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Bivalent mRNA booster encoding virus-like particles elicits potent polyclass receptor-binding domain antibodies in pre-vaccinated mice

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eLife Assessment

This **useful** and interesting study provides evidence that EABR mRNA is at least as effective as standard S mRNA vaccines for SARS-CoV-2. The authors provide **convincing** justification for the conclusion that the inconsistent statistical significance for Omicron is likely due to immune imprinting or original antigenic sin. In this regard, the significance of the findings is stronger as it points to possible challenges for updated vaccine strategies in overcoming immune imprinting.

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Abstract

mRNA vaccines emerged as a leading vaccine technology during the COVID-19 pandemic. However, their sustained protective efficacies were limited by relatively short-lived antibody responses and the emergence of SARS-CoV-2 variants, necessitating frequent and variant-updated boosters. We recently developed the ESCRT- and ALIX-binding region (EABR) mRNA vaccine platform, which encodes engineered immunogens that induce budding of enveloped virus-like particles (eVLPs) from the plasma membrane, thereby resulting in presentation of immunogens on cell surfaces and eVLPs. Prior studies showed that spike (S)-EABR mRNA-LNP immunizations elicited enhanced neutralizing antibody responses against ancestral and variant SARS-CoV-2 compared with conventional S mRNA-LNP in naïve mice, but the effectiveness of S-EABR mRNA-LNP boosters in the context of pre-existing immunity has not been investigated. Here, we evaluated monovalent Wuhan-Hu-1 (Wu1) and bivalent (Wu1/BA.5) S-EABR mRNA-LNP boosters in mice pre-vaccinated with conventional Wu1 S mRNA-LNP. Compared to conventional S mRNA-LNP boosters, the EABR approach enhanced monovalent and bivalent mRNA-LNP booster-induced neutralizing responses against Omicron subvariants BA.1, BA.5, BQ.1.1, and XBB.1, with bivalent S-EABR mRNA-LNP consistently eliciting the highest titers. Epitope mapping of polyclonal antisera by deep mutational scanning revealed that bivalent S-EABR mRNA-LNP boosted diverse “polyclass” anti-RBD responses, suggesting balanced targeting of multiple RBD epitope classes. In contrast, monovalent S, bivalent S, and monovalent S-EABR mRNA-LNP boosters elicited less diverse polyclonal serum responses primarily targeting immunodominant RBD epitopes. Cryo-EM structures demonstrated that bivalent mRNA immunizations promote S heterotrimer formation, potentially enhancing bivalent S-EABR mRNA-LNP booster-induced antibody breadth and

polyclass epitope targeting by activating cross-reactive B cells through intra-S crosslinking. These findings support the future design of bivalent or multivalent S-EABR mRNA-LNP boosters as a promising strategy to confer broader, and therefore potentially more durable, protection against emerging SARS-CoV-2 variants and other rapidly evolving viruses.

Introduction

mRNA vaccines emerged as a leading platform for the rapid and scalable development of effective vaccines during the COVID-19 pandemic¹. Currently approved mRNA vaccines encode the SARS-CoV-2 spike (S) trimer², the main target for neutralizing antibodies (Abs) produced during natural infection³. S-encoding mRNAs formulated in lipid nanoparticles (LNP) are taken up by cells, which promotes translation and cell surface expression of S trimers to activate B cells⁴. Translation of mRNA-encoded S protein within host cells also provides viral peptides for presentation on MHC class I molecules, which are recognized by cytotoxic T cells^{5,6}.

Initial two-shot COVID-19 mRNA vaccination series became available in December 2020 after clinical studies demonstrated protection from severe disease after SARS-CoV-2 infection^{7,8}. However, the durability of mRNA vaccine-induced immune protection was limited by two factors. First, serum Ab levels contracted over several months⁹. Second, SARS-CoV-2 variants acquired mutations in the S protein, which reduced their sensitivity to vaccine-induced neutralizing Abs¹⁰. As a result, a third dose of the original mRNA vaccine encoding the ancestral Wuhan-Hu-1 (Wu1) S protein was offered starting in fall 2021¹¹.

The emergence of the Omicron BA.1 variant in November 2021¹² sparked concern because of its levels of substitution in S, especially in the receptor-binding domain (RBD), resulting in effective evasion of Ab-mediated immunity^{13,14} and increased rates of symptomatic infections in previously vaccinated individuals^{15,16}. Although undetectable after the initial mRNA vaccine series, a third dose of the original mRNA vaccine elicited neutralizing Abs against BA.1, but BA.1 neutralizing titers were lower compared to titers against the ancestral Wu1 variant¹⁷. The subsequent emergence of Omicron subvariants such as BA.5, BQ.1.1, and XBB.1, which exhibited progressively reduced sensitivities to vaccine-induced neutralizing Abs¹⁸, prompted the development of bivalent mRNA boosters, which included mRNAs encoding both the Wu1 and the BA.5 S proteins¹⁹. Preclinical and clinical studies found that bivalent boosters elicited modestly improved neutralizing Ab responses against Omicron subvariants compared to a fourth dose of the original monovalent vaccine^{19–23}, although some studies found no differences between monovalent and bivalent boosters following initial vaccination with the original Wu1 S-encoding mRNA vaccine^{24,25}. Several studies^{21,22,24,26–28} showed that the effectiveness of bivalent boosters was limited by immune imprinting, which occurs when prior exposure to an antigen shapes immunological responses to related antigens during subsequent exposures²⁹. Instead of generating *de novo* Omicron-specific immune responses, bivalent boosters primarily activated pre-existing cross-reactive memory B cells from previous vaccinations and/or infections^{26–28}, leading to lower neutralizing activity against Omicron subvariants compared to the ancestral variant^{21,22,24}.

Continued development of updated boosters in response to emerging variants was slowed by the need for additional clinical testing, regulatory approvals, and adjustments to the manufacturing process. As a result, bivalent boosters became available in fall 2022, almost one year after the emergence of Omicron³⁰. These challenges highlight the urgent need for innovative vaccine strategies that achieve more lasting immune protection against rapidly-evolving viruses such as SARS-CoV-2, thereby reducing the need for frequent and updated boosters.

We recently developed the ESCRT- and ALIX-binding region (EABR) mRNA vaccine platform that combines features of mRNA and protein nanoparticle (NP) vaccines through delivery of mRNA-encoded self-assembling enveloped virus-like particles (eVLPs)³¹. eVLP assembly is achieved by fusing a short EABR sequence to the cytoplasmic tail of viral surface proteins, which recruits proteins from the Endosomal Sorting Complex Required for Transport (ESCRT) pathway. Thus, EABR mRNA vaccines promote dual presentation of S trimers on cell surfaces and eVLPs that bud from the plasma membrane. Immunizations in mice showed that an EABR mRNA vaccine

encoding a Wu1 SARS-CoV-2 S elicited superior neutralizing Ab potency and breadth against ancestral and Omicron SARS-CoV-2 variants compared to conventional mRNA and protein NP vaccines³¹. Similar to conventional mRNA vaccines, the EABR mRNA vaccine also induced potent T cell responses, consistent with the presence of S-specific cytotoxic T cells³¹. These findings suggest that the EABR mRNA vaccine approach has the potential to enhance monovalent or bivalent booster-induced Ab responses against Omicron subvariants in the context of pre-existing immunity induced by prior vaccination, infection, or both.

Here, we evaluated the ability of EABR mRNA boost immunogens to elicit potent and broad humoral immune responses against SARS-CoV-2 variants in pre-vaccinated mice. We demonstrate that the EABR mRNA vaccine approach improves monovalent and bivalent booster-mediated neutralizing Ab responses against Omicron subvariants compared to conventional mRNA boosters. Epitope mapping of polyclonal antisera further showed that a bivalent EABR mRNA booster elicited a more diversified anti-RBD IgG response than other tested boosting immunogens. These findings support the use of the EABR mRNA vaccine platform for the design of effective boosters that could confer more lasting protection against SARS-CoV-2 variants and potentially other rapidly-evolving viruses. In addition, our work provides structural evidence that bivalent mRNA immunizations promote heterotrimer formation, which could enhance activation of cross-reactive B cells through intra-S crosslinking. These insights inform the future design of bivalent or multivalent COVID-19 boosters and highlight the need for further investigation into potentially beneficial effects of S heterotrimer formation on vaccine-induced immune responses against trimeric viral Ss.

Results

To expand upon our previous characterizations of a monovalent SARS-CoV-2 S-EABR mRNA vaccine³¹, we evaluated the potential of the EABR mRNA vaccine approach for monovalent and bivalent SARS-CoV-2 immunizations in pre-vaccinated mice. Four cohorts of 10 BALB/c mice each initially received prime-boost intramuscular (IM) vaccinations on days 0 and 28 with 2 µg of a conventional mRNA vaccine encoding the ancestral S protein from the Wu1 variant, similar to the immunogens used in initially approved COVID-19 mRNA vaccines (Wu1 S mRNA-LNP) (Fig. 1A-C). To simulate the timing of the third available COVID-19 mRNA vaccine dose in humans, booster immunizations were administered on day 230, nearly 7 months after the primary vaccination series, with each cohort receiving a different boost immunogen (Fig. 1A-C). Consistent with the COVID-19 booster that became available in fall 2021¹¹, cohort 1 received a third dose of 2 µg Wu1 S mRNA-LNP (Fig. 1B-C). To evaluate whether formation of mRNA-encoded S-EABR eVLPs enhances booster immunogenicity in pre-vaccinated mice, cohort 2 received 2 µg Wu1 S-EABR mRNA-LNP (Fig. 1B-C). Cohort 3 received a bivalent Wu1/BA.5 S mRNA-LNP booster (1 µg Wu1 S mRNA-LNP combined with 1 µg BA.5 S mRNA-LNP), which resembled the COVID-19 booster that became available in fall 2022³⁰ (Fig. 1B-C). Lastly, cohort 4 received a bivalent Wu1/BA.5 S-EABR mRNA-LNP booster to promote the formation of eVLPs that co-display Wu1 and BA.5 S trimers (Fig. 1B-C). The Wu1 and BA.5 S-EABR constructs were designed as previously described by fusing an endocytosis-preventing motif (EPM), a short Gly-Ser linker, and an EABR sequence to the end of the cytoplasmic domain of Wu1 and BA.5 S trimers³¹ (Fig. S1A). The Wu1 and BA.5 S-EABR constructs induced similar levels of eVLP budding in transiently transfected HEK293T cells (Fig. S1B).

Serum binding Ab responses were measured by enzyme-linked immunosorbent assay (ELISA) four and 18 weeks after the primary vaccination series (days 56 and 154) and two weeks after the booster immunizations (day 244). As previously observed, the initial Wu1 S mRNA-LNP vaccinations elicited high binding Ab titers against soluble Wu1 S-6P trimers³², and as expected, these titers were similar for all cohorts on day 56 (Fig. 1D). Robust binding Ab responses were also detected for all cohorts against Omicron BA.5, BQ.1.1, and XBB.1 S-6P trimers on day 56 (Fig. 1E-G). On day 154, binding Ab levels against all variants dropped slightly, which was consistent

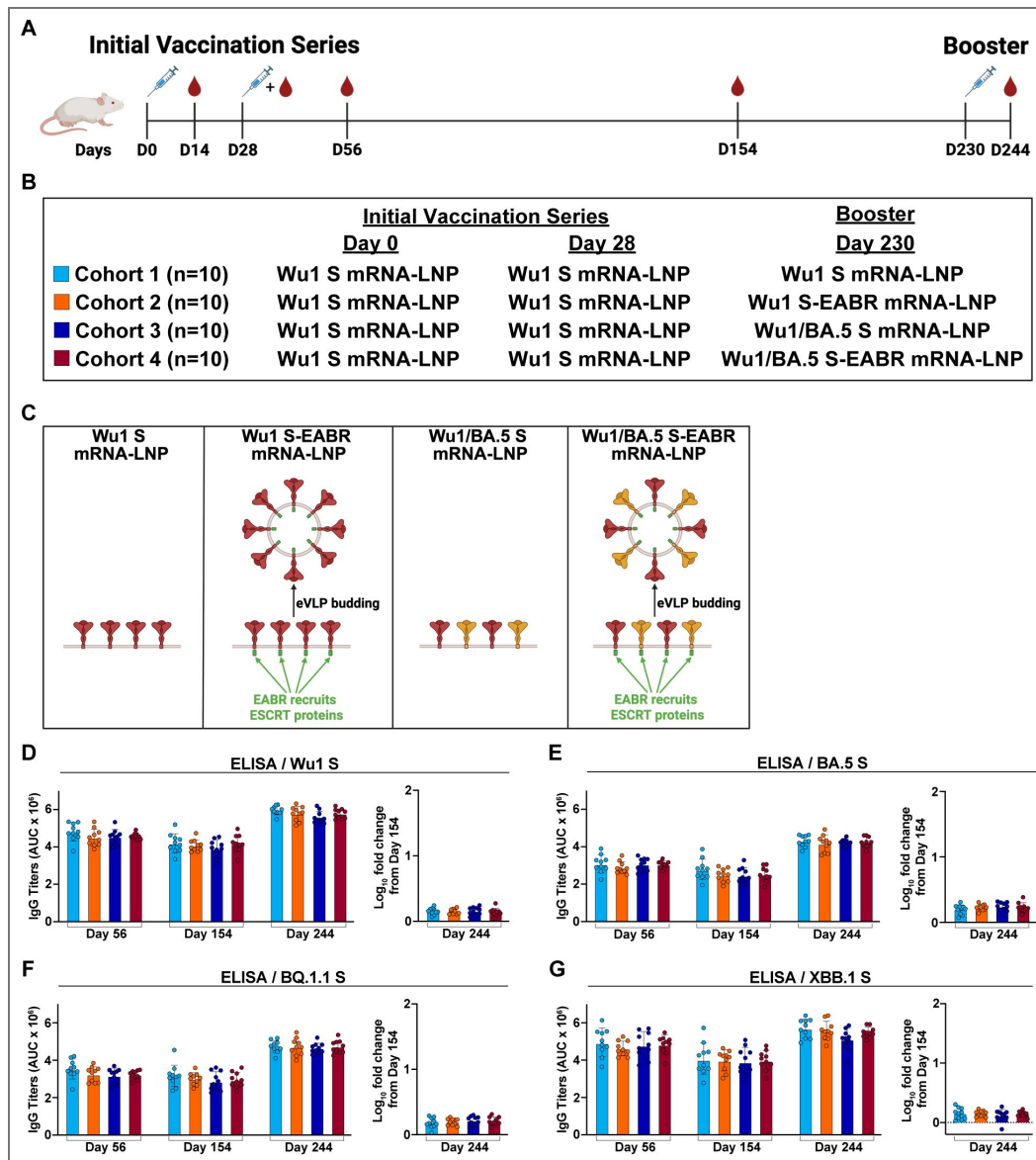


Figure 1. Monovalent and bivalent boosters elicit similar Ab binding responses against variant S trimers in pre-vaccinated mice.

A) Immunization schedule in BALB/c mice. Blood samples were collected on days 14, 56, 154, and 244. This panel was created using *BioRender.com*. **B)** Immunization regimens for each cohort. All cohorts were initially vaccinated with 2 μ g of Wu1 S mRNA-LNP on days 0 and 28 by IM injection. Pre-vaccinated mice received different boost immunogens on day 230 by IM injection. Cohorts 1-4 received 2 μ g of monovalent Wu1 S mRNA-LNP (cyan), monovalent Wu1 S-EABR mRNA-LNP (orange), bivalent Wu1/BA.5 S mRNA-LNP (blue), or bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon), respectively. **C)** Schematic showing the different boost immunogens that were administered on day 230. Wu1 S mRNA-LNP immunization results in cell surface presentation of membrane-anchored Wu1 S trimers. Wu1 S-EABR mRNA-LNP immunization results in dual presentation of Wu1 S trimers on cell surfaces and on eVLPs that are secreted after budding from the plasma membrane. eVLP formation is induced by EABR-mediated recruitment of host cell proteins from the ESCRT pathway. Bivalent Wu1/BA.5 S mRNA-LNP immunization results in cell surface presentation of membrane-anchored Wu1 and BA.5 S trimers. Wu1/BA.5 S-EABR mRNA-LNP immunization results in dual presentation of Wu1 and BA.5 S trimers on cell surfaces and eVLPs that bud from the plasma membrane. S trimers are shown as homotrimers for both monovalent and bivalent boost immunogens. This panel was created using *BioRender.com*. **D-G)** ELISA data from indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of area under the curve (AUC) values. ELISAs evaluated binding of purified Ss from the **D)** Wu1, **E)** BA.5, **F)** BQ.1.1, and **G)** XBB.1 variants. The $\log_{10}$ -fold change in binding titers from day 154 to day 244 is also shown for each S (plotted as mean (bars) and standard deviation (horizontal lines)).

between cohorts (Fig. 1D-G). On day 244, all boost immunogens increased binding Ab titers against Wu1, BA.5, BQ.1.1, and XBB.1 S-6P trimers compared to titers measured on days 56 and 154, but no differences were observed between cohorts for all timepoints (Fig. 1D-G).

Bivalent S-EABR mRNA-LNP booster elicits potent Ab responses targeting Omicron RBDs

We next evaluated the impact of the different boost immunizations on Ab responses against the RBD, the primary target of neutralizing Abs³. The initial Wu1 S mRNA-LNP prime-boost vaccinations elicited robust and similar Ab responses against the Wu1 RBD for all cohorts on day 56 (Fig. 2A). The BA.5, BQ.1.1, and XBB.1 variants harbor multiple RBD substitutions, particularly within the class 1 and 2 epitopes at the tip of the RBD³³ (Fig. S2), promoting effective escape from neutralizing Abs¹⁸. Compared to Wu1 RBD binding, Ab binding titers were weaker against the BA.5, BQ.1.1, and XBB.1 RBDs on day 56 (Fig. 2B-D). The robust Ab binding against the BA.5, BQ.1.1, and XBB.1 S trimers (Fig. 1E-G) in the absence of strong RBD responses was at least in part driven by potent responses against the S2 region of S (Fig. S3), which is relatively conserved in SARS-CoV-2 variants³⁴ (Fig. S2). Similar to binding titers against S-6P trimers (Fig. 1D-G), Wu1, BA.5, BQ.1.1, and XBB.1 RBD binding slightly dropped on day 154 without statistically significant differences between cohorts (Fig. 2A-D). The booster immunizations increased Wu1 RBD binding on day 244 compared to day 56 and 154 levels, but no differences were detected between the four boosting immunogens (Fig. 2A). In contrast, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster elicited the highest BA.5, BQ.1.1, and XBB.1 RBD responses on day 244 (Fig. 2B-D), which were significantly higher than Wu1 S mRNA-LNP booster-induced responses against BQ.1.1 RBD ($p = 0.0499$) (Fig. 2C) and narrowly failed to reach statistical significance compared to Wu1 S mRNA-LNP booster-induced responses against BA.5 RBD ($p = 0.07$) (Fig. 2B). Compared to baseline titers measured prior to boost immunizations on day 154, the bivalent Wu1/BA.5 S-EABR mRNA-LNP immunization elicited significantly larger increases in BA.5 RBD binding than the monovalent Wu1 S mRNA-LNP ($p = 0.02$) and the bivalent Wu1/BA.5 S mRNA-LNP boosters ($p = 0.01$) (Fig. 2B), as well as significantly larger increases in BQ.1.1 RBD binding than the Wu1 S mRNA-LNP booster ($p = 0.002$) (Fig. 2C). While overall titers were similar on day 244, the monovalent Wu1 S-EABR mRNA-LNP booster consistently induced larger increases in Ab binding to the BA.5, BQ.1.1, and XBB.1 RBDs than the monovalent Wu1 S mRNA-LNP booster (Fig. 2B-D), although these differences failed to reach statistical significance. The monovalent Wu1 and bivalent Wu1/BA.5 S mRNA-LNP boosters elicited similar binding titers against the BA.5 and XBB.1 RBDs (Fig. 2B and D), but the bivalent booster induced slightly higher responses and a larger increase compared to day 154 titers against BQ.1.1 RBD (not statistically significant) (Fig. 2C). Overall, these results demonstrate that booster immunizations with the bivalent Wu1/BA.5 S-EABR mRNA-LNP elicited the highest Ab responses against Omicron RBDs in pre-vaccinated mice. Notably, post-boost Ab binding against Omicron RBDs remained lower than binding titers against Wu1 RBD in all cohorts, suggesting that booster responses were affected by immune imprinting induced by the primary vaccination series.

