, and dilutions yielding 3×10^5 RLUs were used for neutralization. For neutralization, heat-inactivated sera were serially diluted (starting at 1:80), incubated with pseudovirus for 30 min at 37°C, and then added to Vero E6 cells. Luciferase activity was measured 48 h post-infection, and infective dose (ID_{50}) values were determined by linear interpolation.

Statistical analysis

Data were analyzed using GraphPad Prism (v.10.1). Comparisons between groups were performed using two-tailed *t* tests or one-way ANOVA with Tukey's post hoc correction. $p < 0.05$ was considered statistically significant.

DATA AND CODE AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

A.D.C., conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, and writing; M.V.L., formal analysis, investigation, methodology, and validation; C.G., investigation and methodology; M.F.P., data curation, formal analysis, investigation, methodology, and validation; A.C., investigation, methodology, and validation; E.P., G.F., and A.S., methodology; A.G., methodology and validation; C.F. and R.P., investigation, methodology, and validation; A.D.S., investigation, methodology, validation, and visualization; S.C., conceptualization; Z.P., investigation, methodology, validation, and visualization; D.N., conceptualization, formal analysis, methodology, validation, visualization, and writing – review and editing; A.D.C., conceptualization, methodology, validation, visualization, and writing – review and editing; A.N., conceptualization, funding acquisition, and project administration; E.S., conceptualization, funding acquisition, project administration, supervision, and writing – review and editing.

DECLARATION OF INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

SUPPLEMENTAL INFORMATION

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