An Engineered Receptor-Binding Domain Improves the Immunogenicity of Multivalent SARS-CoV-2 Vaccines

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ABSTRACT The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike (S) protein mediates viral entry into cells expressing angiotensin-converting enzyme 2 (ACE2). The S protein engages ACE2 through its receptor-binding domain (RBD), an independently folded 197-amino-acid fragment of the 1,273-amino-acid S-protein protomer. The RBD is the primary SARS-CoV-2 neutralizing epitope and a critical target of any SARS-CoV-2 vaccine. Here, we show that this RBD conjugated to each of two carrier proteins elicited more potent neutralizing responses in immunized rodents than did a similarly conjugated proline-stabilized S-protein ectodomain. Nonetheless, the native RBD is expressed inefficiently, limiting its usefulness as a vaccine antigen. However, we show that an RBD engineered with four novel glycosylation sites (gRBD) is expressed markedly more efficiently and generates a more potent neutralizing responses as a DNA vaccine antigen than the wild-type RBD or the full-length S protein, especially when fused to multivalent carriers, such as a *Helicobacter pylori* ferritin 24-mer. Further, gRBD is more immunogenic than the wild-type RBD when administered as a subunit protein vaccine. Our data suggest that multivalent gRBD antigens can reduce costs and doses, and improve the immunogenicity, of all major classes of SARS-CoV-2 vaccines.

IMPORTANCE All available vaccines for coronavirus disease 2019 (COVID-19) express or deliver the full-length SARS-CoV-2 spike (S) protein. We show that this antigen is not optimal, consistent with observations that the vast majority of the neutralizing response to the virus is focused on the S-protein receptor-binding domain (RBD). However, this RBD is not expressed well as an independent domain, especially when expressed as a fusion protein with a multivalent scaffold. We therefore engineered a more highly expressed form of the SARS-CoV-2 RBD by introducing four glycosylation sites into a face of the RBD normally occluded in the full S protein. We show that this engineered protein, gRBD, is more immunogenic than the wild-type RBD or the full-length S protein in both genetic and protein-delivered vaccines.

KEYWORDS ACE2, COVID-19, RBD, receptor-binding domain, SARS-CoV-2, ferritin, vaccine

Coronaviruses are enveloped single-stranded, positive-strand RNA viruses of the family *Coronaviridae* (1). At least seven coronaviruses infect humans: the α-coronaviruses human coronavirus 229E (HCoV-229E) and HCoV-OC43 and the β-coronaviruses severe

acute respiratory syndrome coronavirus (SARS-CoV-1), HCoV-NL63, CoV-HKU1, Middle East respiratory syndrome coronavirus (MERS-CoV), and the recently described SARS-CoV-2, a β-coronavirus closely related to human SARS-CoV-1 (79.0% nucleotide identity) and to SARS-CoV-like variants isolated from bats (2–4). SARS-CoV-2 infection causes flu-like symptoms in many patients, but in other cases, it develops into an acute pulmonary syndrome (3, 5). SARS-CoV-1 causes severe acute respiratory syndrome (SARS), whereas disease associated with SARS-CoV-2 has been named coronavirus disease 2019 (COVID-19). SARS-CoV-2, like SARS-CoV-1, requires expression of the cellular receptor angiotensin-converting enzyme 2 (ACE2) to infect cells (6–8).

Entry of SARS-CoV-2 into ACE2-expressing cells is mediated by its spike (S) protein (7, 8). The coronavirus S protein is a type I viral entry protein similar to influenza virus hemagglutinin and the HIV-1 envelope glycoprotein (9). Like these entry proteins, the S protein is processed into two domains, S1 and S2 (7). S1 binds ACE2, whereas S2 anchors the S protein to the viral membrane. The SARS-CoV-2 S protein has an efficient furin cleavage site at its S1/S2 boundary, and this site is processed in virus-producing cells (10). In contrast, the SARS-CoV-1 S1/S2 junction is cleaved by extracellular or target cell proteases, including TMPRSS2 and cathepsin L (11–13). Both S proteins require processing at a second site, S2′, within the S2 domain to mediate fusion of the viral and target cell membranes (14).

The receptor-binding domains (RBDs; also described as S^B) of SARS-CoV-1 and SARS-CoV-2 directly bind ACE2 (7, 15–17). These RBDs are structurally and functionally distinct from the remainder of the S1 domain, and express and fold as independent domains (15). Both RBDs are highly stable and held together by four disulfide bonds. Structural studies of the SARS-CoV-2 RBD bound to ACE2 have identified a variable region, termed the receptor-binding motif (RBM), which directly engages ACE2 (16). This region is divergent between SARS-CoV-1 and SARS-CoV-2, although both RBDs bind ACE2 in the same orientation and rely on conserved, mostly aromatic, residues to engage this receptor. The divergence between the SARS-CoV-1 and SARS-CoV-2 RBM domains suggest that this region is subject to ongoing positive selection from the humoral response in various hosts. However, some 10 months into the COVID-19 pandemic, changes in the SARS-CoV-2 RBD remain exceedingly rare, consistent with a relatively low overall rate of viral mutation throughout the genome.

Because the S protein is the major protein exposed on the virion, and because its activity can be impeded with antibodies, it is likely the major target of any SARS-CoV-2 vaccine. Soluble trimeric S proteins, including those stabilized through various mechanisms, have been tested as SARS-CoV-1 vaccines, and similar approaches are now being taken against SARS-CoV-2 (7, 17, 18). In fact, all of the vaccines likely to be available in the first half of 2021 express or deliver a full-length or ectodomain S protein, typically engineered with a pair of prolines designed to enhance the stability of these constructs (19). Nonetheless, the neutralizing activity of these vaccines correlates with RBD recognition, and the vast majority of potent neutralizing antibodies described to date, including those in late-stage clinical trials, target the RBD (20–24).

A different approach, immunizing with the RBD alone, has been shown to raise potent neutralizing antibodies against SARS-CoV-1 in rodents (25, 26). Although the RBD presents fewer epitopes than the S-protein trimer, this approach may have key advantages. First, a much larger fraction of RBD epitopes, essentially all RBD epitopes exposed on the native trimer, are neutralizing. Thus, the RBD has fewer decoy epitopes and a greater fraction of the antibodies elicited will be neutralizing. Second, the 197-amino-acid RBD (S-protein residues 331 to 527) is much easier to produce than the full S-protein trimer. Thus, the costs of production of a subunit vaccine will be lower, and expression from an mRNA or adenoviral vaccine will be greater, allowing dose sparing and limiting side effects. Third, an RBD-based vaccine is less likely to include linear or conformational epitopes that, in rare cases, might promote autoimmune disorders through molecular mimicry. Similarly, fewer epitopes reduce residual concerns about antibody-dependent enhancement, observed with other coronaviruses and primarily mediated

through nonneutralizing epitopes. Finally, multivalent antigens are typically more immunogenic than dimeric or trimeric vaccines, and multivalency is much more easily obtained with RBD-based vaccines compared with those based on S-protein trimers.