Bivalent S-EABR mRNA-LNP booster elicits higher neutralizing responses against Omicron variants

The ability of boost immunogens to elicit neutralizing Ab responses in pre-vaccinated mice was evaluated by in vitro pseudovirus neutralization assays^{35,36}. The primary vaccination series with Wu1 S mRNA-LNP elicited potent and consistent autologous geometric mean neutralizing titers as assessed by half-maximal serum inhibitory dilution (ID₅₀) values against Wu1 pseudovirus in all cohorts on day 56 (Fig. 3A; Table S1). Neutralizing activity was >100-fold lower against the BA.5 variant and undetectable in most animals against the BQ.1.1 and XBB.1 variants on day 56 for all cohorts (Fig. 3B-D; Table S1). On day 154, neutralizing titers against the Wu1 and BA.5 variants were consistent in all cohorts and slightly lower compared to day 56 titers (Fig. 3A-B; Table S1). Neutralizing activity against the BQ.1.1 and XBB.1 variants was undetectable in all but one animal on day 154 (Fig. 3C-D; Table S1). Booster immunizations on day 230 increased

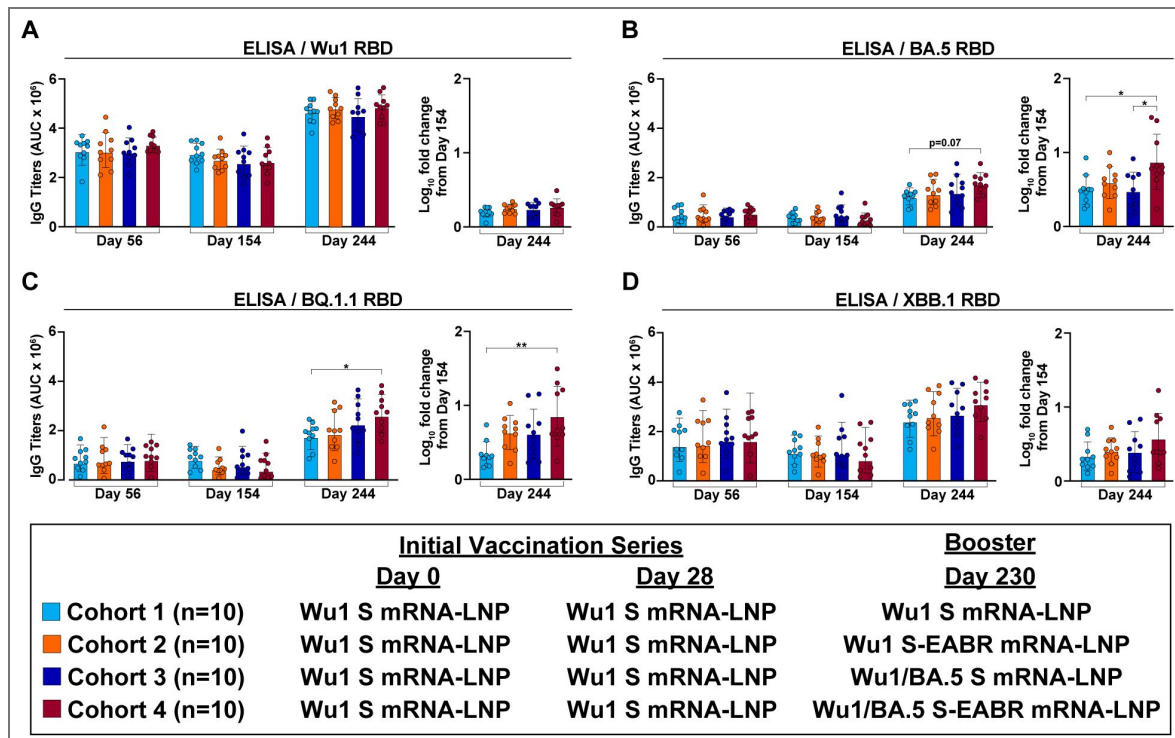


Figure 2. Bivalent S-EABR mRNA-LNP booster elicits potent Ab responses targeting Omicron RBDs.

A-D) RBD binding titers for antisera from pre-vaccinated mice that received booster immunizations with 2 µg of monovalent Wu1 S mRNA-LNP (cyan), 2 µg of monovalent Wu1 S-EABR mRNA-LNP (orange), 2 µg of bivalent Wu1/BA.5 S mRNA-LNP (blue), or 2 µg of bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon) (immunization schedule in Fig. 1A-B). ELISA data are shown for indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of area under the curve (AUC) values. ELISAs evaluated binding of RBDs from the **A)** Wu1, **B)** BA.5, **C)** BQ.1.1, and **D)** XBB.1 SARS-CoV-2 variants. The log₁₀-fold change in binding titers from day 154 to day 244 is also shown for each RBD (plotted as mean (bars) and standard deviation (horizontal lines)). Significant differences between cohorts linked by horizontal lines are indicated by asterisks: p<0.05 = *, p<0.01 = **.

autologous neutralizing titers against Wu1 pseudovirus on day 244 compared to day 154 in all cohorts (Fig. 3A; Table S1). Monovalent Wu1 S mRNA-LNP and S-EABR mRNA-LNP immunizations elicited the smallest and largest increases in Wu1 neutralization (4.2-fold versus 6.6-fold), respectively. All boost immunogens elicited increased BA.5 neutralizing activity on day 244 compared to day 56 and 154 titers (Fig. 3B; Table S1). Boosting with monovalent Wu1 S-EABR mRNA-LNP induced 4.8-fold higher BA.5 neutralizing titers than a third Wu1 S mRNA-LNP immunization, but this difference did not reach statistical significance. Using the respective day 154 titers as baseline, the Wu1 S-EABR mRNA-LNP booster induced a 23.8-fold increase in BA.5 neutralization on day 244 compared to only a 6.3-fold increase detected after boosting with Wu1 S mRNA-LNP (not statistically significant) (Fig. 3B; Table S1). Compared to day 56 baseline titers, the Wu1 S-EABR mRNA-LNP booster induced a significantly higher increase in BA.5 neutralization on day 244 than the conventional Wu1 S mRNA-LNP booster (16.2-fold versus 3.1-fold; $p = 0.048$) (Fig. S4; Table S1). The Wu1 S-EABR mRNA-LNP booster also elicited 3.4-fold higher neutralizing responses than the Wu1 S mRNA-LNP booster against the Omicron BA.1 variant on day 244 ($p = 0.03$) (Fig. S5). Based on day 154 titers, boosting with monovalent Wu1 S-EABR mRNA-LNP induced significantly higher increases in BA.1 neutralization compared to monovalent Wu1 S mRNA-LNP (8.8-fold versus 2.5-fold; $p = 0.009$) (Fig. S5). Interestingly, the monovalent Wu1 S-EABR mRNA-LNP and the bivalent Wu1/BA.5 S mRNA-LNP boosters elicited similar BA.5 neutralizing responses on day 244 (Fig. 3B; Table S1), suggesting that the EABR mRNA vaccine approach might reduce the need for updating boost immunogens to achieve robust Ab responses against emerging SARS-CoV-2 variants. The highest BA.5 neutralizing titers were elicited by the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster, which were 12.3-fold higher than titers induced by the monovalent Wu1 S mRNA-LNP booster ($p = 0.02$) and 2.4-fold higher than titers induced by the bivalent Wu1/BA.5 S mRNA-LNP booster (not statistically significant). Based on titers measured on day 154, the Wu1/BA.5 S-EABR mRNA-LNP booster induced a 67-fold increase in BA.5 neutralization, which was significantly higher than the increase elicited by boosting with monovalent Wu1 S mRNA-LNP ($p = 0.003$).

While the BA.5 neutralizing activity induced by the monovalent Wu1 S and S-EABR mRNA-LNP boosters can likely be attributed to cross-neutralizing Abs that neutralize both the Wu1 and BA.5 variants, it is possible that the bivalent Wu1/BA.5 S and S-EABR mRNA-LNP boosters also elicited *de novo* strain-specific Abs that neutralize BA.5 but not Wu1. Day 244 serum samples from mice that received bivalent Wu1/BA.5 S or S-EABR mRNA-LNP boosters were depleted of Abs that bind to Wu1 RBD. Sera were passaged over a column presenting either a mock protein (HIV-1 gp120) as a control or the Wu1 RBD to remove cross-neutralizing anti-RBD Abs while retaining BA.5 RBD-specific Abs. Following depletions, residual neutralization activity was measured against the BA.5 variant. Interestingly, Wu1 RBD depletion resulted in 17- and 36-fold lower geometric mean neutralizing titers compared to mock depletion for the bivalent Wu1/BA.5 S and S-EABR mRNA-LNP boosters, respectively (Fig. S6). In both cohorts, BA.5 neutralizing titers in all but two mice dropped below ID₅₀ values of 1:120 following Wu1 RBD depletion, consistent with the neutralizing activity in these samples being primarily driven by cross-neutralizing Abs targeting the RBD induced by the primary vaccination series. The potent residual neutralizing activity observed in two mice in each cohort after Wu1 RBD depletion implies either the presence of boost-induced *de novo* BA.5-specific RBD Abs or neutralizing non-RBD Abs that target another region of S, e.g., the N-terminal domain³⁷ or S2³⁴.

Booster immunizations had less pronounced effects on neutralization activity against the BQ.1.1 and XBB.1 variants. Against BQ.1.1 pseudovirus, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster elicited the most potent neutralizing activity on day 244, which was significantly higher than responses induced by a third immunization with the monovalent Wu1 S mRNA-LNP booster ($p = 0.0495$) (Fig. 3C; Table S1). The monovalent Wu1 S-EABR mRNA-LNP and bivalent Wu1/BA.5 S mRNA-LNP boosters elicited 1.8- and 2.5-fold higher BQ.1.1 titers than the monovalent Wu1 S mRNA-LNP booster, respectively, the latter of which narrowly failed to reach statistical significance ($p = 0.07$). Based on day 154 BQ.1.1 neutralizing titers, the monovalent Wu1 S mRNA-LNP and bivalent Wu1/BA.5 S-EABR mRNA-LNP boosters induced the smallest and largest increases in neutralizing activity on day 244, respectively (1.4-fold versus 4.7-fold; $p = 0.0495$). The

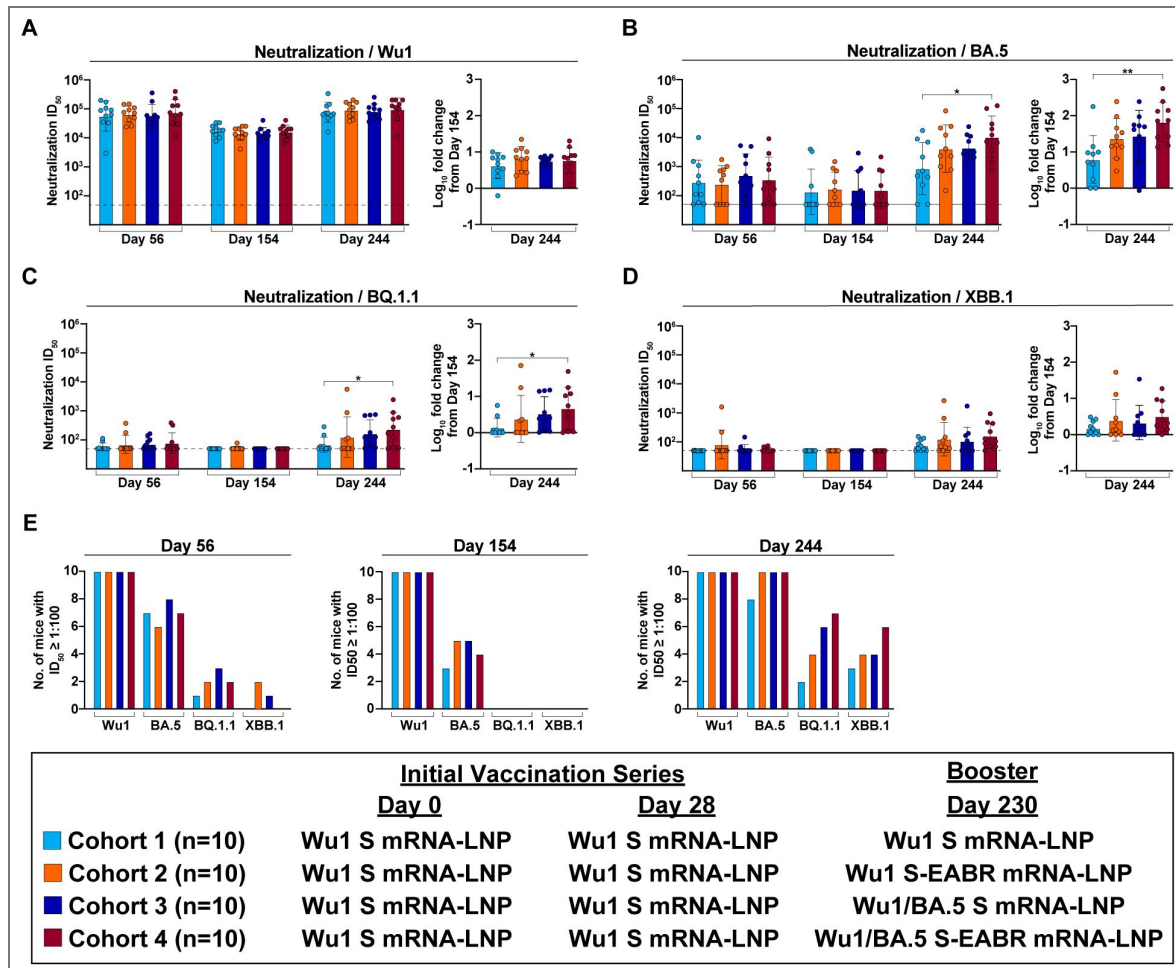


Figure 3. Bivalent Wu1/BA.5 S-EABR mRNA-LNP booster elicits higher neutralizing responses against Omicron subvariants.

A-D) Neutralization titers for antisera from pre-vaccinated mice that received booster immunizations with 2 µg of monovalent Wu1 S mRNA-LNP (cyan), 2 µg of monovalent Wu1 S-EABR mRNA-LNP (orange), 2 µg of bivalent Wu1/BA.5 S mRNA-LNP (blue), or 2 µg of bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon) (immunization schedule in Fig. 1A-B). Neutralization data are shown for indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of ID₅₀ values. Results are shown for **A)** Wu1, **B)** BA.5, **C)** BQ.1.1, and **D)** XBB.1 pseudoviruses. Dashed horizontal lines correspond to the background values representing the limit of detection for neutralization assays (ID₅₀ = 1:50). The log₁₀-fold change in neutralization titers from day 154 to day 244 is also shown for each pseudovirus (plotted as mean (bars) and standard deviation (horizontal lines)). Significant differences between cohorts linked by horizontal lines are indicated by asterisks: p<0.05 = *, p<0.01 = **.

E) Number of mice in each cohort that reached an ID₅₀ ≥ 1:100 against each variant on days 56 (left), 154 (middle), and 244 (right).

monovalent Wu1 S-EABR mRNA-LNP and bivalent Wu1/BA.5 S mRNA-LNP boosters elicited 2.4- and 3.4-fold increases in neutralizing BQ.1.1 titers, respectively. Notably, day 244 neutralizing titers against BQ.1.1 were substantially lower than BA.5 neutralizing titers for all boost immunogens (Fig. 3B-C; Table S1), consistent with previous reports^{18,38}. Notably, Ab binding responses against the BA.5 and BQ.1.1 RBDs were similar (Fig. 2B-C), implying that RBD binding responses are not always predictive of neutralizing activity against Omicron subvariants.

Neutralizing responses against the XBB.1 variant were slightly lower than titers against BQ.1.1 pseudovirus (Fig. 3D; Table S1). Once again, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster elicited the highest XBB.1 neutralizing titers, which were 2.1-fold higher than titers induced by the monovalent Wu1 S mRNA-LNP booster (not statistically significant). Based on day 154 titers, the monovalent Wu1 and bivalent Wu1/BA.5 S-EABR mRNA-LNP boosters increased XBB.1 neutralization by 2.5- and 3.2-fold, respectively, while conventional monovalent and bivalent S mRNA-LNP boosters only induced 1.5- and 2.2-fold increases, respectively.

Notable differences were also observed in the number of mice in each cohort that reached robust neutralizing titers (defined as ID₅₀s exceeding 1:100) against Omicron subvariants following booster immunizations, titers that are likely important for the prevention of symptomatic infections³⁹. The primary vaccination series with Wu1 S mRNA-LNP elicited robust neutralizing titers against the Wu1 variant in all mice on day 56 (Fig. 3F). However, the initial Wu1 S mRNA-LNP vaccinations elicited robust titers in only 6 to 8, 1 to 3, and 0 to 2 of 10 mice against the BA.5, BQ.1.1, and XBB.1 variants on day 56, respectively. On day 154, ID₅₀s over 1:100 were measured for 3 to 5 mice in each cohort against BA.5, but for none of the animals against BQ.1.1 and XBB.1. While a third immunization with Wu1 S mRNA-LNP induced robust titers against BA.5 in only 8 of 10 mice on day 244, the monovalent Wu1 S-EABR mRNA-LNP booster induced robust titers against BA.5 in all 10 mice, including the 4 mice that did not have detectable BA.5 neutralizing activity on day 56 (Fig. 3F). As expected, robust neutralizing titers against the BA.5 variant were also observed in all mice that received the bivalent Wu1/BA.5 S or S-EABR mRNA-LNP boosters. Against the BQ.1.1 and XBB.1 variants, a third immunization with Wu1 S mRNA-LNP induced robust neutralizing titers in only 2 and 3 mice, respectively (Fig. 3F). In comparison, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster induced ID₅₀s over 1:100 in 7 and 6 mice against the BQ.1.1 and XBB.1 variants, respectively.

Overall, these results demonstrate that the EABR mRNA vaccine approach improves the neutralizing activity against Omicron subvariants induced by monovalent and bivalent boost immunogens in pre-vaccinated mice.

Bivalent S-EABR mRNA-LNP booster induces polyclonal Ab responses

Several distinct Ab-binding epitopes have been defined on RBDs, including the variable class 1 and 2 RBD epitopes and the more conserved class 3, 4, 1/4, and 5 RBD epitopes^{33,40,41} (Fig. S2). We determined which RBD epitopes were targeted by booster-induced Abs in pre-vaccinated mice using deep mutational scanning (DMS) of yeast display libraries derived from Wu1⁴² and XBB.1.5 RBDs⁴³. In previous studies, we showed that equimolar mixtures of monoclonal Abs targeting distinct RBD epitopes produced less pronounced DMS escape peaks, which we defined as a polyclonal Ab response⁴⁴. This suggests that polyclonal serum that includes roughly equal distributions of Abs targeting multiple classes of RBD epitopes would exhibit relatively flat DMS profiles, while more defined DMS escape peaks would be observed for less diverse polyclonal serum responses containing Abs that primarily target particular RBD epitopes⁴⁴. For this study, we used DMS escape fractions to define polyclonal responses. Escape fractions for each RBD mutation range from 0 (no cells with this mutation are sorted into the serum Ab escape bin) to 1 (all cells with this mutation are sorted into the serum Ab escape bin)⁴⁵. Total escape peaks represent the sum of escape fractions for all mutations at a specific site⁴⁵. DMS profiles with total escape peaks below 0.5 at all sites were classified as polyclonal responses. In contrast, DMS profiles with total escape peaks of 0.5-1, 1-2, or above 2 at one or more sites were classified as weak, moderate, or strong escape profiles, respectively, indicating weak, moderate, or strong targeting of particular

RBD epitopes (Table S2 [↗](#)). DMS was performed for 6 serum samples for each cohort, which were selected based on XBB.1 neutralization activity (1st, 2nd, 3rd, 5th, 6th, and 10th best neutralizer from each cohort). For the Wu1 RBD library, only 5 DMS profiles were obtained due to poor sequencing quality for one sample in each cohort.

Against the Wu1 RBD library, immunizations with the monovalent Wu1 S and S-EABR mRNA-LNP boosters or the bivalent Wu1/BA.5 S mRNA-LNP booster resulted in DMS profiles consistent with moderate targeting of the variable class 2 epitope primarily inducing escape at RBD residue 484 (Fig. 4A-B [↗](#)). The bivalent Wu1/BA.5 S mRNA-LNP booster also elicited weak to moderate responses that mapped to the variable class 1 and class 3 RBD epitopes. In contrast, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster DMS profiles were consistent with polyclonal Ab induction, suggesting a more diverse response targeting multiple RBD epitopes (Fig. 4A-B [↗](#)). Notably, samples from 4 out of 5 mice in this cohort exhibited polyclonal DMS profiles, whereas moderate to strong escape fractions for class 1, 2, or 3 epitopes were observed in 3-4 mice in each of the other cohorts (Fig. S7 [↗](#); Table S2 [↗](#)).

As expected, no class 1 and 2 DMS profiles were observed against the XBB.1.5 RBD library⁴³ since these epitopes are highly substituted in Omicron subvariants (Fig. S2 [↗](#)). All booster immunizations induced strong class 5 escape peaks targeting RBD residues 352, 357, 396, and 468 (Fig. 4C-D [↗](#)). Importantly, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster induced a less pronounced overall class 5 profile than the other boost immunogens, which was primarily driven by a strong class 5 response observed in a single animal in this cohort (Fig. S8 [↗](#); Table S2 [↗](#)). The other mice that received the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster exhibited polyclonal or weak to moderate responses against class 4 and 5 epitopes. In contrast, strong class 5 profiles were detected in 4-5 mice in cohorts that received the monovalent Wu1 S or S-EABR mRNA-LNP boosters or the bivalent Wu1/BA.5 S mRNA-LNP booster (Fig. S8 [↗](#); Table S2 [↗](#)). Overall, these results suggest that the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster induced a more diversified polyclonal Ab response that likely contributed to the higher neutralizing activity observed against Omicron subvariants, which could result in more effective recognition of future variants.

Co-expression of Wu1 and BA.5 S results in heterotrimer formation

The results from this and previous studies^{19–22} demonstrated that bivalent booster immunizations improve the potency and breadth of Ab responses against Omicron subvariants compared to monovalent boosters. Serum depletion assays also showed that the BA.5 neutralizing activity elicited by the bivalent Wu1/BA.5 S and S-EABR mRNA-LNP boosters was primarily driven by cross-reactive Abs that bind to the Wu1 and BA.5 RBDs (Fig. S6 [↗](#)). Preferential activation of cross-reactive B cells could be achieved through co-display of Wu1 and BA.5 S trimers on the surface of a cell and/or an eVLP (Fig. 1C [↗](#)), which could result in inter-S crosslinking between cross-reactive B cell receptors (BCRs) and neighboring homotrimeric Wu1 and BA.5 S trimers (Fig. S9 [↗](#)). As an additional mechanism for bivalent booster-induced activation of cross-reactive B cells, we hypothesized that co-expression of ancestral Wu1 and Omicron S in the same cell could result in the formation of S heterotrimers consisting of Wu1 and Omicron S protomers and could promote intra-S crosslinking, i.e., simultaneous binding of cross-reactive BCRs to two neighboring RBDs within the same S trimer (Fig. S9 [↗](#)). We previously postulated that intra-S crosslinking is possible for several anti-RBD IgGs based on modeling of intact IgG coordinates into single-particle cryo-electron microscopy (cryo-EM) structures of fragment antigen-binding (Fab)-S complexes^{33,40}, and intra-S crosslinking was observed in EM studies of IgG-S trimer complexes⁴⁶. However, it is currently unknown whether co-expression of Wu1 and Omicron S induces heterotrimerization, and if so, whether heterotrimer formation affects the overall structure of the S ectodomain and influences bivalent booster-induced immune responses.

To investigate whether S heterotrimers could form with different S protomers, we designed 6P versions³² of Wu1/BA.1 and Wu1/BA.1/Delta soluble heterotrimer Ss (HT2 and HT3, respectively) by co-transfection with specific C-terminally-tagged constructs. HT2 was generated by co-transfecting StrepII-tagged Wu1 S and His-tagged BA.1 S, while HT3 was generated using StrepII-

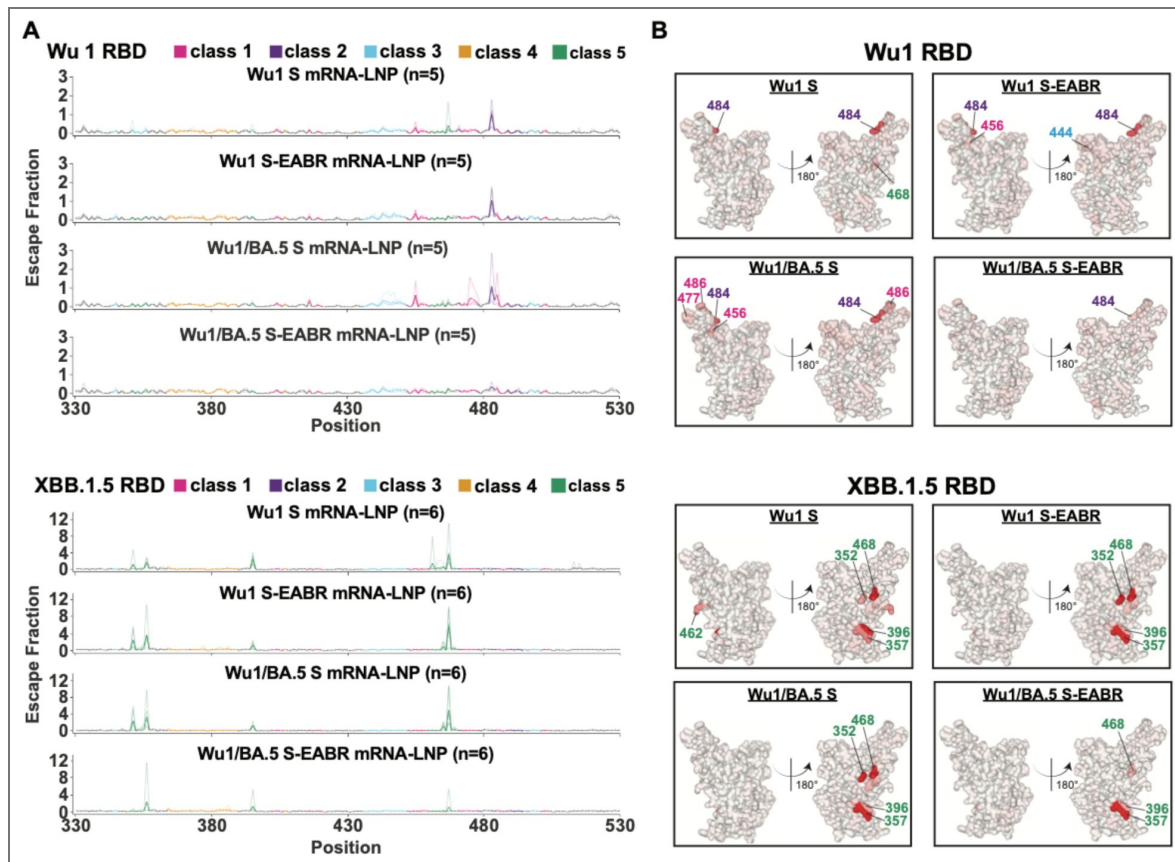


Figure 4. Bivalent S-EABR mRNA-LNP booster induces polyclonal Ab responses.

Sera for DMS studies were derived from pre-vaccinated mice that received booster immunizations with 2 µg of monovalent Wu1 S mRNA-LNP, monovalent Wu1 S-EABR mRNA-LNP, bivalent Wu1/BA.5 S mRNA-LNP, or bivalent Wu1/BA.5 S-EABR mRNA-LNP (immunization schedule in Fig. 1A-B). DMS was performed for 6 serum samples for each cohort, which were selected based on XBB.1 neutralization activity (1st, 2nd, 3rd, 5th, 6th, and 10th best neutralizer from each cohort). For the Wu1 RBD library, only five DMS profiles were obtained for each cohort. **A**) Line plots for DMS results using Wu1 (top) or XBB.1.5 (bottom) RBD libraries for sera from pre-vaccinated mice that received the indicated boost immunogens. The x axes display the RBD residue numbers, and the y axes show the sum of the Ab escape of all mutations at a site (larger numbers indicating increased Ab escape). Each faint line shows one antiserum with heavy lines representing the average across the n = 5 (top) or n = 6 (bottom) sera in each cohort. Lines are colored differently for RBD epitopes within different RBD epitope classes^{33,40} (color definitions shown in legend above line plots; epitopes are shown in Fig. S2); gray color for residues not assigned to an epitope). **B**) The average site-total Ab escape for the Wu1 (top) or XBB.1.5 (bottom) RBD libraries for pre-vaccinated mice that received the indicated boost immunogens mapped to the Wu1 RBD surface (PDB: 6M0J). Gray coloring indicates no escape, and red coloring indicates escape. Lines with numbers colored according to epitope class indicate RBD positions with the most escape.

tagged Wu1 S, His-tagged BA.1 S, and D7324⁴⁷-tagged Delta S (Fig. 5A). Each heterotrimer was subsequently purified using sequential affinity purification specific to each tag, confirming the inclusion of distinct protomers in the purified heterotrimers (Fig. 5A).

HT2 and HT3 S proteins were then evaluated using single-particle cryo-electron microscopy (cryo-EM) to determine whether heterotrimeric Ss resembling their homotrimeric counterparts were formed. To ensure that C-terminal tags did not disrupt the formation of trimers, we included the following controls: (1) three homotrimers with single tags (StrepII-tagged Wu1 S, His-tagged BA.1 S, and D7324-tagged Delta S), and (2) a homotrimer comprised of His- and StrepII-tagged protomers (Wu1-His/Wu1-StrepII).

Single-particle cryo-EM analysis confirmed that all control S proteins formed trimers, indicating that C-terminal tags did not disrupt trimerization (Fig. S10). Because most of the EM constructions for homotrimeric S controls exhibited preferred orientations, we determined overall conformational states (Fig. 5) for the control structures but did not fit atomic models. Structural analysis also revealed trimerized HT2 and HT3 Ss (Fig. 5B–C, Fig. S10). Although atomic models could not be built for the HT2 and HT3 trimer reconstructions because the identities of individual protomers (Wu1 or BA.1 for HT2, and Wu1, BA.1, or Delta for HT3) could not be conclusively determined at relatively low resolution, the EM densities of S heterotrimers and control homotrimers showed no major conformational differences in domain organization (Fig. 5B–C, Fig. S10). As previously seen in SARS-CoV-2 S trimer structures^{48,49}, we observed two trimer populations in all datasets—one with all three RBDs in the ‘down’ conformation and one with a single RBD in an ‘up’ conformation (Fig. 5D). These data demonstrate heterotrimeric S formation for soluble forms of SARS-CoV-2 S proteins. This result suggests that the bivalent S or S-EABR mRNA-LNP boost immunogens likely induce heterotrimerization, which could promote activation of cross-reactive B cells through intra-S cross-linking, a potential mechanism warranting further investigation.

Discussion

The EABR mRNA vaccine platform delivers mRNA encoding engineered immunogens that induce budding of enveloped virus-like particles (eVLPs) from the plasma membrane to promote presentation of immunogens on cell surfaces and eVLPs³¹. This approach elicited superior neutralizing Ab responses in naïve mice compared to a conventional mRNA vaccine against original and variant SARS-CoV-2³¹. However, the majority of the human population has already mounted immune responses against SARS-CoV-2 through prior vaccinations, natural infections, or a combination of both. Here, we demonstrate that the EABR mRNA vaccine approach improves humoral immune responses elicited by monovalent and bivalent mRNA-LNP booster immunizations against Omicron subvariants in pre-vaccinated mice.

The possibility of receiving a third dose of the original COVID-19 mRNA vaccine in fall 2021¹¹ coincided with the emergence of the Omicron variants in November 2021¹². These variants had acquired many substitutions, especially in the RBD, which allowed efficient evasion of Ab-mediated immunity^{13,14} and rapid global spread. Similar to the effects of a third vaccination in humans¹⁷, a third immunization in pre-vaccinated mice with a monovalent Wu1 S mRNA-LNP booster elicited modest neutralizing activity against the early Omicron variants BA.1 and BA.5. However, addition of an EABR sequence³¹ to the boosting immunogen resulted in higher Ab neutralizing titers against BA.1 and BA.5, suggesting that a monovalent Wu1 S-EABR booster could have promoted enhanced protection against early Omicron variants. Importantly, the monovalent Wu1 S-EABR mRNA-LNP booster elicited similar neutralizing responses against the BA.5, BQ.1.1, and XBB.1 subvariants compared to the conventional bivalent S mRNA-LNP booster that contained both Wu1 and BA.5 S immunogens.

While updating of mRNA vaccines can be done relatively quickly, the development, clinical testing, approval, and large-scale manufacturing of a bivalent booster that includes updated immunogens based on new circulating variants cannot be accomplished fast enough to keep up with viral evolution during a global pandemic such as COVID-19. Indeed, the Wu1/BA.5 bivalent booster became available in fall 2022, almost one year after the emergence of Omicron variants³⁰. The

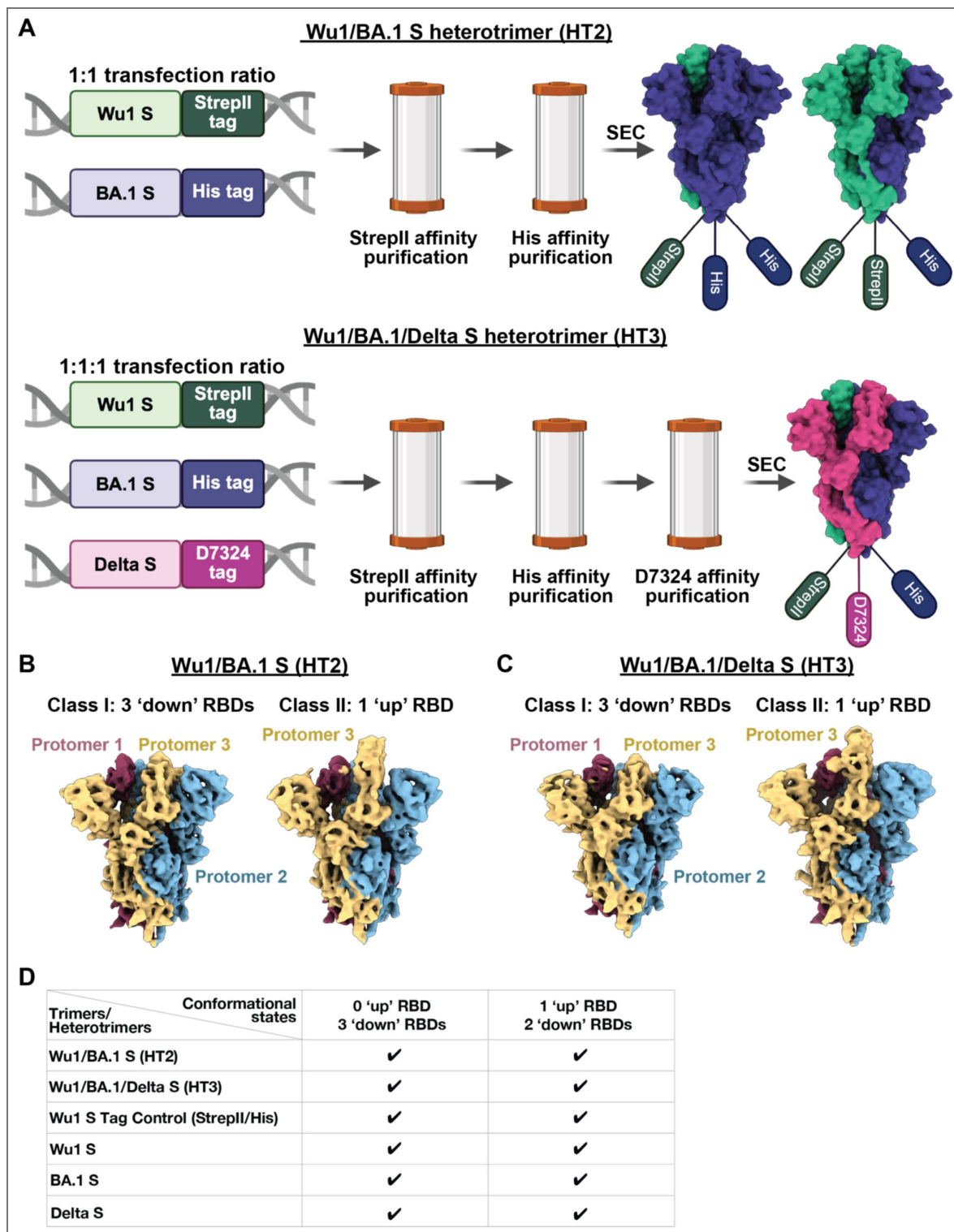


Figure 5. Heterotrimer S formation occurs for soluble constructs.

A) Schematic for the generation and purification of soluble SARS-CoV-2 Wu1/BA.1 (HT2) and Wu1/BA.1/Delta (HT3) S heterotrimers. HT2 heterotrimers were generated by co-transfection of StreptII-tagged Wu1 S and His-tagged BA.1 S constructs at a 1:1 DNA ratio, while HT3 heterotrimers were produced by co-transfecting StreptII-tagged Wu1 S, His-tagged BA.1 S, and D7324-tagged Delta S constructs at a 1:1:1 ratio. Each heterotrimer was sequentially purified using affinity chromatography specific for each tag (StreptII, His, or JR-52) to ensure incorporation of distinct protomers. This panel was created using *BioRender.com*. **B**) Cryo-EM density maps for Wu1/BA.1 HT2 S class I with 3 'down' RBDs (3.8 Å resolution) and class II with 1 'up' RBD (3.8 Å resolution). **C**) 4.0 Å and 3.9 Å resolution cryo-EM density maps for Wu1/BA.1/Delta HT3 S with 3 'down' RBDs and 1 'up' RBD. **D**) Table summarizing S heterotrimer and control homotrimer conformational states.

results reported here suggest that the EABR mRNA vaccine approach presents a potential solution that could reduce the need for frequent development of updated COVID-19 boosters and potentially future pandemic viruses. Combined with our previous finding that an initial two-shot vaccination series with a Wu1 S-EABR mRNA-LNP immunogen elicited substantially higher neutralizing activity against Omicron variants than conventional Wu1 S mRNA-LNP immunizations in naïve mice³¹, our results demonstrate that the EABR mRNA vaccine approach has the potential to induce more effective and lasting humoral immunity against emerging SARS-CoV-2 variants and represents a promising platform for future pandemic preparedness.