Here, we show that when equal amounts of the SARS-CoV-2 S-protein ectodomain or the RBD alone were conjugated to each of two carrier proteins, the RBD generated neutralizing responses equal to or greater than those of the S protein. We nonetheless noted that the RBD was expressed inefficiently, especially as a fusion protein with a range of multivalent carrier proteins. We therefore engineered the RBD to correct this deficiency and showed that this modified RBD, fused to five different multivalent carrier proteins and expressed as DNA vaccines, elicited a more potent neutralizing antibody responses than the wild-type RBD or the full-length proline-stabilized S-protein antigen used in several prominent COVID-19 vaccines. Finally, we show that our modified RBD is more inherently immunogenic than the wild-type RBD when administered at equal dosage as a subunit protein vaccine. These data suggest that future vaccines against COVID-19 should include multivalent forms of engineered RBD antigens.

RESULTS

The SARS-CoV-2 RBD can elicit potent neutralizing antisera. The SARS-CoV-2 RBD, like that of SARS-CoV-1, is exposed in both known states of the S-protein trimer, namely, a closed state, where each RBD contacts its analogues symmetrically on the other protomer, and an open state, in which at least one RBD domain is extended to contact ACE2. We have previously shown that the SARS-CoV-1 RBD folds independently and expresses efficiently and that an immunoadhesin form of this RBD bound ACE2 more efficiently than constructs based on the S1 domain (15). This construct, RBD-Fc, also efficiently raised antibodies in mice capable of neutralizing SARS-CoV-1 variants, including those with distinct RBD sequences (25, 26). Moreover, the vast majority of well characterized neutralizing antibodies against SARS-CoV-2, including those in late-stage clinical trials, target the RBD. These data suggest that an RBD-based SARS-CoV-2 vaccine could be effective against virus throughout the current COVID-19 pandemic.

To initially evaluate this possibility under optimal conditions, we evaluated the immunogenicity of the SARS-CoV-2 RBD fused to the Fc domain as an expedient for rapid purification. RBD-Fc was chemically conjugated to a keyhole limpet hemocyanin (KLH) carrier protein and mixed with the AS01 adjuvant formulation now used in at least two human vaccines (Fig. 1). This antigen/adjuvant combination was inoculated intramuscularly into four female Sprague-Dawley rats with a schedule of seven increasing (2.5-fold) doses, one each day, ultimately administering a total of $500\,\mu g$ of the SARS-CoV-2 RBD-Fc. Thirty days after the first administration, RBD fused to a 4-amino-acid C tag was purified with a C-tag affinity column and administered as before. Blood was harvested from each of the four rats (R15, R16, R17, and R18) immediately before inoculation (day 0) and 40 days after the first inoculation. Serial dilutions of day 0 and day 40 sera were measured for their ability to neutralize retroviruses pseudotyped with the SARS-CoV-2 S protein (SARS2-PV). To estimate neutralization potency, these sera were also compared with a mixture of all four day 0 preimmune sera, further combined with an immunoadhesin form of ACE2 (ACE2-Fc) at concentrations of 10, 100, and 1,000 $\mu g/$ ml before dilution. As anticipated, some baseline inhibition could be observed in heat-inactivated rat sera (gray lines in Fig. 1A). However, day 40 serum from each rat, obtained after two sets of immunizations, potently neutralized SARS2-PV entry with an efficiency comparable to or greater than that of day 0 preimmune sera mixed with ACE2-Fc at a 100-$\mu g/ml$ concentration (Fig. 1A and B). We conclude that the SARS-CoV-2 RBD can elicit a neutralizing response in vaccinated rats comparable to a 100-$\mu g/ml$ (1 μM) concentration of an inhibitor with a 1 nM IC_{50} (50% inhibitory concentration). To confirm that sera from vaccinated rats neutralized SARS2-PV by recognizing the SARS-CoV-2 S protein, we used each pooled serum to prevent binding of an ACE2-Fc variant bearing a rabbit Fc domain (ACE2-rIg) from cells expressing the SARS-CoV-2
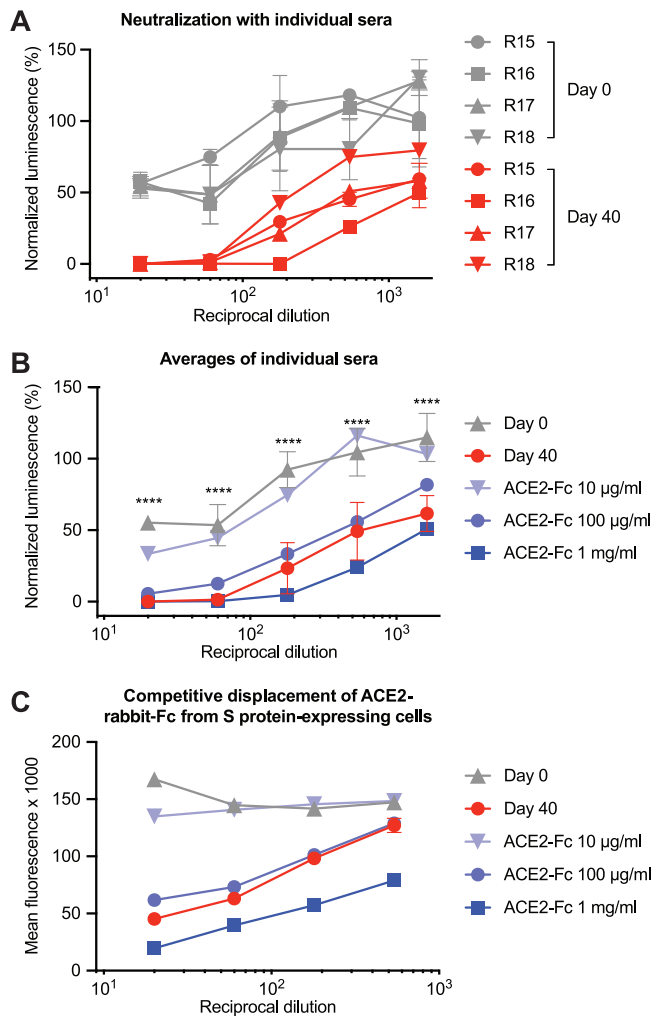
FIG 1 Immunization with the SARS-CoV-2 RBD elicits potently neutralizing antibodies. Four female Sprague-Dawley rats (R15, R16, R17, and R18) were immunized with two sets of escalating doses of RBD conjugated to keyhole limpet hemocyanin. (A) The indicated dilutions of preimmune sera (day 0, gray) were compared to dilutions of sera harvested from immunized rats at day 40 and to the same dilutions of preimmune sera mixed to achieve the indicated ACE2-Fc concentrations before dilution. Each serum and serum-ACE2-Fc mixture was compared for its ability to neutralize S-protein-pseudotyped retroviruses (SARS2-PV) by measuring the activity of a firefly luciferase reporter expressed by these pseudoviruses. The figure shows entry of SARS2-PV as a percentage of that observed without added rat serum. Error bars indicate the ranges of two neutralization studies. (B) The data from each rat in panel A are averaged for clarity. Error bars indicate standard deviations (SD), with each rat considered a different experiment. Differences between day 0 and day 40 serum are significant at all dilutions (****, $P < 0.001$; two-way analysis of variance [ANOVA]). (C) Pooled sera and pooled preimmune sera mixed with the indicated concentrations of ACE2-Fc were further combined with an ACE2-Fc variant bearing a rabbit-derived Fc domain. Binding of the ACE2-Fc was monitored with an anti-rabbit Fc secondary antibody, as determined by flow cytometry. Error bars indicate the ranges of two such measurements. Differences between day 0 and day 40 serum are significant ($P < 0.001$; two-way ANOVA) at all dilutions.