Clinical studies showed that the bivalent COVID-19 mRNA booster outperformed the monovalent mRNA booster in terms of preventing symptomatic illness, hospitalizations, and deaths when administered as a fourth dose in humans^{30,50}. Consistent with other studies^{19–23}, the bivalent conventional and EABR-based boosters evaluated in this study elicited higher neutralizing responses against Omicron subvariants than their monovalent counterparts in pre-vaccinated mice, although improvements were relatively modest. However, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster consistently elicited the highest RBD binding and neutralizing titers against all tested Omicron subvariants and induced robust neutralizing responses in the highest number of animals against the BQ.1.1 and XBB.1 variants that are particularly difficult to neutralize^{18,38}. These results highlight that the EABR mRNA vaccine approach and the bivalent booster strategy work synergistically to induce more effective humoral immune responses.

In addition to eliciting the most potent neutralizing responses against Omicron subvariants, the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster also induced distinct DMS signatures compared to the other boost immunogens, consistent with differences in epitope mapping of polyclonal anti-RBD Ab responses. While the monovalent Wu1 S and S-EABR mRNA-LNP and the bivalent Wu1/BA.5 S mRNA-LNP boosters elicited predominantly class 1 and class 2 responses against Wu1 RBD and strong class 5 responses against XBB.1.5 RBD, boost immunizations with bivalent Wu1/BA.5 S-EABR mRNA-LNP elicited polyclonal serum Ab responses against Wu1 RBD and mostly polyclonal or moderate polyclonal class 4 and class 5 responses against XBB.1.5 RBD. These results imply that combining the bivalent booster strategy with the EABR mRNA vaccine approach elicited a more diversified Ab response, resulting in simultaneous and balanced targeting of multiple RBD epitopes. A more diverse anti-RBD Ab response might be desirable as viral immune evasion would require mutations in multiple sites, including the more conserved class 4 and 5 RBD epitopes. As a potential mechanism for the more frequent induction of polyclonal-like Ab responses, we hypothesize that EABR eVLP formation promotes close proximity of Wu1 and BA.5 S trimers on densely-coated eVLPs, consistent with cryo-electron tomography imaging of purified eVLPs showing that the density of S trimers was equivalent or higher than on SARS-CoV-2 virions (20–40 S trimers on 40–60 nm diameter eVLPs)³¹. The high density of co-displayed Wu1 and BA.5 S trimers on eVLPs should facilitate inter-S crosslinking by B cell receptors that recognize epitopes that are conserved between Wu1 and BA.5 S trimers, leading to preferential activation of cross-reactive B cells. By comparison, conventional bivalent mRNA booster-mediated co-expression of Wu1 and BA.5 S trimers could result in lower S trimer density on cell surfaces that are crowded with endogenous membrane proteins, which could prevent efficient inter-S crosslinking.

As an additional mechanism for bivalent booster-induced preferential activation of cross-reactive B cells, we provide evidence that co-expression of ancestral and Omicron S results in heterotrimer formation that could promote intra-S crosslinking, i.e., simultaneous binding of both Fabs of a cross-reactive BCR to neighboring RBDs within the same S trimer. We previously postulated that intra-S crosslinking is possible for several anti-RBD Abs that target various RBD epitopes based on modeling of intact IgG structures onto cryo-EM structures of Fab-S complexes^{33,40}. The conventional mRNA- and the EABR mRNA-based bivalent boost immunogens should promote similar degrees of heterotrimerization and intra-S crosslinking. While our RBD serum depletion assays showed that both bivalent boosters primarily elicited cross-neutralizing Abs that bind to the Wu1 and BA.5 RBDs, only the EABR mRNA-based bivalent booster induced predominantly polyclonal DMS signatures. Thus, it is possible that the diversification of the anti-RBD Ab response observed for the bivalent EABR mRNA booster was primarily driven by inter-S crosslinking.

possibly in conjunction with intra-S crosslinking. Future studies are needed to investigate the impact of inter- and intra-S crosslinking on the activation of cross-reactive B cells and to evaluate whether the EABR mRNA vaccine approach can improve other types of bivalent and multivalent vaccines.

Although our results highlight the potential of the EABR mRNA vaccine approach in the context of pre-existing immunity, we observed that monovalent and bivalent EABR mRNA booster-induced humoral immune responses were still largely dictated by immunological imprinting resulting from the initial vaccination series. Post-boost BA.5 neutralizing responses were roughly an order of magnitude lower than Wu1 neutralization titers, even for bivalent boosters that included BA.5 S immunogens. In addition, despite eliciting consistent polyclonal anti-RBD Ab responses, bivalent EABR mRNA booster-induced neutralizing activity against the BQ.1.1 and XBB.1 variants dropped over 10-fold compared to BA.5 neutralization. Notably, Ab binding responses to BQ.1.1 and XBB.1 RBDs were similar compared to BA.5 RBD binding for all boost immunogens. This discrepancy between RBD binding responses and neutralization titers indicates that only a relatively small fraction of binding Abs has neutralizing activity and/or the Abs still bind to the BQ.1.1 and XBB.1 RBDs, but fail to effectively prevent interactions with ACE2 on the surface of target cells. The initial two-shot COVID-19 mRNA vaccination series and all booster immunizations elicited potent binding responses against the S2 region, which is relatively conserved among SARS-CoV-2 variants³⁴. Consistent with previous reports⁵¹, our depletion studies showed that anti-S2 Abs had little impact on virus neutralization, which was instead predominantly driven by anti-RBD Abs. However, anti-S2 Abs could still provide valuable immune protection against symptomatic Omicron infections through Fc-mediated effector functions⁵². In addition, mRNA vaccines induce potent cytotoxic T cell responses that cross-recognize Omicron variants⁵³ and reduce COVID-19 disease severity⁵⁴.

Taken together, our results demonstrate that the EABR mRNA vaccine approach improves monovalent and bivalent booster-mediated humoral immune responses against Omicron subvariants in pre-vaccinated mice. DMS epitope mapping studies further showed that the bivalent EABR mRNA booster elicited a more diversified anti-RBD Ab response than all other tested boost immunogens. Our work also provides evidence that bivalent mRNA booster immunizations promote heterotrimer formation, which could activate cross-reactive B cells through intra-S crosslinking. Despite these advances, future work is needed to develop booster strategies that overcome immunological imprinting more effectively and confer lasting immunity against emerging variants.

Limitations of the study

Due to the substantial time required to conduct immunization studies in pre-vaccinated mice, this proof-of-concept study to evaluate the potential of EABR-modified boosters in the context of pre-existing immunity was performed using outdated SARS-CoV-2 variants. Future studies are needed to assess EABR booster responses against currently circulating variants. While the monovalent Wu1 S-EABR mRNA-LNP booster elicited robust improvements in neutralization titers against BA.1 and BA.5 compared to the conventional Wu1 S mRNA-LNP booster, consistent with previously reported results in naïve mice³¹, only modest and statistically non-significant increases were observed for later Omicron variants, such as BQ.1.1 and XBB.1. Moreover, although the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster consistently induced the highest neutralizing titers across all tested variants, these increases were statistically significant only relative to the conventional monovalent Wu1 S mRNA-LNP and not the bivalent Wu1/BA.5 S mRNA-LNP booster. Larger mouse cohorts could have increased the statistical power of the study, such that the observed trends might have reached statistical significance. Another factor contributing to the relatively modest improvements elicited by the monovalent and bivalent EABR boosters against BQ.1.1 and XBB.1 was the generally low levels of neutralizing responses observed against these variants. Although the low neutralization activity against BQ.1.1 and XBB.1 was likely the result of immune imprinting caused by the initial vaccination series, addition of more cohorts might have excluded the possibility that the ancestral Wu1 S immunogen is more immunogenic than the Omicron BA.5 S immunogen, e.g., three immunizations with monovalent BA.5 S or bivalent Wu1/BA.5 S mRNA-

LNP. However, this is unlikely since prior studies reported that ancestral and Omicron S immunogens are similarly immunogenic^{19,55,56}. Finally, it remains unclear why the polyclonal RBD epitope-targeting response elicited by the bivalent Wu1/BA.5 S-EABR mRNA-LNP booster translated into only modest gains in neutralizing activity against Omicron variants. It is possible that a longer post-boost follow-up period or an additional booster incorporating an XBB.1 S immunogen would have revealed further maturation and expansion of these responses, and thereby additional benefits of the bivalent EABR booster.

Methods

Design of EABR constructs

SARS-CoV-2 S-EABR constructs were designed as described³¹. The EABR domain (residues 160-217) of the human CEP55 protein was fused to the C-terminus of the SARS-CoV-2 S protein obtained from the Wu1 (GenBank: QTA38991.1) or BA.5 (GenBank: UPP14409.1) variants separated by a 4-residue (Gly)₃Ser (GS) linker (Fig. S1A [↗](#)). These constructs contained the native furin cleavage site, 2P stabilizing mutations⁵⁷, and the C-terminal 21 residues were truncated to remove an endoplasmic reticulum retention signal⁵⁸. The D614G substitution⁵⁹, naturally present in BA.5, was inserted into Wu1 S and S-EABR constructs. To prevent endocytosis and enhance the cell surface expression of S-EABR constructs, an EPM (residues 243-290 of mouse FcγRII-B1)^{31,60} was inserted upstream of the 4-residue Gly-Ser linker and the EABR domain as described³¹ (Fig. S1A [↗](#)).

In vitro transfection

To verify that the designed constructs induce eVLP budding, HEK293T were transiently transfected with DNA plasmids encoding Wu1 or BA.5 S-EABR using Fugene HD (Promega). After 48 hours, transfected cell supernatants were harvested, and eVLPs were purified by ultracentrifugation on a 20% sucrose cushion as described³¹. The purified eVLP fractions were 3-fold serially diluted in 0.1 M NaHCO₃ pH 9.6, directly coated onto Costar high binding 96-well plates (Corning), and eVLP-associated S protein levels were quantified by ELISA. After 1 hour blocking in Tris-buffered saline with 0.1% TWEEN 20 (TBS-T) and 3% bovine serum albumin (BSA) (TBS-T/3% BSA) at room temperature, the monoclonal anti-S2 Ab S2P6⁶¹ was diluted to a concentration of 5 µg/mL in TBS-T/3% BSA and added to plates for 1 hour at room temperature. After washing with TBS-T, HRP-conjugated goat anti-human IgG (2015-05; SouthernBiotech) was diluted 1:8,000 in TBS-T/3% BSA and added to plates for 30 min at room temperature. After washing with TBS-T, plates were developed using 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) and absorbance was measured at 450 nm.

mRNA synthesis

Codon-optimized mRNAs encoding Wu1 S, Wu1 S-EABR, BA.5 S, and BA.5 S-EABR constructs were synthesized by RNAcore (<https://www.houstonmethodist.org/research-cores/rnacore/> [↗](#)) using proprietary manufacturing protocols as described³¹. In brief, mRNAs were synthesized by T7 RNA polymerase-mediated in vitro transcription using DNA templates encoding the immunogen open reading frame flanked by 5' and 3' untranslated regions and terminated by an encoded polyA tail. CleanCap 5' cap structures (TriLink) were co-transcriptionally incorporated into the 5' end. Uridine was completely substituted with N1-methyl-pseudouridine to lower immunogenicity⁶². mRNAs were purified by oligo-dT affinity purification and high-performance liquid chromatography (HPLC) to remove double-stranded RNA contaminants⁶³. Purified mRNAs were stored at -80 °C.

LNP encapsulation of mRNAs

Purified mRNAs were formulated in LNP as described^{31,64}. In brief, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, cholesterol, a PEG lipid, and an ionizable cationic lipid dissolved in ethanol were rapidly mixed with an aqueous acidic solution containing the mRNAs using an in-line mixer. The ionizable lipid and LNP composition are described in the international patent application

WO2017075531(2017). The post in-line solution was dialyzed with PBS to remove ethanol and displace the acidic solution. Subsequently, mRNA-LNP were measured for size (60–65 nm) and polydispersity (PDI < 0.075) by dynamic light scattering (Malvern Nano ZS Zetasizer). The encapsulation efficiencies were >97% as measured by the Quant-iT Ribogreen Assay (Invitrogen).

Immunizations

All animal procedures in this study were performed in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 2011); and the National Institutes of Health, Office of Laboratory Animal Welfare. Whenever possible, procedures in this study were designed to avoid or minimize discomfort, distress, and pain to animals. Animals were observed for signs of morbidity and/or mortality at least once daily, according to animal welfare act and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi). All mouse procedures were approved by the Labcorp Institutional Animal Care and Use Committee (IACUC).

7–8 week-old female BALB/c mice (Charles River Laboratories) were purchased and housed at Labcorp Drug Development, Denver, PA for immunization studies. All animals were healthy upon receipt and were weighed and monitored during a 7-day acclimation period before the start of the study. Mice were randomly assigned to experimental cohorts of 10 animals. Up to 10 mice were co-housed together, and cages were kept in a climate-controlled room at 63 to 77°C at 50±20% relative humidity. Mice were provided Rodent Diet #5001 (Purina Lab Diet) ad libitum.

2 µg of Wu1 S mRNA-LNP were administered to all cohorts of mice by IM injections on days 0 and 28. On day 230, pre-vaccinated mice received different boosters by IM injection: i) 2 µg Wu1 S mRNA-LNP; ii) 2 µg Wu1 S-EABR mRNA-LNP; iii) 1 µg Wu1 S mRNA-LNP + 1 µg BA.5 S mRNA-LNP; iv) 1 µg Wu1 S-EABR mRNA-LNP + 1 µg BA.5 S-EABR mRNA-LNP. For bivalent boosters (iii and iv), the Wu1 and BA.5 S or S-EABR mRNA-LNP were mixed prior to the IM injections. Serum samples for ELISAs and neutralization assays were obtained on indicated days by retro-orbital bleeding. One small drop of either 0.5% proparacaine hydrochloride ophthalmic solution or 0.5% tetracaine hydrochloride ophthalmic solution was applied in the eye of the mouse prior to performing retro-orbital blood collection. Eyes were alternated for each blood collection. Euthanasia was performed in accordance with American Veterinary Medical Association (AVMA) guidelines for euthanasia. Mice were individually placed in a CO₂ chamber and rendered unconscious. Mice were verified as unconscious by lack of response when toe was pinched. Unconscious mice were subjected to bilateral jugular vein severance for terminal exsanguination. Mice were placed back in the CO₂ chamber until death occurred. A double thoracotomy was performed as a second confirmation of death.

Protein expression and purification

Soluble SARS-CoV-2 S trimers (Wu1/D614G, BA.5, BQ.1.1, XBB.1)³², RBDs (Wu1, BA.5, BQ.1.1, XBB.1), and a prefusion-stabilized S2 protein⁶⁵ were expressed as described^{37,66}. Briefly, Avi/His-tagged S trimers and His-tagged RBD and S2 proteins were purified from transiently-transfected Expi293F cells (Gibco) by nickel affinity chromatography (HisTrap, Cytiva) and size-exclusion chromatography (SEC) (Superose 6 Increase 10/300, Cytiva)^{33,37,66}. Peak fractions corresponding to S, RBD, or S2 proteins were pooled, concentrated, and stored at 4°C. Biotinylated S trimers for ELISAs were generated by co-transfection of Avi/His-tagged S constructs with a plasmid encoding an endoplasmic reticulum-directed BirA enzyme (kind gift from Michael Anaya, Caltech).

For heterotrimeric constructs, Wu1/BA.1 (HT2) and Wu1/BA.1/Delta (HT3) heterotrimers were expressed via the addition of C-terminal tags and co-transfection of Expi293F cells (Gibco) with specific plasmid DNA ratios. HT2 heterotrimers were produced by co-transfecting a 1:1 plasmid DNA ratio of StrepII-tagged Wu1 S and His-tagged BA.1 S constructs. HT3 heterotrimers were produced by co-transfecting a 1:1:1 plasmid DNA ratio of StrepII-tagged Wu1 S, His-tagged BA.1 S, and D7324⁴⁷-tagged Delta S constructs. HT2 was sequentially purified using StrepTrap (Cytiva),

HisTrap (Cytiva), and SEC, while HT3 also underwent immunoaffinity chromatography using the D7324-specific monoclonal Ab JR-52⁴⁷ (kind gift from James Robinson, Tulane University) before SEC.

The Wu1-His/Wu1-StrepII S homotrimer was generated by co-transfecting Expi293F cells with a 1:1 plasmid DNA ratio of His-tagged Wu1 S and StrepII-tagged Wu1 S, followed by purification as described for the HT2 heterotrimer. Single-tagged S homotrimers included StrepII-tagged Wu1 S, His-tagged BA.1 S, and D7324-tagged Delta S, each purified using their respective affinity chromatography steps followed by SEC.

Single-particle cryo-EM sample preparation

Grids of SARS-CoV-2 S homotrimers and heterotrimers were prepared at a protein concentration of 2 mg/mL. Prior to freezing, fluorinated octylmaltoside solution (Anatrace) was added to the S proteins at a final concentration of 0.02% (w/v). Three μ L of S proteins were immediately applied to Quantifoil 300 mesh 1.2/1.3 grids (Electron Microscopy Sciences) that were freshly glow discharged with the PELCO easiGLOW (Ted Pella) for 60 seconds at 20 mA. Grids were blotted for 3 seconds using Whatman No.1 filter paper with 0 blot force, 100% humidity and room temperature, and vitrified in 100% liquid ethane using a Vitrobot Mark IV system (Thermo Fisher Scientific).

Single-particle cryo-EM data collection and processing

Single-particle cryo-EM datasets for SARS-CoV-2 S homotrimers and heterotrimers were collected using SerialEM automatic data collection software⁶⁷ on a 300 keV Titan Krios (Thermo Fisher Scientific) cryo-electron microscope equipped with a K3 direct electron detector camera (Gatan). Movies were acquired in 40 frames with a defocus range of -1 to -3 μ m and a total dosage of 60 electrons per $\text{\AA}^2$ using the 3 by 3 beam image shift pattern with 3 exposures per hole in the super-resolution mode at a pixel size of 0.416 $\text{\AA}$. Data processing workflows are outlined in Figure S10⁶⁸. For all datasets, motion correction was done with patch motion correction with a binning factor of 2 and contrast transfer function (CTF) parameters were estimated with Patch CTF in cryoSPARC⁶⁸. Particles with a diameter of 100 to 200 $\text{\AA}$ were picked with blob picker, inspected, extracted, and finally classified with 2D classification in cryoSPARC⁶⁸. Ice particles, junk particles, and particles with preferred orientations were discarded, and the remaining particles were used for *ab initio* reconstruction of three to four volumes. These reconstructions were further refined with heterogeneous refinement in cryoSPARC⁶⁸. Depending on the state for the receptor-binding domains (RBDs) in the reconstructions, particles were combined for homogeneous and non-uniform refinements to obtain the final reconstructions in cryoSPARC⁶⁸.

ELISAs

ELISAs were performed as described^{31,44}. To analyze serum binding to S trimers, pre-blocked streptavidin-coated Nunc® MaxiSorp™ 384-well plates (ThermoFisher Scientific) were coated with 5 μ g/mL biotinylated S trimers in TBS-T/3% BSA for 1 hour at room temperature. To analyze serum binding to RBDs or S2, Nunc® MaxiSorp™ 384-well plates (Sigma) were coated with 2.5 μ g/mL RBD or S2 in 0.1 M NaHCO_3 pH 9.8 and incubated overnight at 4°C. Plates were blocked with TBS-T/3% BSA for 1 hour at room temperature followed by removal of the blocking buffer by aspiration. Serum samples from immunized mice were diluted 1:100, 4-fold serially diluted in TBS-T/3% BSA, and added to plates. After a 3-hour incubation at room temperature, plates were washed with TBS-T using an automated plate washer. HRP-conjugated goat anti-mouse IgG (715-035-150; Jackson ImmunoResearch) was diluted 1:100,000 in TBS-T/3% BSA and added to plates for 1 hour at room temperature. After washing with TBS-T, plates were developed using SuperSignal™ ELISA Femto Maximal Signal Substrate (Thermo Fisher Scientific) and absorbance was measured at 425 nm. Area under the curve (AUC) calculations for binding curves were performed using GraphPad Prism 9.3.1 assuming a one-site binding model with a Hill coefficient as described⁶⁹.

Neutralization assays

Lentivirus-based SARS-CoV-2 pseudoviruses were generated as described^{35,36} using S proteins from the Wu1/D614G, BA.1, BA.5, BQ.1.1, and XBB.1 variants in which the C-terminal 21 residues of the S cytoplasmic tails were removed³⁵. Serum samples from immunized mice were heat-inactivated for 30 min at 56°C. Three-fold serial dilutions of heat-inactivated samples were incubated with pseudoviruses for 1 hour at 37°C, followed by addition of the serum-virus mixtures to pre-seeded HEK293T-ACE2 target cells. After 48-hour incubation at 37°C, BriteLite Plus substrate (Perkin Elmer) was added and luminescence was measured. Half-maximal inhibitory dilutions (ID₅₀s) were calculated using 4-parameter non-linear regression analysis in AntibodyDatabase⁷⁰ and ID₅₀ values were rounded to three significant figures.

Ab depletions

HiTrap™ NHS-activated resin (Cytiva) was coupled to purified SARS-CoV-2 Wu1 RBD or a mock protein (HIV-1 gp120) at a 10 mg/mL concentration and stored in PBS supplemented with 0.02% sodium azide at 4°C. For Ab depletions, 50 µL dry worth of RBD-coupled resin were aliquoted into microcentrifuge tubes and spun down at 13,000 x g for 2 minutes. The supernatant was discarded. The resin was washed with 100 µL of sterile PBS to remove excess PBS-azide solution. The supernatant was again discarded. 10 µL of mouse serum was then diluted in 90 µL PBS, and added to the resin. The tubes were mixed and incubated at room temperature for 10 minutes. The resin-serum mixture was spun down at 13,000 x g for 7 minutes to allow the resin to settle, and 90 µL of the supernatant was transferred into a fresh microcentrifuge tube. To ensure no resin was present in the final sample, the collection tube was spun down again, and supernatant was transferred to a fresh microcentrifuge tube. Sterile PBS was added for a final volume of 200 µL. Prior to neutralization assays, the Ab depleted serum samples were heat-inactivated at 56°C for 30 minutes.

Deep mutational scanning

DMS studies to map RBD epitopes recognized by serum Abs were performed in biological duplicates using independent mutant RBD yeast libraries (Wu1^{42,71} and XBB.1.5 RBD⁴³ libraries were generously provided by Tyler Starr, University of Utah) as described^{44,66,72,73}. Briefly, yeast libraries were stained with serum Abs, labeled with a secondary Ab (115-605-008, Jackson ImmunoResearch), and then processed using a Sony SH800 cell sorter. Cells were gated to capture RBD mutants that had reduced Ab binding for a relatively high degree of RBD expression. Ab-escaped cells were expanded for DNA extraction and Illumina sequencing was done as described^{44,72,73}. Raw sequencing data are available on the NCBI SRA under BioProject PRJNA1067836. Escape fractions were computed using previously described processing steps^{44,72,73} and implemented using a Swift DMS program available from authors upon request.

Escape map visualizations shown as static line plots, logo plots and structural depictions were all created using Swift DMS as previously described^{41,44,73}. In some visualizations, RBD sites were categorized based on epitope region, which were defined as class 1 (pink) (RBD residues 403, 405, 406, 417, 420, 421, 453, 455-460, 473-478, 486, 487, 489, 503, 504), class 2 (purple) (residues 472, 479, 483-485, 490-495), class 3 (blue) (residues 341, 345, 346, 437-450, 496, 498-501), class 4 (orange) (residues 365-390, 408)⁷⁴, or class 5 (green) (352-357, 396, 462-468)⁴¹. Structural visualizations were performed using an RBD surface (PDB 6M0J) colored by the site-wise escape metric at each site as described⁴⁴.

Statistical analysis

Statistically significant titer differences between immunized cohorts of BALB/c mice (10 mice per cohort) for ELISAs were determined using analysis of variance (ANOVA) test followed by Tukey's multiple comparison post hoc test calculated using Graphpad Prism 9.3.1. Titer differences between immunized cohorts of BALB/c mice for Wu1, BA.5, BQ.1.1, and XBB.1 neutralization assays were determined using the non-parametric Kruskal-Wallis test followed by Dunn's multiple

comparison post hoc test calculated using Graphpad Prism 9.3.1. For BA.1 neutralization titers, statistically significant differences were determined using the non-parametric two-tailed Mann-Whitney test calculated using Graphpad Prism 9.3.1.

Supplementary material

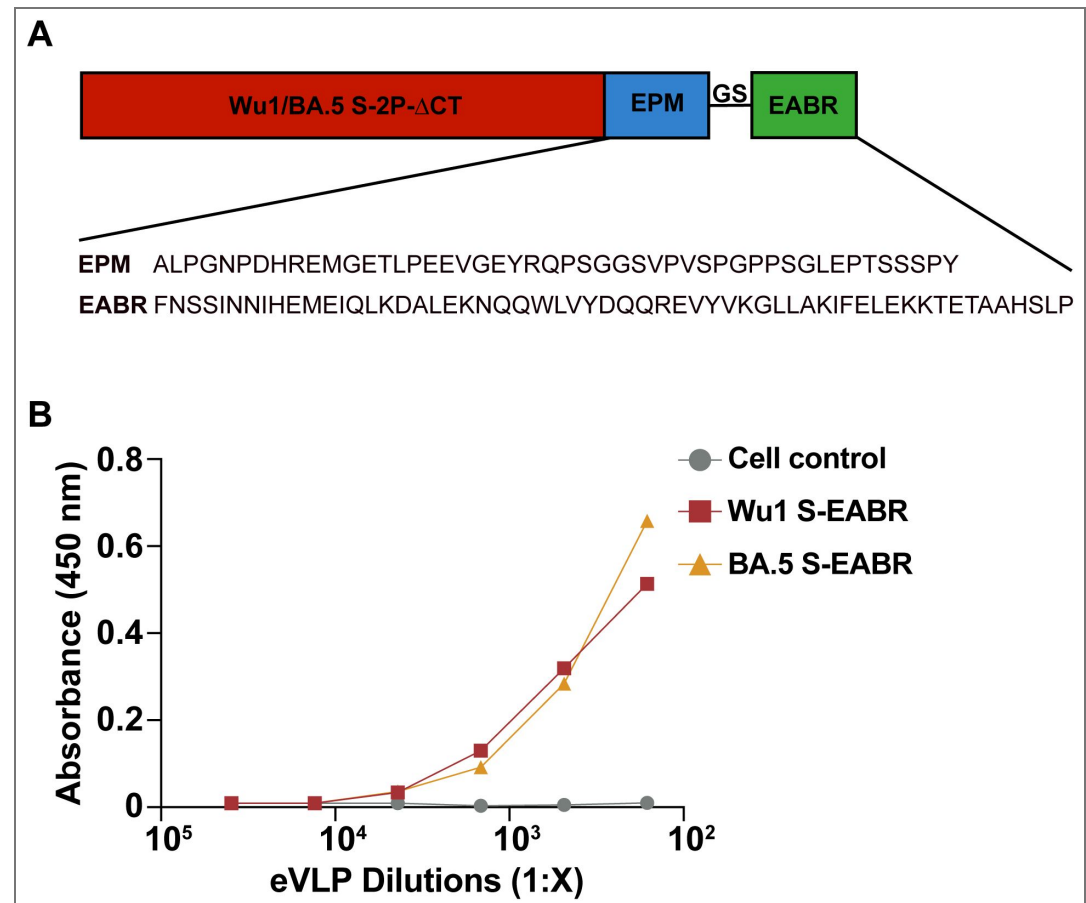


Figure S1. Wu1 and BA.5 S-EABR constructs induce efficient eVLP budding. **A)** Design and sequence information for SARS-CoV-2 S-EABR constructs. Top: The Wu1 and BA.5 S (including a furin cleavage site, 2P stabilizing substitutions, the D614G substitution, and ΔCT, a cytoplasmic tail deletion) were fused to an EPM sequence, a (Gly)₃Ser (GS) spacer, and an EABR sequence. EPM = Endocytosis prevention motif. GS = (Gly)₃Ser linker. EABR = ESCRT- and ALIX-binding region. Bottom: EPM and EABR sequence information. **B)** DNA plasmids encoding Wu1 S-EABR (red) or BA.5 S-EABR (orange) constructs were transfected into HEK293T cells. 48 hours post-transfection, transfected cell culture supernatants were harvested, and eVLPs were purified by ultracentrifugation on a 20% sucrose cushion. Purified eVLP samples were serially diluted, directly coated onto 96-well plates, and eVLP-associated S protein levels were quantified by ELISA.

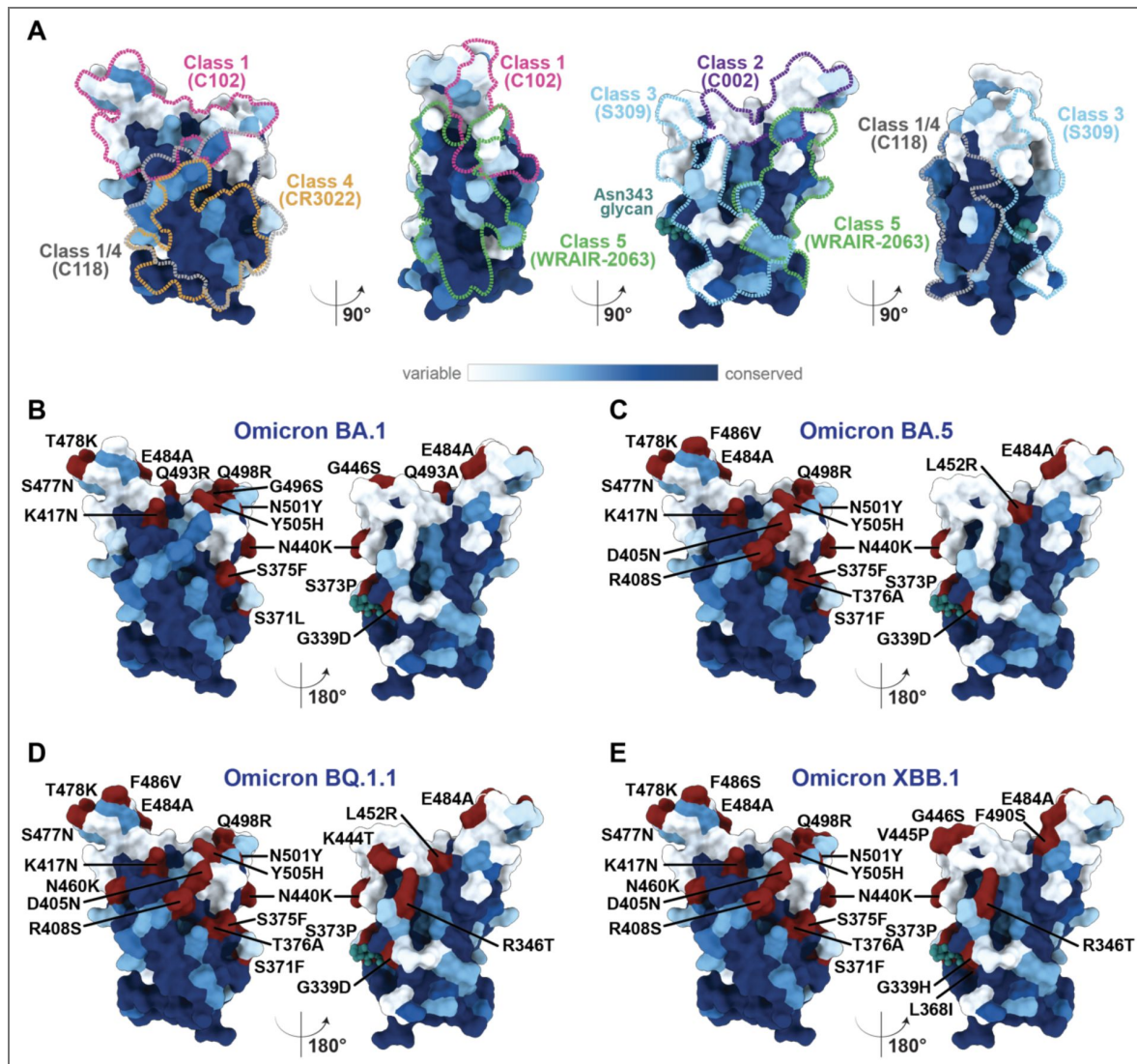


Figure S2. Sarbecovirus RBD sequence conservation.

A) Sequence conservation of 16 sarbecovirus RBDs calculated with ConSurf⁷⁵ and plotted on a surface representation of a SARS-CoV-2 RBD (PDB 7BZ5). Anti-RBD class 1, 2, 3, 4, 1/4, and 5 Ab epitopes are outlined with dotted lines in different colors using structural information from representative structures of mAb bound to SARS-CoV-2 S or RBD (C102: PDB 7K8M; C002: PDB 7K8T; S309: PDB 7JX3; CR3022: PDB 7LOP; C118: PDB 7RKV; WRAIR-2063: PDB 8EOO). SARS-CoV-2 RBD substitutions (colored in red) in Omicron **B)** BA.1, **C)** BA.5, **D)** BQ.1.1, and **E)** XBB.1 variants plotted on the RBD sequence conservation plot.

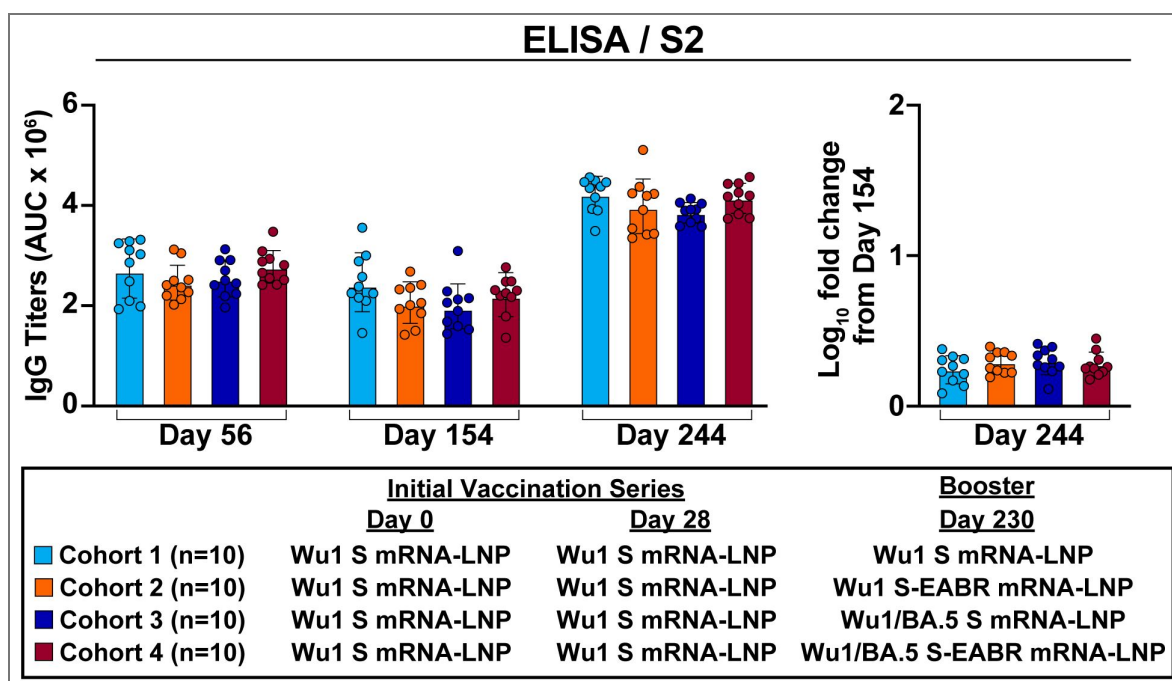


Figure S3. Primary vaccinations and booster immunizations elicit potent anti-S2 binding responses.

S2 binding titers for antisera from pre-vaccinated mice that received booster immunizations with 2 μ g of monovalent Wu1 S mRNA-LNP (cyan), 2 μ g of monovalent Wu1 S-EABR mRNA-LNP (orange), 2 μ g of bivalent Wu1/BA.5 S mRNA-LNP (blue), or 2 μ g of bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon). ELISA data for binding of Wu1 S2 are shown for indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of area under the curve (AUC) values. The $\log_{10}$ -fold change in binding titers from day 154 to day 244 is also shown (plotted as mean (bars) and standard deviation (horizontal lines)).

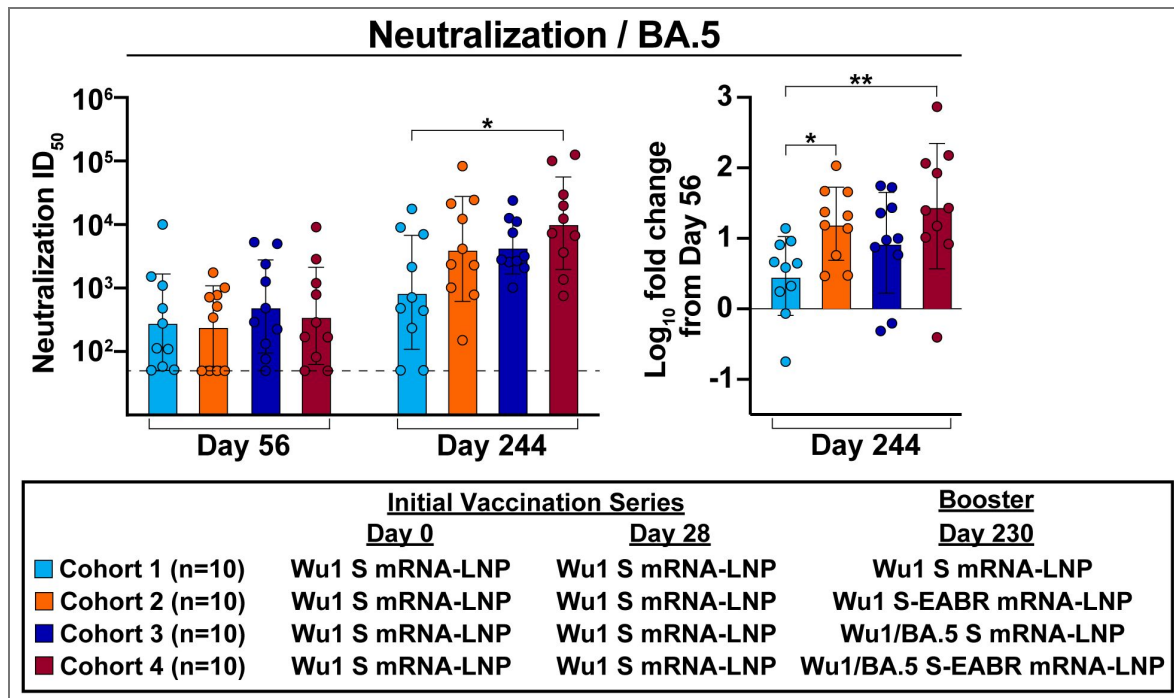


Figure S4. Monovalent and bivalent S-EABR mRNA-LNP boosters elicit higher increases in BA.5 neutralization compared to conventional S mRNA-LNP boosters.

Neutralization titers for antisera from pre-vaccinated mice that received booster immunizations with 2 μ g of monovalent Wu1 S mRNA-LNP (cyan), 2 μ g of monovalent Wu1 S-EABR mRNA-LNP (orange), 2 μ g of bivalent Wu1/BA.5 S mRNA-LNP (blue), or 2 μ g of bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon) (immunization schedule in Fig. 1A-B). BA.5 neutralization data are shown for indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of ID₅₀ values. Dashed horizontal lines correspond to the background values representing the limit of detection for neutralization assays (ID₅₀ = 1:50). The log₁₀-fold change in neutralization titers from day 56 to day 244 is also shown (plotted as mean (bars) and standard deviation (horizontal lines)). Significant differences between cohorts linked by horizontal lines are indicated by asterisks: p<0.05 = *, p<0.01 = **.