S protein (Fig. 1C). The ability of pooled serum to compete with ACE2-rIg indicates that these antisera neutralized SARS2-PV entry by blockading ACE2 association with the S protein. Thus, under ideal conditions, immunization with SARS-CoV-2 RBD elicits antibodies that very potently neutralize SARS2-PV, and these antibodies do so by preventing S-protein association with ACE2.

One concern associated with coronavirus vaccines is the possibility that anti-S-protein antibodies could promote infection of cells, such as alveolar macrophages, expressing Fc receptors, for example FcγRI (CD64) or FcγRII (CD32). This undesirable antibody-dependent enhancement (ADE) has been well characterized in tissue culture studies of several

flaviviruses, including Zika virus (ZIKV) and dengue virus (27). To evaluate this possibility for SARS-CoV-2, SARS2-PV were mixed with pooled day 0 or day 40 serum at the indicated serial dilutions, and the resulting virus-serum mixtures were incubated with HEK293T cells transfected to express rat FcγRI. These cells did not express ACE2, and no infection was observed with day 0 preimmune sera or day 40 immune sera (see Fig. S1A in the supplemental material). In contrast, rat anti-ZIKV antisera or day 0 preimmune sera incubated at the same dilutions with ZIKV virus-like particles (VLP) promoted robust ADE (Fig. S1B). ADE activity peaked at approximately a 3,000-fold dilution, consistent with competition between ADE and neutralizing activities of these antisera. Moreover, no SARS2-PV ADE was observed in the presence of ACE2 (Fig. S2C and D) or with K562 cells that endogenously express FcγRII (Fig. S1E and F) (28). Thus, anti-RBD antisera do not mediate SARS2-PV ADE in the presence or absence of ACE2 under the conditions described.

The RBD is more immunogenic than the S-protein ectodomain. To directly compare the immunogenicity of the RBD and a proline-stabilized S-protein ectodomain, both proteins were fused at their C termini to SpyTag and conjugated to one of two carrier systems (Fig. 2). First, each was conjugated by isopeptide bond formation to SpyCatcher-mi3 60-mer particles (RBD-mi3 and S-mi3) (29, 30). Second, the same amounts (in grams) of proteins were conjugated chemically to KLH as in Fig. 1 (RBD-KLH and S-KLH). Four rats were vaccinated with each antigen/carrier combination as in Fig. 1 except that one-fifth the total antigen (100 μg) was administered for each vaccination round. Sera collected at day 0 and day 45 from each rat were characterized for neutralization with SARS2-PV (Fig. S2), and the results were averaged (Fig. 2A and B). We observed that, with both carrier proteins, RBD conjugates elicited more potent neutralizing responses at day 45 than did S-protein conjugates. We further observed that conjugates to the mi3 60-mer elicited more potent responses than conjugates to the KLH carrier protein. Thus, RBD-mi3 was significantly more immunogenic than S-mi3, RBD-KLH, and S-KLH (Fig. 2C). We conclude that, when equal amounts of the RBD and the S-protein ectodomain are conjugated to a carrier and administered with a potent adjuvant, the RBD elicits a more potent neutralizing response. We also conclude that the mi3 carrier protein elicited more potent responses to both antigens than did KLH.

A glycan-modified RBD, gRBD, administered as a protein, elicits a more potent neutralizing response than does the wild-type RBD. Conjugates of the sort produced for Fig. 2 cannot readily be used with genetic vaccines such as those delivered as DNA, or as mRNA, or through viral vectors. However, fusion proteins that express both the antigen and the carrier as a single polypeptide chain can be used in these formats. Such fusion proteins also simplify the production of subunit protein vaccines. We therefore undertook to produce fusion proteins with the RBD in various formats but observed that these constructs were not efficiently produced in cells and were not efficiently secreted (Fig. S4). To solve this problem, we developed a screening procedure whereby the expression levels of engineered RBD variants, expressed as dimers, were monitored by flow cytometry. We observed that one such variant, modified with four additional glycosylation sites in an RBD region occluded in the S-protein trimer (Fig. 3A to C), was expressed and secreted markedly more efficiently as a fusion with the mi3 60-mer than the unmodified RBD (Fig. 4A). Each of three of these newly engineered glycans—those engineered at residues 370, 428, and 517—markedly increased RBD expression when fused to a multivalent carrier (Fig. S3), with the engineered glycosylation motif at residue 517 contributing most to gRBD expression. A fourth glycan, at residue 394, did not contribute to higher RBD expression, but it was included to further limit RBD aggregation.

We then investigated whether these additional glycans would alter immune responses to the RBD (Fig. 4B to E). Because fusions of the wild-type RBD with higher-order multivalent carrier proteins proved difficult to express and purify, we produced RBD and gRBD as fusion proteins with Fc domains of human IgG1 (wtRBD-Fc and gRBD-Fc). Two doses of 25 μg of RBD-Fc antigen with 25 μg monophosphoryl lipid A (MPLA) and 10 μg Quil-A adjuvants,
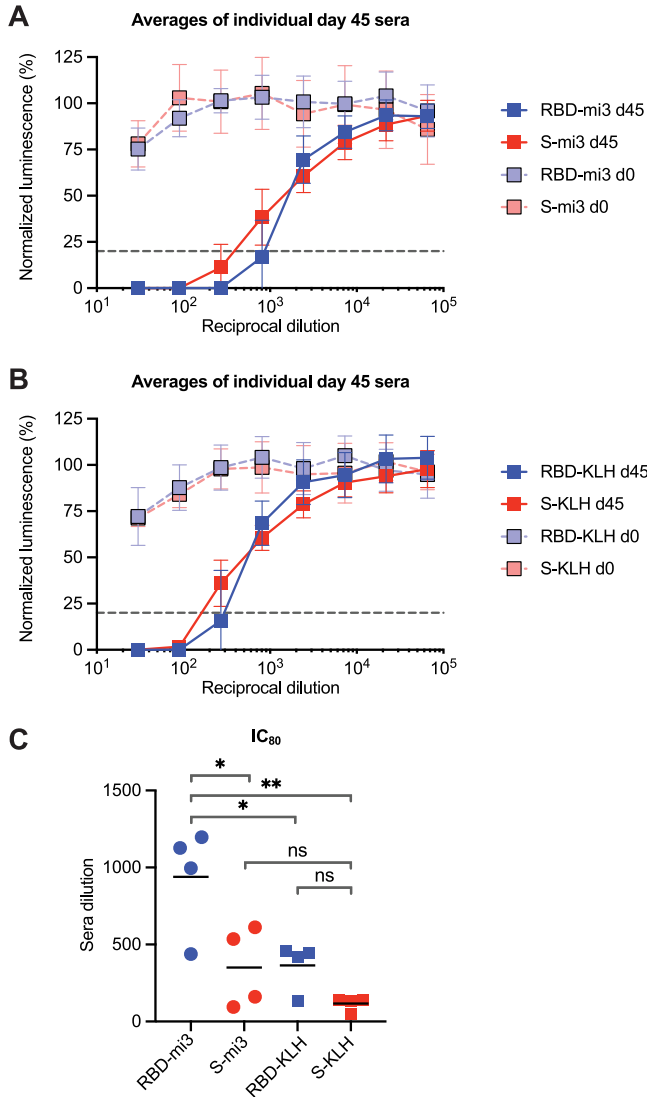
FIG 2 SARS-CoV-2 RBD nanoparticles are more immunogenic than S-protein nanoparticles. Four groups of four female Sprague-Dawley rats were inoculated with either RBD-SpyTag or S protein-SpyTag conjugated to either SpyCatcher-mi3 particles (A) by isopeptide bond formation or KLH (B) by EDC. The indicated dilutions of preimmune serum (day 0) were compared to dilutions of serum harvested from immunized rats at day 40. Each serum was compared for its ability to neutralize S-protein-pseudotyped retroviruses (SARS2-PV) by measuring the activity of a firefly luciferase reporter expressed by these pseudoviruses. The figure shows entry of SARS2-PV as a percentage of that observed without added rat serum. Dashed lines indicate 80% neutralization. Error bars indicate SD for biological replicates. (C) IC_{80} values for each rat at day 40 were calculated in Prism 8, and significance between groups is indicated (*, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$; one-way ANOVA with Tukey's multiple-comparison test).