Figure S5. Monovalent Wu1 S-EABR mRNA-LNP booster elicits improved neutralizing responses against BA.1 variant.

Neutralization titers for antisera from pre-vaccinated mice that received booster immunizations with 2 μ g of monovalent Wu1 S mRNA-LNP (cyan) or monovalent Wu1 S-EABR mRNA-LNP (orange). Neutralization data are shown for indicated time points for antisera from individual mice (colored circles) presented as the geometric mean (bars) and standard deviation (horizontal lines) of ID_{50} values against BA.1 pseudovirus. Dashed horizontal lines correspond to the limit of detection for neutralization assays ($ID_{50} = 1:50$). The $\log_{10}$ -fold change in neutralization titers from day 154 to day 244 is also shown (plotted as mean (bars) and standard deviation (horizontal lines)). Significant differences between cohorts linked by horizontal lines are indicated by asterisks: $p < 0.05 = *$.

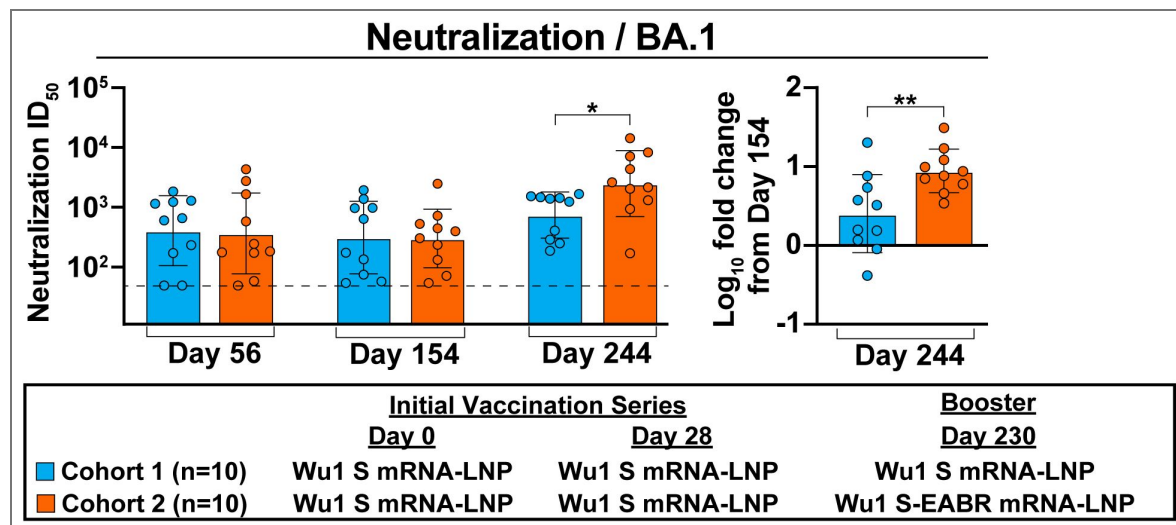
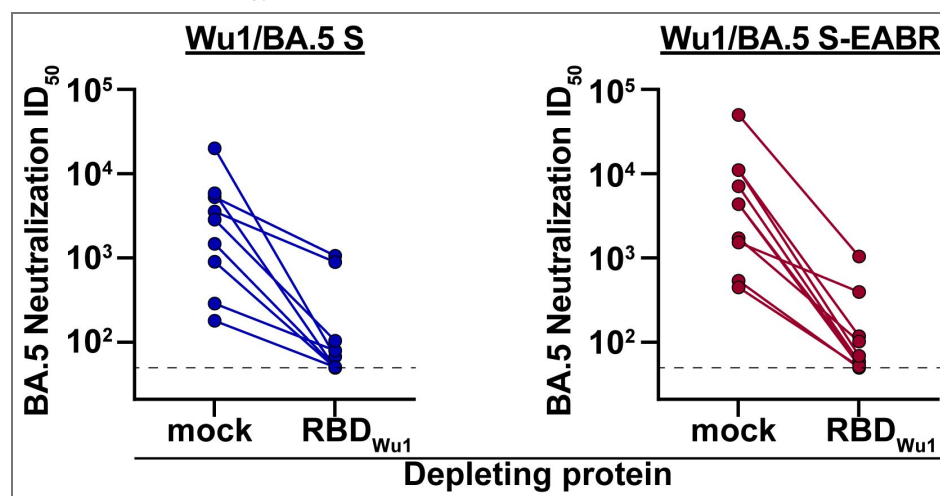


Figure S6. Depleting bivalent booster-induced antisera with Wu1 RBD reduces BA.5 neutralizing activity.

Day 244 serum samples obtained from pre-vaccinated mice that received booster immunizations with 2 μ g of bivalent Wu1/BA.5 S mRNA-LNP (blue) or 2 μ g of bivalent Wu1/BA.5 S-EABR mRNA-LNP (maroon) were depleted using a mock protein (HIV-1 gp120; left) or Wu1 RBD (right). The neutralizing activity of the depleted antisera was measured against BA.5 pseudovirus. Neutralization data are shown as ID_{50} values for individual mice (colored circles). Data points for individual mice following mock or Wu1 RBD depletions are connected by colored lines. Dashed horizontal lines correspond to the limit of detection for neutralization assays ($ID_{50} = 1:50$).



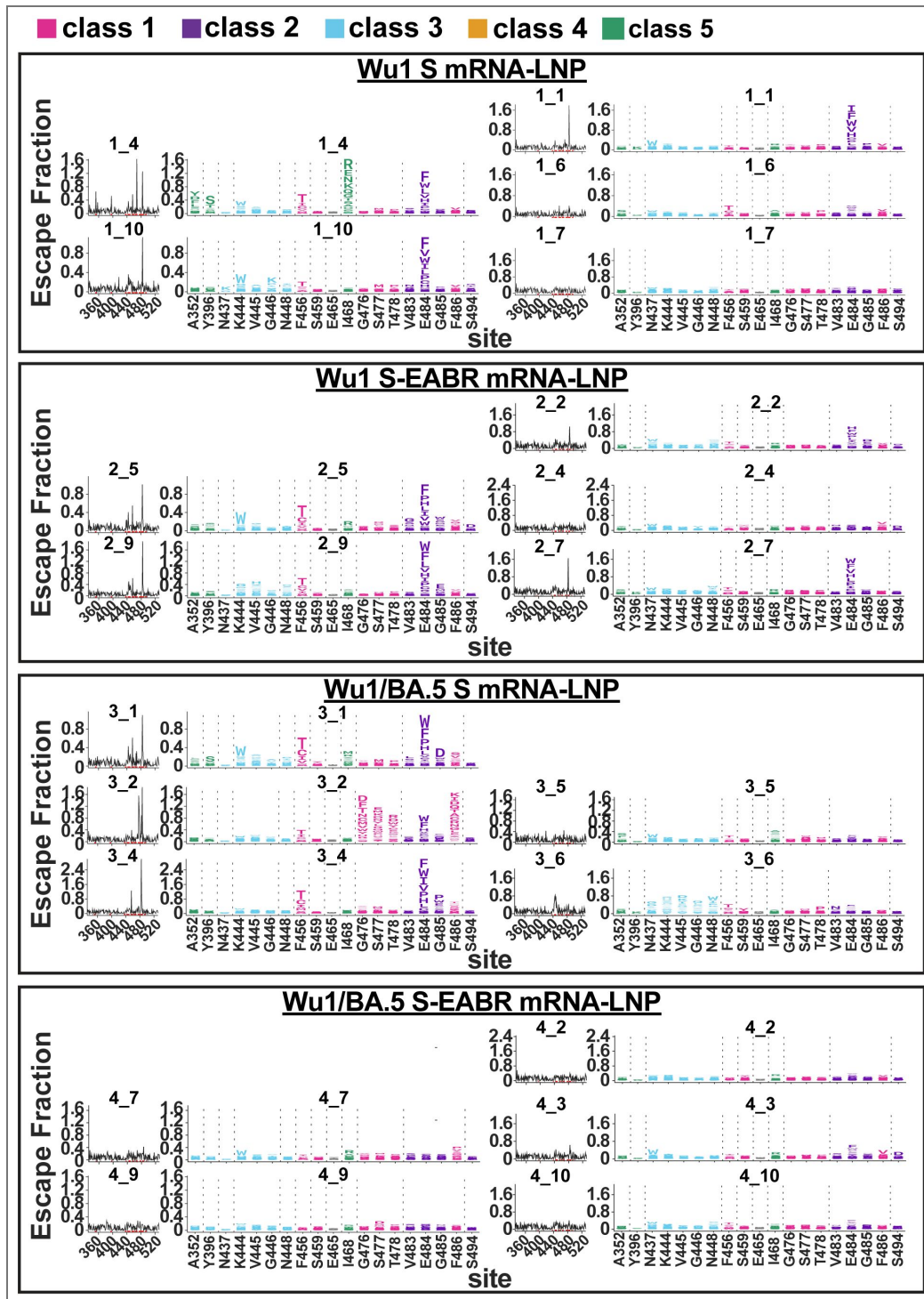


Figure S7. Bivalent S-EABR mRNA-LNP booster induces polyclonal Ab responses against Wu1 RBD.

Sera for DMS studies were derived from pre-vaccinated mice that received booster immunizations with 2 µg of monovalent Wu1 S mRNA-LNP, monovalent Wu1 S-EABR mRNA-LNP, bivalent Wu1/BA.5 S mRNA-LNP, or bivalent Wu1/BA.5 S-EABR mRNA-LNP (immunization schedule in Fig. 1A-B). Line (left) and logo plots (right) are shown for DMS results for individual sera from pre-vaccinated mice that received the indicated boost immunogens. DMS was performed using a Wu1 RBD library. The x axes of the line and logo plots display the RBD residue numbers. The y axis of the line plots shows the sum of the Ab escape of all mutations at a site (larger numbers indicating increased Ab escape). Sites with the strongest Ab escape are shown in logo plots, with tall letters representing the most frequent mutations at a site. Logo plots are colored differently for RBD epitopes within different classes^{33,40,41} (epitopes are shown in Fig. S2; gray color for residues not assigned to an epitope).

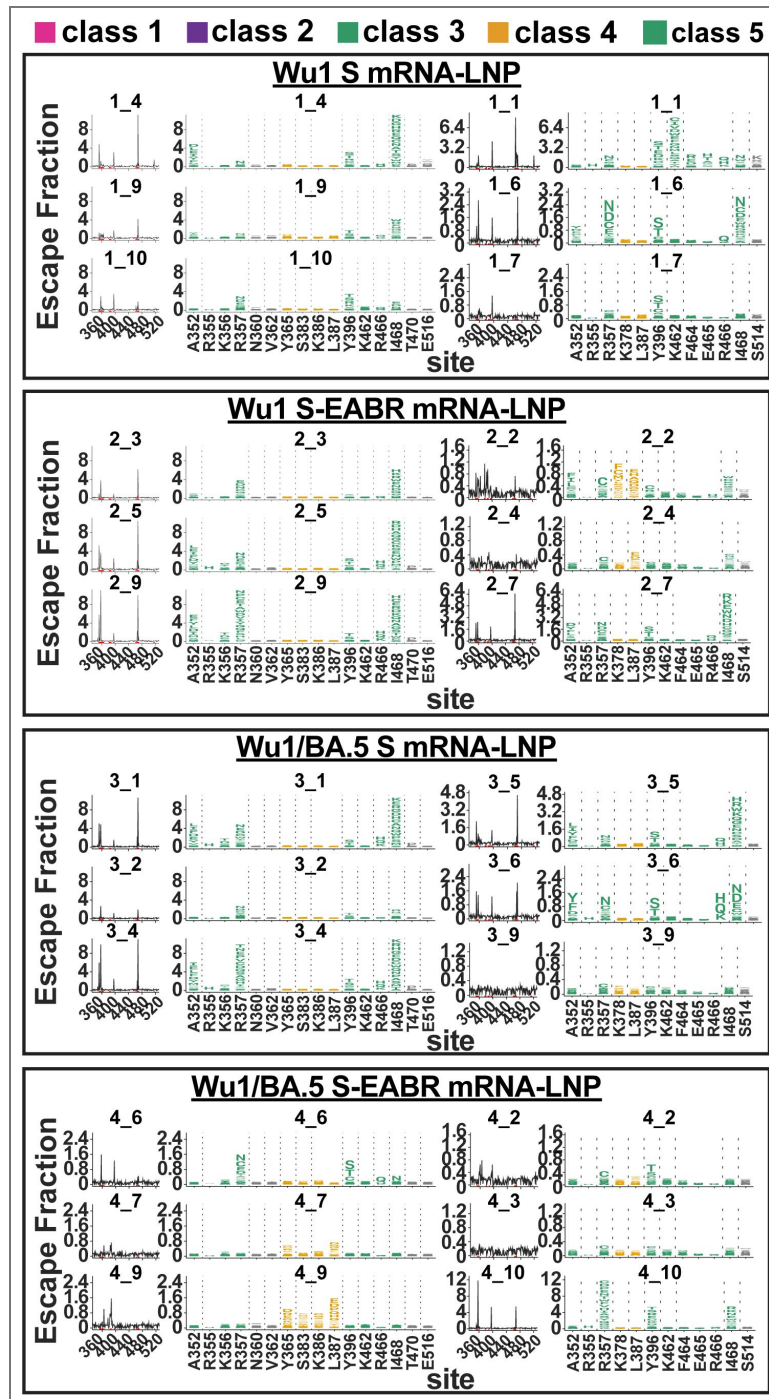


Figure S8. Bivalent S-EABR mRNA-LNP booster induces less pronounced class 3 Ab responses against XBB.1.5 RBD.

Sera for DMS studies were derived from pre-vaccinated mice that received booster immunizations with 2 μ g of monovalent Wu1 S mRNA-LNP, monovalent Wu1 S-EABR mRNA-LNP, bivalent Wu1/BA.5 S mRNA-LNP, or bivalent Wu1/BA.5 S-EABR mRNA-LNP (immunization schedule in Fig. 1A-B). Line (left) and logo plots (right) are shown for DMS results for individual sera from pre-vaccinated mice that received the indicated boost immunogens. DMS was performed using an XBB.1.5 RBD library. The x axes of the line and logo plots display the RBD residue numbers. The y axis of the line plots shows the sum of the Ab escape of all mutations at a site (larger numbers indicating increased Ab escape). Sites with the strongest Ab escape are shown in logo plots, with tall letters representing the most frequent mutations at a site. Logo plots are colored differently for RBD epitopes within different classes^{33,40} (epitopes are shown in Fig. S2; gray color for residues not assigned to an epitope).

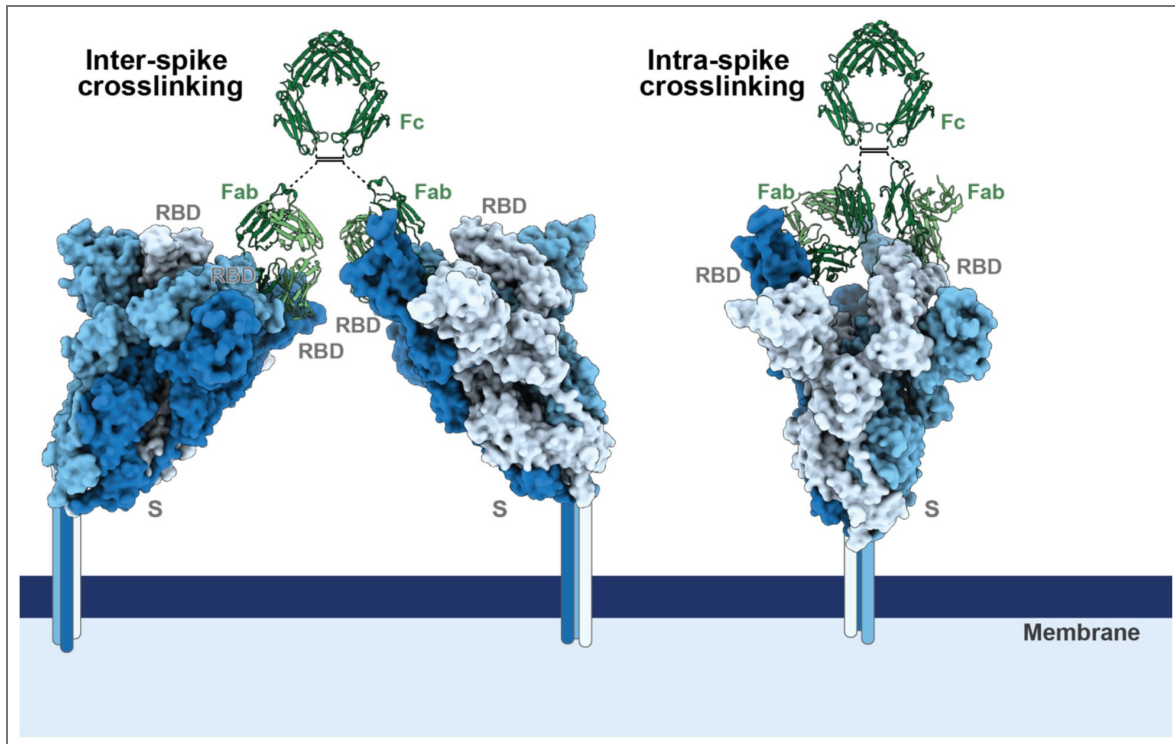
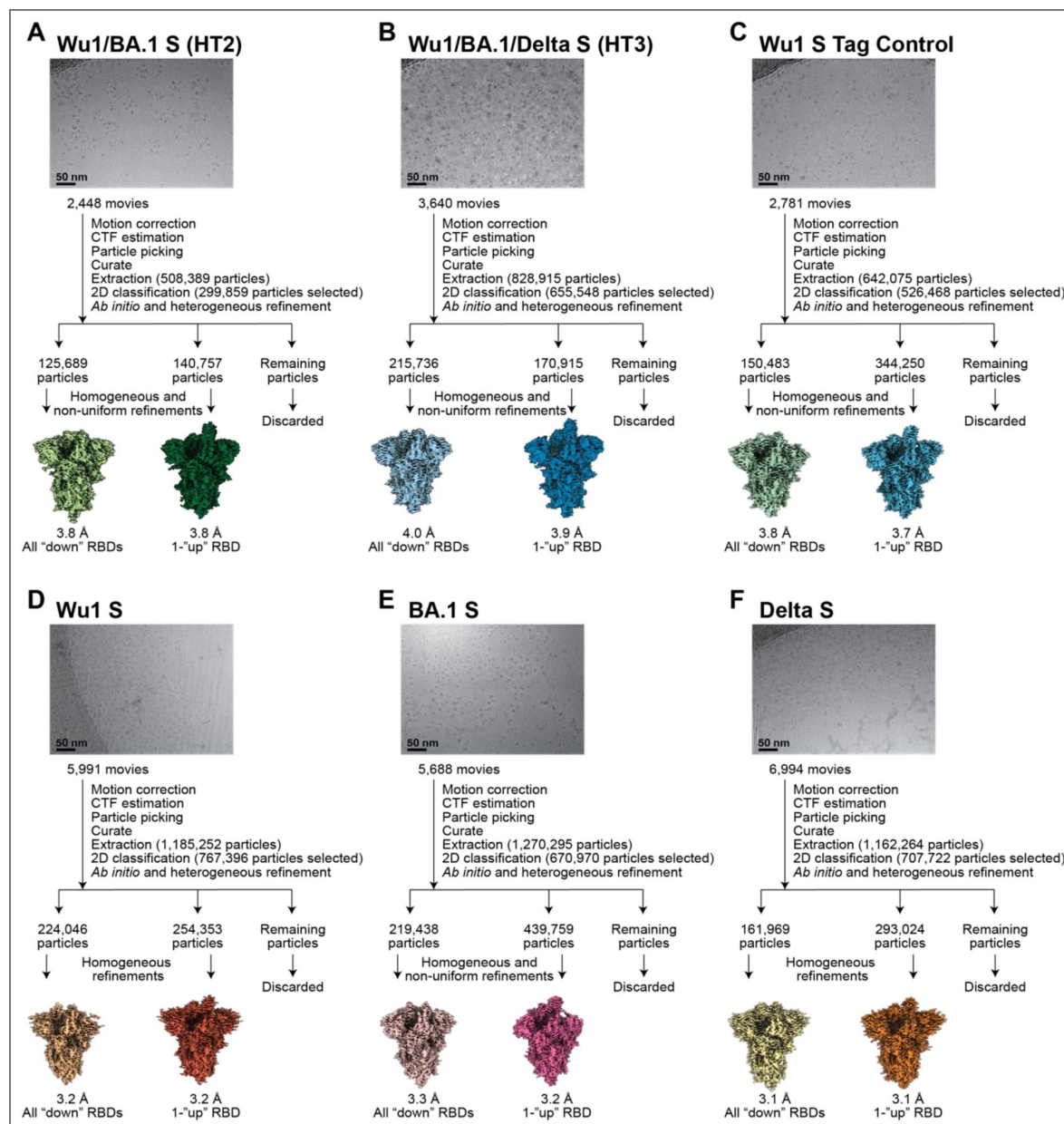


Figure S9. Cartoon diagram illustrating inter- and intra-S crosslinking.