separated by 14 days, were administered intramuscularly to five mice per group. Antisera were harvested 21 days after the first vaccination, and analyzed for its ability to neutralize SARS2-PV or control pseudoviruses expressing the entry (GP) protein of the lymphocytic choriomeningitis virus (LCMV-PV). Sera from inoculated mice was mixed and compared with preimmune sera and preimmune sera mixed at an initial concentration of 200 μg/ml ACE2-Fc. We observed, somewhat unexpectedly, that gRBD-Fc elicited a more potent neutralizing response than did wtRBD-Fc (Fig. 4B and C). Consistent with this observation, sera from gRBD-Fc inoculated mice more efficiently bound cell-expressed S protein (Fig. 4D) and more efficiently blocked binding of fluorescently labeled ACE2-Fc (Fig. 4E) than did sera from wtRBD-Fc-inoculated mice. Thus, the engineered glycans of gRBD do not interfere with and
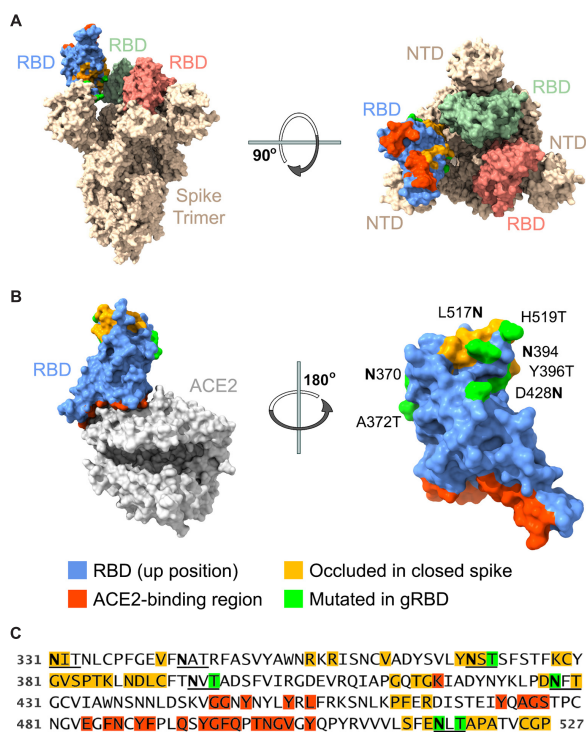
FIG 3 Engineered SARS-CoV-2 RBD glycans enhance expression of multivalent RBD fusion proteins. Views of the RBD (A) in the context of the SARS-CoV-2 S protein in the open one-up conformation, with the ACE2-binding region (red) facing upward, and (B) bound to the ACE2 receptor, with the RBD ACE2-binding region facing downward. Blue indicates surface residues that are neither occluded in the closed conformation (yellow) nor part of the ACE2 interface (red). Green indicates residues whose mutation creates a novel N-glycosylation motif. (C) The sequence of the engineered RBD bearing four novel glycosylation motifs (gRBD) is shown. Numbering indicates S-protein residues. Glycosylation motifs (2 native and 4 engineered) are underlined. Coloring corresponds to that in panel B.

may enhance its ability to raise anti-RBD antibodies in mice. We speculate that gRBD glycans better focus the B-cell response to neutralizing RBD epitopes (31, 32). Alternatively, aggregation of the wild-type RBD may impede access to these epitopes. Collectively, these data suggest that multivalent antigens based on gRBD will be easier to produce than and at least as immunogenic as their wild-type RBD analogues.

DNA vaccines expressing multivalent gRBD fusion proteins elicit more potent neutralizing antisera than do the corresponding wtRBD fusion proteins or the full-length S protein. To evaluate the utility of gRBD in the context of DNA-, mRNA-, or viral vector-based vaccines, we developed plasmids expressing wtRBD and gRBD-fusion proteins with five multivalent carriers (Fig. 5). Specifically, fusions with the IgG1 Fc domain dimer (Fc), the T4 foldon trimerizing domain (33), a dodecameric scaffold based on the *Helicobacter pylori* neutrophil-activating protein (NAP) (34), the *H. pylori* ferritin 24-mer (35), and the engineered mi3 60-mer (29, 30). Complete amino acid sequences of these fusion proteins are provided in Fig. S4A. In each case, the gRBD fusion protein expressed more efficiently than its wild-type RBD analogue (Fig. S4B). We also evaluated a plasmid expressing the full-length proline-stabilized 1,273-amino-acid S protein (S1273-PP, the full-length S protein with prolines introduced at residues 986 and 987) which expressed efficiently on the surface of HEK293T cells, more so than the otherwise identical construct lacking these stabilizing prolines. (Fig. S4C). Five mice per group were electroporated with 120 μg plasmid (60 μg per hind leg) encoding either wtRBD or gRBD antigen fused to each of the aforementioned multivalent scaffolds, or with plasmid expressing the S1273-PP full-length S protein. Mice were again electroporated 14 days later with the same plasmids. Sera were harvested 21 days after the first inoculation. Combined sera for each group were evaluated for their ability to neutralize SARS2-PV or LCMV-PV and compared with preimmune sera or preimmune sera mixed
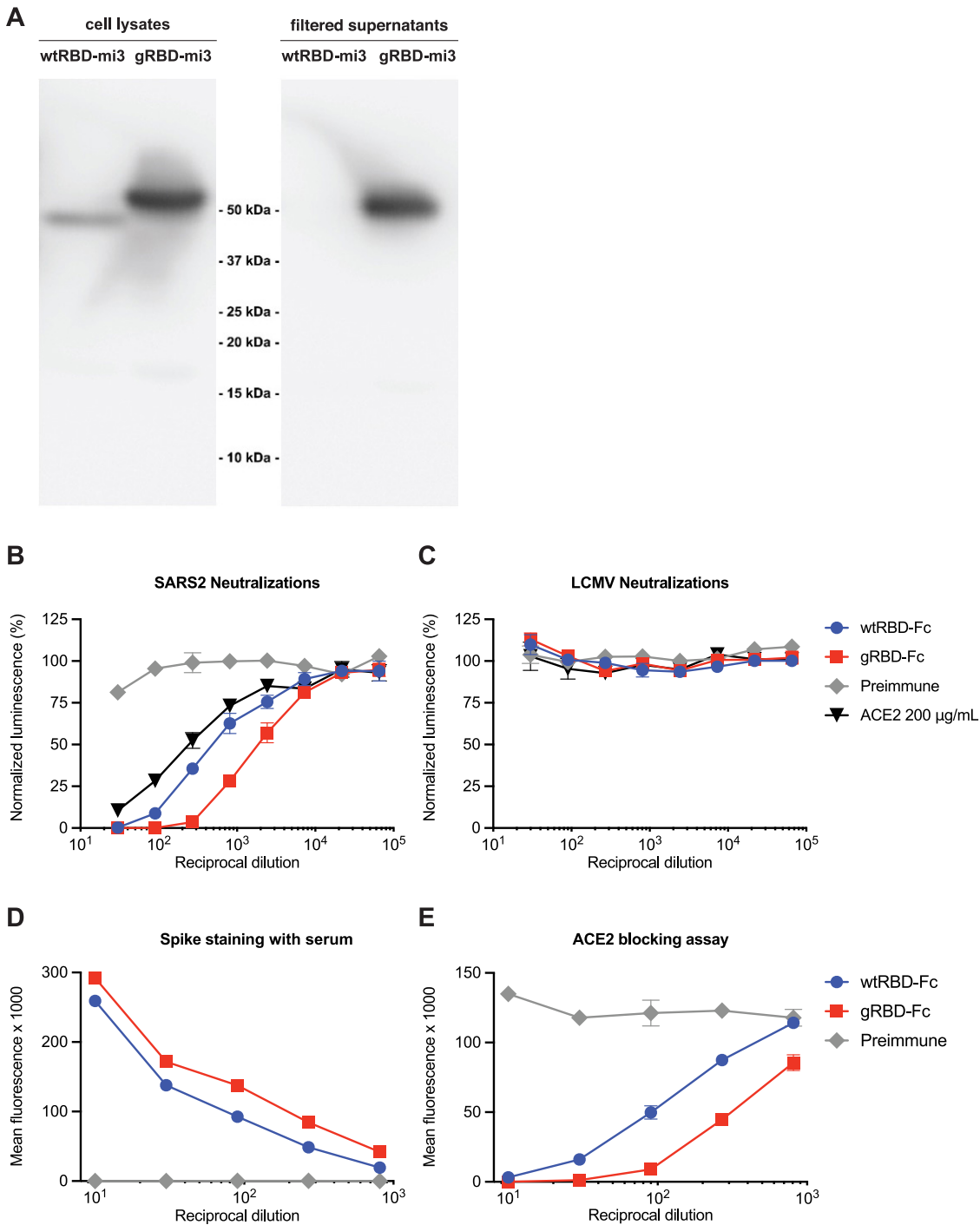
FIG 4 gRBD is expressed efficiently as an mi3 fusion protein and is more immunogenic than wild-type RBD as an adjuvanted protein. (A) For expression, RBD-mi3 60-mer fusion proteins were expressed in Expi293 cells; after 5 days, supernatants and cell lysates were analyzed by SDS-PAGE and anti-C tag Western blotting. Note that no wtRBDmi3 could be detected in the supernatant. For immunogenicity, five mice per group were inoculated with $25\,\mu g$ of protein A/SEC purified wtRBD-Fc or gRBD-Fc adjuvanted with $25\,\mu g$ of MPLA and $10\,\mu g$ Quil-A. Immunizations were conducted day 0 and day 14, and serum was collected and pooled on day 21. Pooled preimmune sera and pooled preimmune sera mixed with $200\,\mu g/ml$ of ACE2-Fc were used as negative and positive controls. Pooled sera were used to neutralize (B) SARS-CoV2 pseudovirus or (C) LCMV pseudovirus control. In parallel, HEK293T cells were transfected with $1\,\mu g/well$ in a six-well plate and stained the next day with pooled preimmune and day 21 sera and then stained with either (D) FITC (fluorescein isothiocyanate)-conjugated anti-mouse immunoglobulin or (E) ACE2-Fc-DyLight650. Error bars indicate standard errors of the means (SEM).
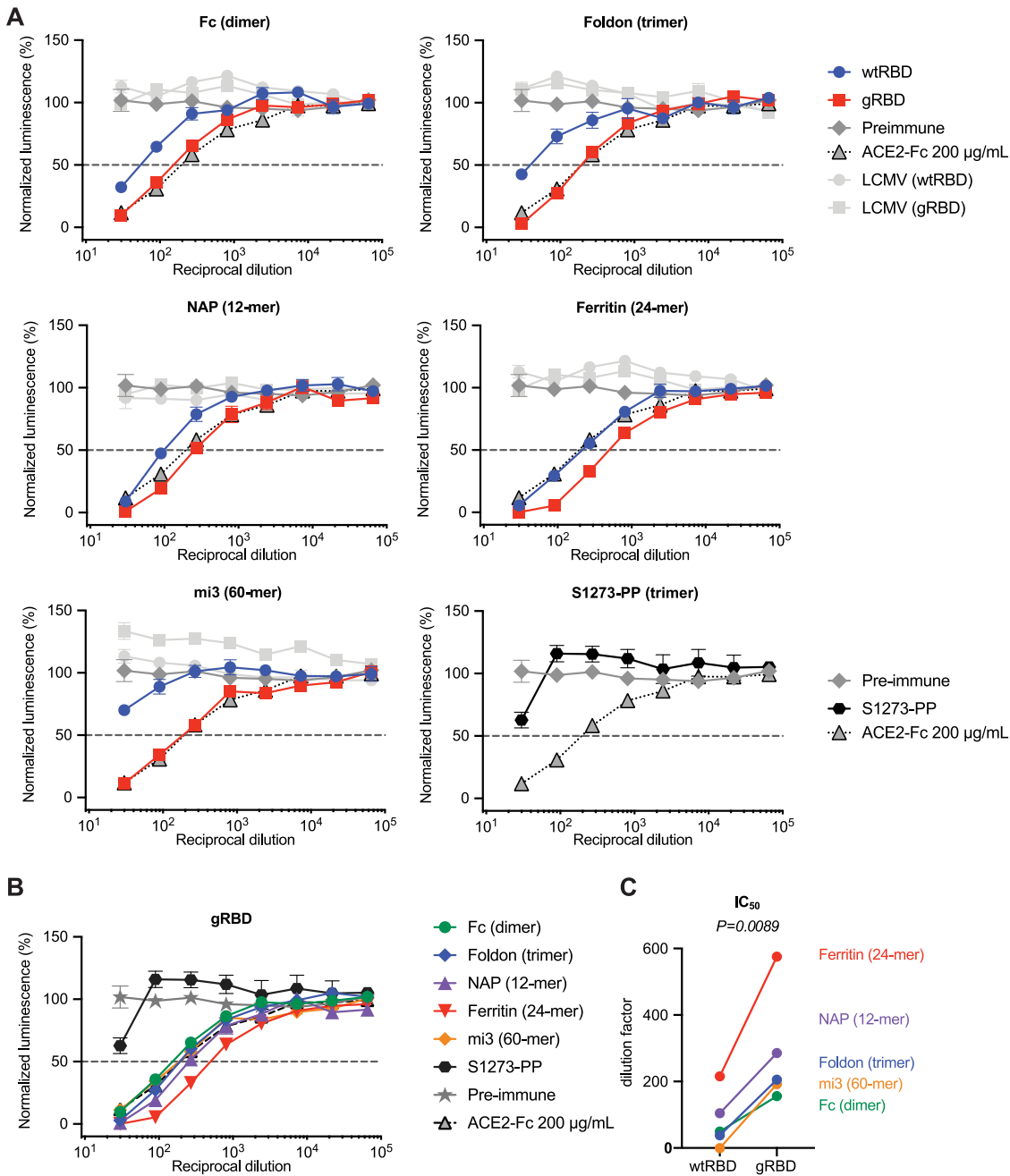
FIG 5 gRBD based DNA vaccines raise neutralizing antibodies more efficiently than those based on wild-type RBD. (A) Five mice per group were electroporated in each hind leg with 60 μg plasmid DNA expressing wtRBD or gRBD fused to human Fc dimer, foldon trimer, *H. pylori* NAP 12-mer, *H. pylori* ferritin 24-mer, and mi3 60-mer. An additional control group was electroporated with plasmid expressing the full-length SARS-CoV-2 spike protein with two stabilizing prolines (S1273-PP). Electroporations were conducted day 0 and day 14, and serum was collected and pooled for neutralization assays on day 21. Pooled preimmune sera and pooled preimmune sera mixed with 200 μg/ml of ACE2-Fc were used as negative and positive controls. (B) The neutralizing potency of gRBD varied by platform. (C) IC_{50}s for wtRBD and gRBD were calculated (Prism 8) against normalized values by least-squares fit. *P* value was calculated by 2-tailed paired *t* test between wtRBD and gRBD pairs.