Left: model showing potential inter-S crosslinking through the interactions between both Fabs of a single IgG and two RBDs of adjacent viral S proteins. Right: model showing intra-S crosslinking through the interaction between both Fabs of a single IgG and two RBDs on the same viral S trimer. The hinge regions between the Fabs and Fc are modeled with flexible linkers, shown as dotted lines, as these regions were not resolved in the crystal structure of an intact IgG⁷⁶. Disulfide bonds in the hinge region are shown as black lines. These diagrams are modeled based on the cryo-EM structure of SARS-CoV-2 S complexed with human C118 Fab (PDB ID 7RKV) and the crystal structure of human Fc (PDB ID 1FC1). IgGs shown in this figure represent either circulating Abs or membrane-bound BCRs.

Figure S10. Cryo-EM data collection and processing for heterotrimeric and control SARS-CoV-2 S structures.

Representative micrographs, workflow for single-particle data processing, and final reconstructions are shown for SARS-CoV-2 (A) Wu1/BA.1 S HT2 heterotrimer, (B) Wu1/BA.1/Delta S HT3 heterotrimer, and homotrimer S controls including (C) Wu1 His/StrepII double-tag control, (D) StrepII-tagged Wu1, (E) His-tagged BA.1, and (F) D7324-tagged Delta constructs.

**Table S1. Geometric mean neutralization titers.**

Day 56, 154, and 244 geometric mean neutralization titers for cohorts 1-4 against the Wu1, BA.5, BQ.1.1, and XBB.1 variants.

	Day 56				Day 154				Day 244			
	Wu1	BA.5	BQ.1.1	XBB.1	Wu1	BA.5	BQ.1.1	XBB.1	Wu1	BA.5	BQ.1.1	XBB.1
Cohort 1	56,855	274	58	50	17,843	136	50	50	74,875	851	69	75
Cohort 2	65,752	253	69	82	14,022	172	52	50	92,492	4,100	125	125
Cohort 3	60,300	513	74	57	14,130	159	50	50	80,978	4,441	169	108
Cohort 4	74,780	365	79	55	16,194	156	50	50	95,515	10,446	236	162

Boosting Immunogen	Animal	Wu1 DMS Profile	XBB.1.5 DMS Profile
Wu1 S mRNA-LNP	1_4	Moderate class 5 and 2	Strong class 5
Wu1 S mRNA-LNP	1_9		Strong class 5
Wu1 S mRNA-LNP	1_10	Moderate class 2	Strong class 5
Wu1 S mRNA-LNP	1_1	Moderate class 2	Strong class 5
Wu1 S mRNA-LNP	1_6	Polyclass	Strong class 5
Wu1 S mRNA-LNP	1_7	Polyclass	Moderate class 5
Wu1 S-EABR mRNA-LNP	2_3		Strong class 5
Wu1 S-EABR mRNA-LNP	2_5	Moderate class 2	Strong class 5
Wu1 S-EABR mRNA-LNP	2_9	Moderate class 2	Strong class 5
Wu1 S-EABR mRNA-LNP	2_2	Moderate class 2	Moderate class 5 and 4
Wu1 S-EABR mRNA-LNP	2_4	Polyclass	Polyclass
Wu1 S-EABR mRNA-LNP	2_7	Moderate class 2	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_1	Moderate class 2	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_2	Moderate class 1	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_4	Strong class 2	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_5	Polyclass	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_6	Weak class 3	Strong class 5
Wu1/BA.5 S mRNA-LNP	3_9		Polyclass
Wu1/BA.5 S-EABR mRNA-LNP	4_6		Moderate class 5
Wu1/BA.5 S-EABR mRNA-LNP	4_7	Polyclass	Weak class 4
Wu1/BA.5 S-EABR mRNA-LNP	4_9	Polyclass	Moderate class 4
Wu1/BA.5 S-EABR mRNA-LNP	4_2	Polyclass	Weak class 5
Wu1/BA.5 S-EABR mRNA-LNP	4_3	Weak class 2	Polyclass
Wu1/BA.5 S-EABR mRNA-LNP	4_10	Polyclass	Strong class 5

	Escape fraction
Polyclass	<0.5
Weak	0.5-1
Moderate	1-2
Strong	>2

Table S2. Bivalent S-EABR mRNA-LNP booster induces consistent polyclass anti-RBD Ab responses.

The DMS profiles for antisera from individual mice against the Wu1 and XBB.1.5 RBD libraries shown in Fig. S6 and S7 were classified as polyclass (total escape peaks <1 at all sites; green), moderate escape (total escape peaks between 1 and 2 at one or more sites; yellow), or strong escape (total escape peaks >2 at one or more sites; red) profiles. Total escape peaks represent the sum of escape fractions for all mutations at a specific site⁴⁵. For moderate and strong escape profiles, the RBD epitope classes with the highest total escape peaks are specified.

Data availability

Materials generated in this study are available upon request through a Materials Transfer Agreement. Code used for data processing and visualization of DMS results is available upon request. Main text and supplementary data will be publicly available. DMS raw sequencing data will be available under an NCBI SRA. Cryo-EM density maps are deposited in the EMDB with accession codes: EMD-71513, EMD-71514, EMD-71515 and EMD-71516. Materials are available upon request to corresponding authors with a signed material transfer agreement, and other information required to analyze the data in this paper is available from the lead contacts upon request. This paper does not report original code.

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Additional information

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Conceptualization: C.F., A.A.C., K.A.D., P.J.B., and M.A.G.H.; Methodology: C.F., A.A.C., K.A.D., A.-C.P.F., P.J.B., and M.A.G.H.; Investigation: C.F., A.A.C., K.A.D., A.V.R., A.-C.P.F., Z.Y., P.N.P.G., K.E.H.T., W.J.M., P.J.C.L., and M.A.G.H.; Writing – original draft: C.F., A.A.C., K.A.D., P.J.B., and M.A.G.H.; Writing – review and editing: C.F., A.A.C., K.A.D., A.-C.P.F., P.J.C.L., P.J.B., and M.A.G.H.; Visualization: C.F., A.A.C., K.A.D.; Supervision: P.J.B., and M.A.G.H.; Funding: P.J.B., and M.A.G.H.

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Competing interests: M.A.G.H. and P.J.B. are inventors on a US patent application filed by the California Institute of Technology that covers the EABR technology described in this work. P.J.B. is a scientific advisor for Vaccine Company, Inc. M.A.G.H. is a scientific consultant for Vaccine Company, Inc. W.J.M. and P.J.C.L. are employees of Acuitas Therapeutics, a company developing lipid nanoparticle delivery technology; P.J.C.L. holds equity in Acuitas Therapeutics.

References

- 1 **Corbett K. S., et al.** (2020) SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. *Nature* **586**:567-571 <https://doi.org/10.1038/s41586-020-2622-0>
- 2 **Zheng C., et al.** (2022) Real-world effectiveness of COVID-19 vaccines: a literature review and meta-analysis. *Int J Infect Dis* **114**:252-260 <https://doi.org/10.1016/j.ijid.2021.11.009>
- 3 **Chen Y., et al.** (2023) Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. *Nat Rev Immunol* **23**:189-199 <https://doi.org/10.1038/s41577-022-00784-3>
- 4 **Hogan M. J., Pardi N** (2022) mRNA Vaccines in the COVID-19 Pandemic and Beyond. *Annu Rev Med* **73**:17-39 <https://doi.org/10.1146/annurev-med-042420-112725>
- 5 **Painter M. M., et al.** (2021) Rapid induction of antigen-specific CD4(+) T cells is associated with coordinated humoral and cellular immunity to SARS-CoV-2 mRNA vaccination. *Immunity* **54**:2133-2142 e2133 <https://doi.org/10.1016/j.immuni.2021.08.001>
- 6 **Oberhardt V., et al.** (2021) Rapid and stable mobilization of CD8(+) T cells by SARS-CoV-2 mRNA vaccine. *Nature* **597**:268-273 <https://doi.org/10.1038/s41586-021-03841-4>
- 7 **Polack F. P., et al.** (2020) Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* **383**:2603-2615 <https://doi.org/10.1056/NEJMoa2034577>
- 8 **Baden L. R., et al.** (2021) Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med* **384**:403-416 <https://doi.org/10.1056/NEJMoa2035389>
- 9 **Zhang Z., et al.** (2022) Humoral and cellular immune memory to four COVID-19 vaccines. *Cell* **185**:2434-2451 e2417 <https://doi.org/10.1016/j.cell.2022.05.022>
- 10 **Planas D., et al.** (2021) Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature* **596**:276-280 <https://doi.org/10.1038/s41586-021-03777-9>
- 11 **Andrews N., et al.** (2022) Effectiveness of COVID-19 booster vaccines against COVID-19-related symptoms, hospitalization and death in England. *Nat Med* **28**:831-837 <https://doi.org/10.1038/s41591-022-01699-1>
- 12 **WHO** (2021) Classification of Omicron (B.1.1.529): SARS-CoV-2 variant of concern. World Health Organization.
- 13 **Liu L., et al.** (2022) Striking antibody evasion manifested by the Omicron variant of SARS-CoV-2. *Nature* **602**:676-681 <https://doi.org/10.1038/s41586-021-04388-0>

- 14 Cele S., et al. (2022) Omicron extensively but incompletely escapes Pfizer BNT162b2 neutralization. *Nature* **602**:654-656 <https://doi.org/10.1038/s41586-021-04387-1>
- 15 Andrews N., et al. (2022) Covid-19 Vaccine Effectiveness against the Omicron (B.1.1.529) Variant. *N Engl J Med* **386**:1532-1546 <https://doi.org/10.1056/NEJMoa2119451>
- 16 Tseng H. F., et al. (2022) Effectiveness of mRNA-1273 against SARS-CoV-2 Omicron and Delta variants. *Nat Med* **28**:1063-1071 <https://doi.org/10.1038/s41591-022-01753-y>
- 17 Gruell H., et al. (2022) mRNA booster immunization elicits potent neutralizing serum activity against the SARS-CoV-2 Omicron variant. *Nat Med* **28**:477-480 <https://doi.org/10.1038/s41591-021-01676-0>
- 18 Wang Q., et al. (2023) Alarming antibody evasion properties of rising SARS-CoV-2 BQ and XBB subvariants. *Cell* **186**:279-286 e278 <https://doi.org/10.1016/j.cell.2022.12.018>
- 19 Scheaffer S. M., et al. (2023) Bivalent SARS-CoV-2 mRNA vaccines increase breadth of neutralization and protect against the BA.5 Omicron variant in mice. *Nat Med* **29**:247-257 <https://doi.org/10.1038/s41591-022-02092-8>
- 20 Khoury D. S., et al. (2023) Predicting the efficacy of variant-modified COVID-19 vaccine boosters. *Nat Med* **29**:574-578 <https://doi.org/10.1038/s41591-023-02228-4>
- 21 Chalkias S., et al. (2022) A Bivalent Omicron-Containing Booster Vaccine against Covid-19. *N Engl J Med* **387**:1279-1291 <https://doi.org/10.1056/NEJMoa2208343>
- 22 Collier A. Y., et al. (2023) Immunogenicity of BA.5 Bivalent mRNA Vaccine Boosters. *N Engl J Med* **388**:565-567 <https://doi.org/10.1056/NEJMc2213948>
- 23 Davis-Gardner M. E., et al. (2023) Neutralization against BA.2.75.2, BQ.1.1, and XBB from mRNA Bivalent Booster. *N Engl J Med* **388**:183-185 <https://doi.org/10.1056/NEJMc2214293>
- 24 Wang Q., et al. (2023) Deep immunological imprinting due to the ancestral spike in the current bivalent COVID-19 vaccine. *Cell Rep Med* **4**:101258 <https://doi.org/10.1016/j.xcrm.2023.101258>
- 25 Wang Q., et al. (2023) SARS-CoV-2 neutralising antibodies elicit bivalent versus monovalent booster. *Lancet Infect Dis* **23**:527-528 [https://doi.org/10.1016/S1473-3099\(23\)00181-0](https://doi.org/10.1016/S1473-3099(23)00181-0)
- 26 Alsoussi W. B., et al. (2023) SARS-CoV-2 Omicron boosting induces de novo B cell response in humans. *Nature* **617**:592-598 <https://doi.org/10.1038/s41586-023-06025-4>
- 27 Park Y. J., et al. (2022) Imprinted antibody responses against SARS-CoV-2 Omicron sublineages. *Science* **378**:619-627 <https://doi.org/10.1126/science.adc9127>
- 28 Carreno J. M., Singh G., Simon V., Krammer F., PVI study group (2023) Bivalent COVID-19 booster vaccines and the absence of BA.5-specific antibodies. *Lancet Microbe* **4**:e569 [https://doi.org/10.1016/S2666-5247\(23\)00118-0](https://doi.org/10.1016/S2666-5247(23)00118-0)
- 29 Cobey S., Hensley S. E (2017) Immune history and influenza virus susceptibility. *Curr Opin Virol* **22**:105-111 <https://doi.org/10.1016/j.coviro.2016.12.004>
- 30 Song S., et al. (2024) A systematic review and meta-analysis on the effectiveness of bivalent mRNA booster vaccines against Omicron variants. *Vaccine* **42**:3389-3396 <https://doi.org/10.1016/j.vaccine.2024.04.049>
- 31 Hoffmann M. A. G., et al. (2023) ESCRT recruitment to SARS-CoV-2 spike induces virus-like particles that improve mRNA vaccines. *Cell* <https://doi.org/10.1016/j.cell.2023.04.024>
- 32 Hsieh C. L., et al. (2020) Structure-based design of prefusion-stabilized SARS-CoV-2 spikes. *Science* **369**:1501-1505 <https://doi.org/10.1126/science.abd0826>
- 33 Barnes C. O., et al. (2020) SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature* **588**:682-687 <https://doi.org/10.1038/s41586-020-2852-1>
- 34 Li C. J., Chang S. C (2023) SARS-CoV-2 spike S2-specific neutralizing antibodies. *Emerg Microbes Infect* **12**:2220582 <https://doi.org/10.1080/22221751.2023.2220582>
- 35 Crawford K. H. D., et al. (2020) Protocol and Reagents for Pseudotyping Lentiviral Particles with SARS-CoV-2 Spike Protein for Neutralization Assays. *Viruses* **12** <https://doi.org/10.3390/v12050513>

- 36 Robbiani D. F., et al. (2020) Convergent antibody responses to SARS-CoV-2 in convalescent individuals. *Nature* **584**:437-442 <https://doi.org/10.1038/s41586-020-2456-9>
- 37 Wang Z., et al. (2022) Analysis of memory B cells identifies conserved neutralizing epitopes on the N-terminal domain of variant SARS-Cov-2 spike proteins. *Immunity* **55**:998-1012 e1018 <https://doi.org/10.1016/j.immuni.2022.04.003>
- 38 Miller J., et al. (2023) Substantial Neutralization Escape by SARS-CoV-2 Omicron Variants BQ.1.1 and XBB.1. *N Engl J Med* **388**:662-664 <https://doi.org/10.1056/NEJMc2214314>
- 39 Feng S., et al. (2021) Correlates of protection against symptomatic and asymptomatic SARS-CoV-2 infection. *Nat Med* **27**:2032-2040 <https://doi.org/10.1038/s41591-021-01540-1>
- 40 Fan C., et al. (2022) Neutralizing monoclonal antibodies elicited by mosaic RBD nanoparticles bind conserved sarbecovirus epitopes. *Immunity* **55**:2419-2435 e2410 <https://doi.org/10.1016/j.immuni.2022.10.019>
- 41 Fan C., et al. (2025) Cross-reactive sarbecovirus antibodies induced by mosaic RBD nanoparticles. *Proc Natl Acad Sci U S A* **122**:e2501637122 <https://doi.org/10.1073/pnas.2501637122>
- 42 Starr T. N., et al. (2020) Deep Mutational Scanning of SARS-CoV-2 Receptor Binding Domain Reveals Constraints on Folding and ACE2 Binding. *Cell* **182**:1295-1310 e1220 <https://doi.org/10.1016/j.cell.2020.08.012>
- 43 Taylor A. L., Starr T. N. (2023) Deep mutational scans of XBB.1.5 and BQ.1.1 reveal ongoing epistatic drill during SARS-CoV-2 evolution. *PLoS Pathog* **19**:e1011901 <https://doi.org/10.1371/journal.ppat.1011901>
- 44 Cohen A. A., et al. (2024) Mosaic sarbecovirus nanoparticles elicit cross-reactive responses in pre-vaccinated animals. *Cell* **187**:5554-5571 e5519 <https://doi.org/10.1016/j.cell.2024.07.052>
- 45 Greaney A. J., et al. (2021) Mapping mutations to the SARS-CoV-2 RBD that escape binding by different classes of antibodies. *Nat Commun* **12**:4196 <https://doi.org/10.1038/s41467-021-24435-8>
- 46 Callaway H. M., et al. (2023) Bivalent intra-spike binding provides durability against emergent Omicron lineages: Results from a global consortium. *Cell Rep* **42**:112014 <https://doi.org/10.1016/j.celrep.2023.112014>
- 47 Sanders R. W., et al. (2013) A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog* **9**:e1003618 <https://doi.org/10.1371/journal.ppat.1003618>
- 48 Walls A. C., et al. (2020) Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* **181**:281-292 e286 <https://doi.org/10.1016/j.cell.2020.02.058>
- 49 Wrapp D., et al. (2020) Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* **367**:1260-1263 <https://doi.org/10.1126/science.abb2507>
- 50 Tan C. Y., et al. (2023) Effectiveness of bivalent mRNA vaccines against medically attended symptomatic SARS-CoV-2 infection and COVID-19-related hospital admission among SARS-CoV-2-naïve and previously infected individuals: a retrospective cohort study. *Lancet Infect Dis* **23**:1343-1348 [https://doi.org/10.1016/S1473-3099\(23\)00373-0](https://doi.org/10.1016/S1473-3099(23)00373-0)
- 51 Bowen J. E., et al. (2022) SARS-CoV-2 spike conformation determines plasma neutralizing activity elicited by a wide panel of human vaccines. *Sci Immunol* **7**:eadf1421 <https://doi.org/10.1126/sciimmunol.adf1421>
- 52 Balinsky C. A., et al. (2023) Antibodies to S2 domain of SARS-CoV-2 spike protein in Moderna mRNA vaccinated subjects sustain antibody-dependent NK cell-mediated cell cytotoxicity against Omicron BA.1. *Front Immunol* **14**:1266829 <https://doi.org/10.3389/fimmu.2023.1266829>
- 53 Keeton R., et al. (2022) T cell responses to SARS-CoV-2 spike cross-recognize Omicron. *Nature* **603**:488-492 <https://doi.org/10.1038/s41586-022-04460-3>
- 54 Moderbacher Rydzynski, et al. (2020) Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations with Age and Disease Severity. *Cell* **183**:996-1012 e1019 <https://doi.org/10.1016/j.cell.2020.09.038>