with ACE2-Fc at an initial concentration of 200 μg/ml. In each case, sera from gRBD-fusion constructs neutralized SARS2-PV more efficiently than their wtRBD analogues, and more efficiently than sera from mice electroporated with S1273-PP (Fig. 5A). Among the various scaffolds, the *H. pylori* ferritin 24-mer elicited the most potent neutralizing antisera (Fig. 5B and C). We conclude that gRBD consistently and significantly (*P* = 0.0089) (Fig. 5C) improves the immunogenicity of multivalent fusion proteins relative to the same construct fused to the wild-type RBD.

DISCUSSION

For several reasons, a vaccine against SARS-CoV-2 is easier to develop than those against many other viruses (9, 36). First, coronaviruses have exceptionally large genomes compared to other RNA viruses, and, to avoid error catastrophe, their viral polymerase has acquired a proofreading function. Thus, although a number of mutations of concern have been described, the number of total mutations remains lower than that typically observed in a single HIV-1-infected person. Second, coronaviruses in general, and clearly SARS-CoV-2 in particular, are transmitted to new hosts more rapidly than an adaptive immune response can emerge. A likely consequence of this strategy is that one of its most critical epitopes, namely, its RBD, is exposed on the virion (7, 9, 15, 17), favoring transmission efficiency over antibody resistance. Finally, the stability and compactness of the SARS-CoV-1 and SARS-CoV-2 RBD suggest that it can be easily manufactured and presented to the immune system using many production technologies, presentation scaffolds, and delivery systems (15). Here, we repeated studies of the SARS-CoV-1 RBD showing that the SARS-CoV-2 RBD is sufficient to raise potent neutralizing antibodies (Fig. 1) and that it does so more efficiently than the SARS-CoV-2 S protein ectodomain when conjugated to a KLH or mi3 carrier and administered as a protein (Fig. 2). One attractive hypothesis for why this may be so is that the S protein has decoy epitopes that serve to focus the immune response away from its best neutralizing targets.

However, the wild-type SARS-CoV-2 RBD suffers from one major deficiency. When expressed as a fusion protein in a multivalent scaffold or carrier, it is expressed inefficiently in cells, it is secreted poorly, and it tends to aggregate (Fig. 4A; Fig. S4B). This tendency to aggregate appears to impair its immunogenicity, even when administered as a reasonably well-behaved Fc dimer (Fig. 4B). Through trial-and-error screening of RBD variants modified in a region normally occluded on the S-protein trimer, we identified a construct with four novel glycosylation motifs (Fig. 3) that substantially improved or rescued expression of all five multivalent protein carriers assayed (Fig. S4B). Each of these glycosylation motifs improved expression or RBD solubility (Fig. S3). The resulting engineered RBD, which we call gRBD to reflect its additional glycans, elicited more potent neutralizing sera when administered as an adjuvanted protein (Fig. 4B to E) or when electroporated as a DNA vaccine expressing each of five carrier proteins (Fig. 5A to C). Importantly, these gRBD fusion proteins were more immunogenic than the proline-stabilized S proteins used as antigens in most prominent SARS-CoV-2 vaccines (Fig. 5B).

Our data therefore show that (i) the SARS-CoV-2 RBD can be more immunogenic than the S protein, (ii) an RBD engineered with four glycans can be more immunogenic than the wild-type RBD, and (iii) multivalent forms of the RBD and gRBD, and in particular those fused with the *H. pylori* ferritin 24-mer, can be more immunogenic than dimeric or trimeric constructs. Why is the RBD more immunogenic than the S protein? The answer appears straightforward: the RBD is the dominant neutralizing epitope. Expression of the remainder of the protein taxes cellular resources and exposes potential decoy epitopes. Why is gRBD more immunogenic than the wild-type RBD? We speculate that poor folding or solubility of the wild-type RBD helps occlude its major neutralizing epitopes or limit its access to the lymph nodes (37). It is also possible that the glycans of gRBD mask dominant but nonneutralizing RBD epitopes. Finally, why is the ferritin 24-mer more immunogenic than the other scaffolds? Immunogenicity is likely improved by multivalency, by higher expression, and by preexisting T cell help (37, 38). The mi3 60-mer is maximally multivalent, but it is expressed relatively poorly and may include fewer epitopes recognized by established memory T cells. The NAP 12-mer is expressed much more efficiently, but its size or the arrangement of gRBD domains may be suboptimal. The Fc dimer and foldon trimer may also be insufficiently multivalent and, again, include fewer T-cell epitopes. The *H. pylori* ferritin 24-mer combines high expression, a larger particle, and high valency, and it provides T-cell epitopes similar to those in many bacterial antigens. Further work identifying and

engineering high-expressing multivalent scaffolds capable of presenting gRBD may yield even more potent antigens. In addition, investigation into whether multivalent, multivariant constructs can be more effect at broadening the immune response will also be important.

In short, we have engineered a SARS-CoV-2 RBD antigen that is expressed more efficiently than the wild-type RBD as a fusion with multivalent carrier proteins, and these fusion proteins are more immunogenic as protein or DNA vaccines than commonly used S-protein antigens. We propose, therefore, that multivalent gRBD fusion proteins could improve production efficiencies of protein-based SARS-CoV-2 vaccines and limit the doses necessary for all vaccine classes.

MATERIALS AND METHODS

Production of SARS-CoV-2 and LCMV pseudoviruses and ZIKV virus-like particles. Retroviruses pseudotyped with the SARS-CoV-2 S protein or lymphocytic choriomeningitis virus GP protein (SARS2-PV and LCMV-PV) were produced as previously described (40) with modest modifications as described. HEK293T cells were transfected by polyethylenimine (PEI) transfection at a ratio of 5:5:1 with a plasmid encoding murine leukemia virus (MLV) gag/pol proteins, a retroviral vector pQCXIX expressing firefly luciferase, and a plasmid expressing the spike protein of SARS-CoV-2 (GenBank no. YP_009724390) or LCMV GP (GenBank no. AHZ55917.1). Cells were washed 6 h later, and the culture supernatant containing pseudoviruses was harvested at 48 to 72 h posttransfection. ZIKV VLP were produced by transfecting HEK293T cells by the calcium phosphate transfection method with a ZIKV replicon (strain FS13025; GenBank no. KU955593.1) whose expression is controlled by tetracycline, a plasmid encoding ZIKV capsid, prM, and E proteins (strain FSS13025; GenBank no. KU955593.1) and the pTet-On plasmid expressing a reverse Tet-responsive transcriptional activator (rtTA) at a ratio of 2:1:1. Cells were washed 6 h later and replenished with fresh medium containing 1 μg/ml doxycycline. The VLP-containing culture supernatant was harvested 48 h posttransfection. ZIKV replicon was generated by replacing the region spanning the 39th through 763rd amino acids of the polyprotein of a ZIKV molecular clone we previously generated (39) with *Renilla* luciferase with the 2A self-cleaving peptide fused at its C terminus. This construct contains the tetracycline-responsive P tight promoter that drives ZIKV RNA transcription. The pseudovirus- or VLP-containing culture supernatants were cleared by 0.45-μm filtration. SARS2-PV and ZIKV-VLP titers were assessed by RT-qPCR targeting the cytomegalovirus (CMV) promoter in the retroviral vector pQCXIX and ZIKV NS3 gene, respectively. In some cases, clarified pseudovirus and VLP stocks were stored at $-80°C$ for long-term storage and reuse.

Generation of human ACE2-expressing cell lines HEK293T cells expressing human ACE2 (hACE2) were generated by transduction with murine leukemia virus (MLV) pseudotyped with the vesicular stomatitis virus G protein and expressing myc-hACE2-c9, as previously described (41). Briefly, HEK293T cells were cotransfected by PEI with three plasmids, pMLV-gag-pol, pCAGGS-VSV-G, and pQCXIP-myc- hACE2-c9 at a ratio of 3:2:1, and medium was refreshed after overnight incubation of transfection mix. The supernatant with produced virus was harvested 72 h posttransfection and clarified by passing through a 0.45-μm filter. 293T-hACE2 cells transduced with MLV vectors were selected and maintained with medium containing puromycin (Sigma). hACE2 expression was confirmed by SARS1-PV and SARS2-PV entry assays and by immunofluorescence staining using mouse monoclonal antibody recognizing c-Myc.

Protein production. Expi293 cells (Thermo Fisher) were transiently transfected using FectoPRO (Polyplus) with plasmids encoding SARS-CoV-2 RBD with a human or rabbit Fc fusion or a C-terminal C tag (-EPEA, where each letter indicates the single-letter amino acid code for the four-amino-acid tag). After 5 days in shaker culture, media were collected and cleared of debris for 10 min at 3,000 $\times$ *g* and filtered using 0.45-μm flasks (Nalgene). Proteins were isolated using MabSelect SuRe (GE Lifesciences) or CaptureSelect C-TagXL (Thermo Fisher) columns according to the manufacturers' instructions. Eluates were buffer exchanged four times with phosphate-buffered saline (PBS) and concentrated using Amicon ultrafiltration devices (Millipore Sigma), except for S protein-SpyTag, which was buffer exchanged by dialysis (Pierce) 3 times, and concentrated. For wtRBD-Fc and gRBD-Fc used in mouse protein immunizations, further purification was performed by size exclusion chromatography (SEC) on a HiPrep 16/60 Sephacryl S-400 HR column connected to an ÄKTA fast protein liquid chromatograph (FPLC). Fractions were isolated with PBS buffer, verified by SDS-PAGE, pooled, and concentrated. All purified proteins were stored at 4°C prior to use.

For RBD-multimer fusion proteins, supernatant pH was adjusted to 8.5 by addition of 1/20 volume 1 M Tris, pH 9.0 (G-Biosciences; 786-476), and the supernatant was filtered with a 0.45-μm filter prior to purification on CaptureSelect C-tag XL columns. In the case of RBD-ferritin fusions, 0.5% Tween 20 was also added to supernatants prior to filtration, and supernatants were mixed with anti-Flag M2 agarose affinity gel (1 ml slurry for 25 ml culture; Sigma-Aldrich; A2220-10ML) and incubated on a rotary shaker overnight at 4°C. The mixture was packed into columns (Agela Technologies; AZ-IC-1T). Columns (C tag or M2) were washed with 10 column volumes (CV) of Tris-buffered saline (TBS) (25 mM Tris [pH 8.5], 150 mM NaCl), and eluted with 5 CV Gentle Ag/Ab elution buffer, pH 6.6 (Thermo Scientific Pierce; 21027). Buffer was exchanged 4 times with TBS (pH 8.5) for yield studies, or 3 times with TBS (pH 8.5), and once with PBS for subsequent SEC polishing.

Protein immunizations and serum collection in rats. All animals used in these studies were handled and maintained in accordance with NIH guidelines and approved by Institutional Animal Care

and Use Committee (IACUC) of Scripps Research (Protocol 18-025). Female Sprague-Dawley rats were immunized with incrementally increasing doses of antigen over 7 days starting at day 0 and given boosters with a similar regimen at day 30.

Rats were inoculated in the first set with the SARS-CoV-2 RBD fused to the Fc domain of human IgG1 and in the second set with the RBD fused to a four-amino-acid C tag. In both cases, RBD fusions were conjugated at a 1:1 ratio to mariculture keyhole limpet hemocyanin (mcKLH; Thermo Fisher Pierce) by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Thermo Fisher Pierce) according to the manufacturer's protocols. Each set of seven injections were performed in the following manner. RBD-KLH conjugates were administered intramuscularly into the rear quadriceps. Inoculations were initiated with 2.2 μg RBD-KLH (equivalent to 1.1 μg RBD antigen) adjuvanted with 0.1 μg MPLA and 0.1 μg Quil-A, and this inoculum was increased by 2.55-fold for each of the next 6 days, for a total of 500 μg RBD-Fc or RBD–C-tag fusion protein and 40 μg of each adjuvant component administered. Sera were collected before inoculation (day 0 preimmune sera) and every 5 days starting on the 10th day after the first injection. All sera were heat inactivated for 30 min at 56°C and stored at -80°C for reuse.

For the mi3-versus-KLH round of inoculations, rats were inoculated with either RBD-SpyTag or S protein-SpyTag, conjugated either to equal quantities of either Spycatcher-mi3 (mixed and incubated 4°C overnight) or mcKLH (EDC conjugation). Incrementally increasing injections were conducted as described above but with an initial inoculation of 0.4 μg (0.2 μg each antigen and carrier) for a total of 200 μg protein over both rounds of vaccination and 40 μg of each adjuvant component.

Protein immunizations and serum collection in mice. Female 8- to 9-week-old BALB/cJ mice were immunized with 25 μg protein antigen, 25 μg MPLA, and 10 μg of Quil-A on day 0 and day 14. Sera were collected before the initial inoculation (preimmune sera) and on day 21. All sera were heat inactivated for 30 min at 56°C and stored at -80°C for reuse.

DNA immunizations in mice. CMV/R expression plasmids encoding wtRBD or gRBD fused to multimerization platforms (Fig. S3A) were prepared using NucleoBond PC 2000 (TaKaRa Bio USA Inc.) and confirmed to be essentially endotoxin free using Pierce Quant chromogenic endotoxin quantification kit (Thermo Scientific) according to the manufacturers' instructions. Female 8- to 9-week-old BALB/cJ mice were electroporated with 60 μg DNA in each hindquarter for a total dose of 120 μg on day 0 and day 14. Electroporations were conducted on a Harvard Apparatus ECM 839 BTX system in LV mode at 40 V using 8 pulses, with a pulse length of 100 ms, at 100-ms intervals with unipolar polarity. Sera were collected before the initial inoculation (preimmune sera) and on day 21. All sera were heat inactivated for 30 min at 56°C and stored at -80°C for reuse.

Neutralization studies of SARS-CoV-2 and LCMV pseudoviruses. Individual sera or pooled sera collected at day 0 (preimmune sera) and, at the indicated day after the first inoculation, were serially diluted in Dulbecco's modified Eagle medium (DMEM). In some cases, day 0 sera were mixed with ACE2-Fc to a concentration of 10, 100, or 1,000 μg/ml before dilution and then diluted in the same manner. Individual, pooled, or pooled-ACE2-Fc serum was mixed with SARS2-PV and incubated at 37°C for 1 h. For rat studies, 1 h later, 104 ACE2-239T cells were added along with DEAE-dextran (final concentration, 5 μg/ml), and medium was exchanged 6 h later with fresh medium without rat serum. For mouse studies, 1 h later, 10^4 ACE2-239T cells were added and spun at 3,000 × g for 30 min at 4°C; the mixture was then returned to 37°C, and medium was exchanged 2 h later with fresh medium without mouse serum. At least two independently mixed replicates were measured for each experiment. Firefly luciferase activity was measured (Britelite) 48 h postinfection. All neutralization studies were performed at least twice with similar results.

Competitive displacement of ACE2-rabbit Fc from S-protein-expressing cells. Serial dilutions of pooled sera or pooled preimmune sera mixed with ACE2-Fc (with the human Fc domain) at initial concentrations of 10, 100, and 1,000 μg/ml were then mixed with 1 μg/ml ACE2-rabbit Fc. Independent mixtures were made for each replicate. Pooled sera and serum mixtures were used to stain HEK293T cells transfected by the jetPRIME transfection reagent (Polyplus) to express the full-length SARS-CoV-2 S protein. Specifically, 10^5 cells per well were placed in a 96-well V-bottom plate and incubated for 45 min at 4°C with 100 μl of the serially diluted serum mixed with ACE2-Fc. After washing, cells were stained with anti-rabbit IgG-Alexa Fluor 647 antibody for 45 min at 4°C, and mean fluorescence intensities were measured for each well by flow cytometry.

Measurement of antibody-dependent enhancement. The ability of anti-SARS-CoV-2 RBD immune sera to mediate antibody-dependent enhancement (ADE) was measured using HEK293T cells or HEK293T cells stably expressing human ACE2 (293T-hACE2 cells) that had been transfected using the calcium phosphate transfection method to express the rat ortholog of FcγRI (CD64). The human monocytic cell line K562 (ATCC CCL-243), which endogenously expresses FcγRII, was also used for ADE assays. The RBD immune sera, collected from four different rats at day 40 after the first immunization, were mixed at an equal ratio, as was preimmune sera obtained at day 0 from the same rats. As a positive control, sera from ZIKV-infected rats (rats 13 and 15, distinct from the similarly numbered RBD-inoculated rats) were also mixed at an equal ratio. Immune and preimmune serum samples were heat inactivated for 30 min at 56°C and serially diluted in DMEM containing 10% heat-inactivated fetal bovine serum (FBS). SARS2-PV or ZIKV VLP in 50 μl were preincubated for 1 h at 37°C with 50 μl of diluted sera and added to the indicated cells plated on the 96-well plates. Two days later, infection levels were assessed using a Luc-Pair firefly luciferase HS assay kit (Genocopia) for SARS-PV and a Luc-Pair *Renilla* luciferase HS assay kit (Genocopia) for ZIKV-VLP.

SDS-PAGE and Western blotting. Expi293 supernatant samples were centrifuged, separated, and filtered (0.45 μm). Equivalent fractions of cell lysate and clarified supernatants were loaded and subjected to SDS-PAGE on NuPAGE bis-Tris gels. Gels were then transferred to 0.4-μm polyvinylidene difluoride (PVDF) membrane using an XCell blot module under a constant voltage of 25 V for 1 h. The membrane was washed and blocked (5% PBS-Tween [PBST]–milk, 4°C) and then blotted with 100 ng/ml NbSym2-rabbit Fc, recognizing

the C tag, previously conjugated to horseradish peroxidase (Lightning-Link; Novus Biologicals) according to the manufacturer's instructions.

Blue native polyacrylamide gel electrophoresis. RBD multimer proteins and particles were analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE). The proteins were mixed with sample buffer and G250 loading dye and added to a 3 to 12% bis-Tris NativePAGE gel (Life Technologies). BN-PAGE gels were run for 2 h at 150 V using the NativePAGE running buffer (Life Technologies) according to the manufacturer's instructions.

Native Western blotting. Expi293 supernatant samples were centrifuged and filtered (0.45 μm). Clarified supernatants were loaded and analyzed by BN-PAGE. Gels were then transferred to 0.2-μm PVDF membranes using an XCell blot module under a constant voltage of 25 V for 1 h. The membrane was washed and blocked (5% TBST-milk, 4°C) and then blotted with 50 ng/ml ACE2-Fc, previously conjugated to horseradish peroxidase (Lightning-Link; Novus Biologicals), according to the manufacturer's instructions.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.5 MB.
FIG S2, PDF file, 0.5 MB.
FIG S3, PDF file, 0.5 MB.
FIG S4, PDF file, 1.9 MB.

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