- 55 Muik A., et al. (2022) Exposure to BA.4/5 S protein drives neutralization of Omicron BA.1, BA.2, BA.2.12.1, and BA.4/5 in vaccine-experienced humans and mice. *Sci Immunol* **7**:eade9888 <https://doi.org/10.1126/sciimmunol.ade9888>
- 56 Ying B., et al. (2022) Boosting with variant-matched or historical mRNA vaccines protects against Omicron infection in mice. *Cell* **185**:1572-1587 e1511 <https://doi.org/10.1016/j.cell.2022.03.037>
- 57 Pallesen J., et al. (2017) Immunogenicity and structures of a rationally designed prefusion MERS-CoV spike antigen. *Proc Natl Acad Sci U S A* **114**:E7348-E7357 <https://doi.org/10.1073/pnas.1707304114>
- 58 McBride C. E., Li J., Machamer C. E (2007) The cytoplasmic tail of the severe acute respiratory syndrome coronavirus spike protein contains a novel endoplasmic reticulum retrieval signal that binds COPI and promotes interaction with membrane protein. *J Virol* **81**:2418-2428 <https://doi.org/10.1128/JVI.02146-06>
- 59 Korber B., et al. (2020) Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. *Cell* **182**:812-827 e819 <https://doi.org/10.1016/j.cell.2020.06.043>
- 60 Miennen H. M., Rose J. K., Mellman I (1989) Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. *Cell* **58**:317-327 [https://doi.org/10.1016/0092-8674\(89\)90846-5](https://doi.org/10.1016/0092-8674(89)90846-5)
- 61 Pinto D., et al. (2021) Broad betacoronavirus neutralization by a stem helix-specific human antibody. *Science* <https://doi.org/10.1126/science.abj3321>
- 62 Kariko K., et al. (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol Ther* **16**:1833-1840 <https://doi.org/10.1038/mt.2008.200>
- 63 Kariko K., Muramatsu H., Ludwig J., Weissman D (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res* **39**:e142 <https://doi.org/10.1093/nar/gkr695>
- 64 Pardi N., et al. (2015) Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *J Control Release* **217**:345-351 <https://doi.org/10.1016/j.jconrel.2015.08.007>
- 65 Hsieh C. L., et al. (2024) Prefusion-stabilized SARS-CoV-2 S2-only antigen provides protection against SARS-CoV-2 challenge. *Nat Commun* **15**:1553 <https://doi.org/10.1038/s41467-024-45404-x>
- 66 Cohen A. A., et al. (2022) Mosaic RBD nanoparticles protect against challenge by diverse sarbecoviruses in animal models. *Science* **377**:eabq0839 <https://doi.org/10.1126/science.abq0839>
- 67 Mastronarde D. N (2005) Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol* **152**:36-51 <https://doi.org/10.1016/j.jsb.2005.07.007>
- 68 Punjani A., Rubinstein J. L., Fleet D. J., Brubaker M. A (2017) cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* **14**:290-296 <https://doi.org/10.1038/nmeth.4169>
- 69 Cohen A. A., et al. (2021) Mosaic nanoparticles elicit cross-reactive immune responses to zoonotic coronaviruses in mice. *Science* **371**:735-741 <https://doi.org/10.1126/science.abf6840>
- 70 West A. P., et al. (2013) Computational analysis of anP-HIV-1 antibody neutralization panel data to identify potential functional epitope residues. *Proc Natl Acad Sci U S A* **110**:10598-10603 <https://doi.org/10.1073/pnas.1309215110>
- 71 Starr T. N., et al. (2022) Shilling mutational constraints in the SARS-CoV-2 receptor-binding domain during viral evolution. *Science* **377**:420-424 <https://doi.org/10.1126/science.abo7896>
- 72 Greaney A. J., et al. (2021) Comprehensive mapping of mutations in the SARS-CoV-2 receptor-binding domain that affect recognition by polyclonal human plasma antibodies. *Cell Host Microbe* **29**:463-476 e466 <https://doi.org/10.1016/j.chom.2021.02.003>
- 73 Hills R. A., et al. (2024) Proactive vaccination using multiviral Quartet Nanocages to elicit broad anP-coronavirus responses. *Nat Nanotechnol* **19**:1216-1223 <https://doi.org/10.1038/s41565-024-01655-9>

- 74 Barnes C. O., et al. (2020) SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies. *Nature* **588**:682-687 <https://doi.org/10.1038/s41586-020-2852-1>
- 75 Landau M., et al. (2005) ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures. *Nucleic Acids Res* **33**:W299-302 <https://doi.org/10.1093/nar/gki370>
- 76 Saphire E. O., et al. (2001) Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design. *Science* **293**:1155-1159

Peer reviews

Reviewer #1 (Public review):

Summary:

This study investigated the immunogenicity of a novel bivalent EABR mRNA vaccine for SARS-CoV-2 that expresses enveloped virus-like particles in pre-immune mice as a model for boosting the population that is already pre-immune to SARS-CoV-2. The study builds on promising data showing a monovalent EABR mRNA vaccine induced substantially higher antibody responses than a standard S mRNA vaccine in naïve mice. In pre-immune mice, the EABR booster increased the breadth and magnitude of the antibody response, but for Omicron, the effects were modest and often not statistically significant. The authors provide compelling evidence to support this may be due to immune imprinting.

This study also builds on prior work with additional experiments to elucidate the mechanisms that contributed to the EABR increased immunogenicity in naïve mice including evidence that the vaccine is inducing responses to more RBD epitopes and a potential role for heterodimer formation as a mechanism whereby bivalent vaccines induce cross-reactive B cell responses.

Strengths:

Evaluating a novel SARS-CoV-2 vaccine that was substantially superior in naïve mice in pre-immune mice as a model for its potential in the pre-immune population.

Providing insight into a possible role of immune imprinting in shaping immune responses to updated booster immunizations.

Minor weaknesses:

(1) Overall, immune responses against Omicron variants were substantially lower than against the ancestral Wu-1 strain that the mice were primed with. The authors speculate this is evidence of immune imprinting. While parallel controls (mice immunized 3 times with just the bivalent EABR vaccine) were not tested, the authors point to prior published work showing Omicron S antigen is a strong immunogen. This indicates the lower immune responses to Omicron are likely due to immune imprinting (or original antigenic sin) and not due to S immunogen being inherently less immunogenic than the S protein from the ancestral Wu-1 strain.

(2) The authors reported statistically significant increase in antibody responses with the bivalent EABR vaccine booster when compared to the monovalent S mRNA vaccine but consistently failed to show significantly higher responses when compared to the bi-valent S mRNA vaccine suggesting that in pre-immune mice, the EABR vaccine has no apparent advantage over the bivalent S mRNA vaccine which is the current standard. There were, however, some trends indicating the group sizes were insufficiently powered to see a difference. The discussion acknowledges these limitations of their studies and potential limited benefits of the EABR strategy in pre-immune mice vs standard bivalent mRNA vaccine.

(3) The EABR S mRNA vaccine was superior to the conventional mRNA S vaccine in naïve mice but not in pre-immune mice. The authors expanded the discussion to propose a possible role for immune imprinting in this result which is supported by the data.

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Reviewer #3 (Public review):

Summary:

The authors evaluated a novel bivalent (Wu1/BA.5 based) mRNA platform that uses the EABR strategy to produce enveloped virus-like particles for vaccination. These were tested as boosters in the context of pre-existing immunity in mice that received two prior immunizations with conventional Wu1 mRNA vaccines. The animal experimental timeline aimed at mimicking the vaccinations/booster schedule implemented during the COVID-19 pandemic. The authors tested and compared different booster strategies: (1) conventional Wu1 S protein encoding mRNA vaccine, (2) EABR Wu1 S protein encoding mRNA vaccine that produces enveloped virus-like particles, (3) conventional Wu1/BA.5 S protein encoding mRNA vaccine, and (4) EABR Wu1/BA.5 S protein encoding mRNA vaccine that produces enveloped virus-like particles. The EABR approach (monovalent or bivalent) enhanced the antibody response against Wu1 and Omicron subvariants. Interestingly, the bivalent EABR Wu1/BA.5 mRNA (strategy 4) generated polyclonal sera targeting multiple receptor-binding domain epitopes: these sera were more diverse than those generated with the other tested booster strategies (1 to 3).

Strengths:

The monovalent Wu1 S-EABR mRNA booster led to increase in antibody binding to tested Omicron variants (BA.5, BQ.1.1, XBB.1), while the bivalent Wu1/BA.5 S-EABR mRNA booster led to the highest Ab response against Omicron variants (BA.5, BQ.1.1, XBB.1) in pre-vaccinated mice.

Neutralization assays showed that the monovalent Wu1 S-EABR mRNA booster had the highest Wu1 neutralization activity and to a lesser extent the early BA.1 early Omicron variant. The monovalent Wu1 S-EABR mRNA booster and bivalent Wu1/BA.5 S-EABR mRNA booster had similar BA.5 neutralizing activity. Neutralizing activity of the different boosters was less pronounced with later Omicron variants BQ.1.1 and XBB.1. However, of the different boosters tested, the bivalent Wu1/BA.5 S-EABR mRNA booster induced the highest neutralizing titers. These results support that the EABR mRNA vaccine strategy helps improve neutralizing activity against different tested Omicron subvariants: a few (1 or 2) mRNA constructs expressing major antigens in enveloped virus-like particles likely provide a novel strategy to elicit an immune response that has the potential to neutralize subsequent variants.

The EABR enveloped virus-like particle strategy induces a more diverse antibody response, including epitopes not recognized by the other booster strategies: these new epitopes could play a role in neutralizing activity against new future variants.

Moreover, the bivalent Wu1/BA.5 S-EABR mRNA booster could potentially produce heterotrimeric S proteins to help activation of cross-reactive B cells and increase polyclonal antibody responses.

Weaknesses:

When it comes to later Omicron variants (BQ.1.1 and XBB.1), there is a discrepancy between epitope binding response and neutralization titers: only a few binding antibodies have neutralizing activity with these later variants, showing a limitation of the EABR strategy.

The authors showed that the EABR mRNA strategy represents a novel antigen exposing strategy where antigens are produced at the cell surface and also at the surface of enveloped virus-like particles. This allows the production of novel antigens in addition to those that would be typically generated against cell surface exposed antigens. These novel antigens targeting new epitopes could potentially have neutralizing activity.

Using a bivalent EABR mRNA booster led to higher antibody titers and higher neutralizing activity. The challenge is to select the best antigen target/variant to support neutralizing activity against later virus variants.

<https://doi.org/10.7554/eLife.108959.2.sa1>

Author Response:

The following is the authors' response to the original reviews.

eLife Assessment

This report provides useful evidence that EABR mRNA is at least as effective as standard S mRNA vaccines for the SARS-CoV-2 booster vaccine. Although the methodology and the experimental approaches are solid, the inconsistent statistical significance throughout the study presents limitations in interpreting the results. Also, the absence of results showing possible mechanisms underlying the lack of benefit with EABR in the pre-immune makes the findings mostly observational.

Thank you for your assessment of our study. Respectfully, we do not agree that our study shows a lack of benefit of using the EABR approach. For the monovalent boosters, the S-EABR mRNA booster improved neutralizing antibody titers by 3.4-fold against BA.1 ($p = 0.03$; Fig. S5) and 4.8-fold against BA.5 (failed to reach statistical significance; Fig. 3B) compared to the regular S mRNA booster, which is consistent with the findings from our prior study in naïve mice. In addition, the bivalent S-EABR booster consistently elicited the highest neutralizing titers against all tested variants, including significantly higher titers against BA.5 and BQ.1.1 than the monovalent S booster. The bivalent S-EABR booster also induced detectable neutralization activity in a larger number of mice than all other boosters.

Consistent with this analysis, please note that reviewers 1 and 2 commented that “the EABR booster increased the breadth and magnitude of the antibody response, but the effects were modest and often not statistically significant” (reviewer 1) and “the authors found that across both monovalent and bivalent designs, the EABR antigens had improved antibody titers than conventional antigens, although they observed dampened titers against Omicron variants, likely due to immune imprinting” (reviewer 2).

We agree with the reviewers' assessment that the EABR booster-mediated improvements were mostly modest, in particular against the BQ.1.1 and XBB.1 strains. We also acknowledge that the improvements in titers did not reach statistical significance in many cases, which we believe could have been addressed by adding more animals to our cohorts. Unfortunately, that would have been prohibitively expensive and time-consuming given that we already included 10 mice per group, which is standard practice in the vaccine field.

Finally, we also wish to point out that we did include experiments that addressed potential mechanistic differences between booster groups. For example, we conducted deep mutational scanning studies to determine polyclonal antibody epitope mapping profiles,

showing that bivalent S-EABR boosters induced more balanced targeting of multiple RBD epitopes, which likely contributed to the observed improvements in neutralization. Our work also included cryo-EM studies demonstrating that bivalent S mRNA boosters promote heterotrimer formation, which could potentially drive preferential stimulation of cross-reactive B cells via intra-spike crosslinking. This represents a potential mechanism explaining how bivalent boosters outperformed monovalent boosters in our and many prior studies, which warrants further investigation. Finally, we also performed serum depletion assays, showing that the BA.5 neutralizing activity elicited by the bivalent Wu1/BA.5 S and S-EABR mRNA boosters was primarily driven by cross-neutralizing Abs induced by the primary vaccination series.

Public Reviews:

Reviewer #1 (Public review):

Summary:

This study investigated the immunogenicity of a novel bivalent EABR mRNA vaccine for SARS-CoV-2 that expresses enveloped virus-like particles in pre-immune mice as a model for boosting the population that is already pre-immune to SARS-CoV-2. The study builds on promising data showing a monovalent EABR mRNA vaccine induced substantially higher antibody responses than a standard S mRNA vaccine in naïve mice. In pre-immune mice, the EABR booster increased the breadth and magnitude of the antibody response, but the effects were modest and often not statistically significant.

We thank the reviewer for their accurate summary of our study. Please see our comments to the reviewer's individual points below, as well as our responses to the editor's assessment above.

Strengths:

Evaluating a novel SARS-CoV-2 vaccine that was substantially superior in naïve mice in pre-immune mice as a model for its potential in the pre-immune population.

Weaknesses:

(1) Overall, immune responses against Omicron variants were substantially lower than against the ancestral Wu-1 strain that the mice were primed with. The authors speculate this is evidence of immune imprinting, but don't have the appropriate controls (mice immunized 3 times with just the bivalent EABR vaccine) to discern this. Without this control, it's not clear if the lower immune responses to Omicron are due to immune imprinting (or original antigenic sin) or because the Omicron S immunogen is just inherently more poorly immunogenic than the S protein from the ancestral Wu-1 strain.

The reviewer raises an important point, and we agree that including additional groups receiving three immunizations with the bivalent spike and/or spike-EABR mRNA vaccines would have improved the experimental design. However, we believe that several prior studies have already demonstrated that Omicron S immunogens are not inherently poorly immunogenic compared to the ancestral S; e.g., Scheaffer et al., Nat Med (2022); Ying et al., Cell (2022); Muik et al., Sci Immunol (2022). Based on these prior reports, we conclude that the lower neutralizing titers against Omicron variants in our study are most likely driven by immune imprinting as a result of the initial vaccination series with the ancestral S immunogen.

(2) The authors reported a statistically significant increase in antibody responses with the bivalent EABR vaccine booster when compared to the monovalent S mRNA vaccine, but consistently failed to show significantly higher responses when compared to the bivalent

S mRNA vaccine, suggesting that in pre-immune mice, the EABR vaccine has no apparent advantage over the bivalent S mRNA vaccine which is the current standard. There were, however, some trends indicating the group sizes were insufficiently powered to see a difference. This is mostly glossed over throughout the manuscript. The discussion section needs to better acknowledge these limitations of their studies and the limited benefits of the EABR strategy in pre-immune mice vs the standard bivalent mRNA vaccine.

We acknowledge that the improvements in titers did not reach statistical significance in many cases, which we believe could have been addressed by adding more animals to our cohorts. Unfortunately, that would have been prohibitively expensive and timeconsuming given that we already included 10 mice per group, which is standard practice in the vaccine field. We added a “Limitations of the study” section at the end of the discussion to address all of these points in detail (lines 570-598 in the revised version).

(3) The discussion would benefit from additional explanation about why they think the EABR S mRNA vaccine was substantially superior in naïve mice vs the standard S mRNA vaccine in their previously published work, but here, there is not much difference in pre-immune mice.

As we pointed out in our response to the editor’s assessment above, the monovalent SEABR mRNA booster improved neutralizing antibody titers by 3.4-fold against BA.1 ($p = 0.03$; Fig. S5) and 4.8-fold against BA.5 (failed to reach statistical significance; Fig. 3B) compared to the conventional monovalent S mRNA booster, which is largely consistent with the findings from our prior study in naïve mice. Although the bivalent S-EABR mRNA booster consistently elicited higher neutralizing titers than the conventional bivalent S mRNA booster, we agree with the reviewer that these improvements were modest and not statistically significant. Overall, neutralizing activity against later Omicron variants, such as BQ.1.1 and XBB.1 was low. We attributed this finding to immune imprinting (see response to point (1) above) and acknowledged that the EABR approach was not able to effectively overcome this effect (see discussion section of the paper, lines 537-558; and “Limitations of the study” section, lines 570-598 in the revised version).

Reviewer #2 (Public review):

Summary:

In this manuscript, Fan, Cohen, and Dam et al. conducted a follow-up study to their prior work on the ESCRT- and ALIX-binding region (EABR) mRNA vaccine platform that they developed. They tested in mice whether vaccines made in this format will have improved binding/neutralization antibody capacity over conventional antigens when used as a booster. The authors tested this in both monovalent (Wu1 only) or bivalent (Wu1 + BA.5) designs. The authors found that across both monovalent and bivalent designs, the EABR antigens had improved antibody titers than conventional antigens, although they observed dampened titers against Omicron variants, likely due to immune imprinting. Deep mutational scanning experiments suggested that the improvement of the EABR format may be due to a more diversified antibody response. Finally, the authors demonstrate that co-expression of multiple spike proteins within a single cell can result in the formation of heterotrimers, which may have potential further usage as an antigen.

We thank the reviewer for their support and for the accurate summary and evaluation of our study.

Strengths:

(1) The experiments are conducted well and are appropriate to address the questions at hand. Given the significant time that is needed for testing of pre-existing immunity, due

to the requirement of pre-vaccinated animals, it is a strength that the authors have conducted a thorough experiment with appropriate groups.

(2) The improvement in titers associated with EABR antigens bodes well for its potential use as a vaccine platform.

Weaknesses:

As noted above, this type of study requires quite a bit of initial time, so the authors cannot be blamed for this, but unfortunately, the vaccine designs that were tested are quite outdated. BA.5 has long been replaced by other variants, and importantly, bivalent vaccines are no longer used. Testing of contemporaneous strains as well as monovalent variant vaccines would be desirable to support the study.

We thank the reviewer for bringing up this important point. We agree that the variants used for this study are now outdated, and it would have been informative to evaluate conventional and EABR boosters against contemporaneous strains. However, as the reviewer correctly pointed out, this type of study requires a substantial amount of time to conduct and will therefore will likely always be outdated by the time the data are analyzed and prepared for publication. To accurately assess immune responses against recent or current strains in mice, multiple boosters would have been needed to mimic the pre-existing immune context in the human population in 2025. Assuming intervals of 6-7 months between boosters (as used in this study to mimic booster intervals in the human population as closely as possible), this type of study would have been challenging to conduct, especially given the limited lifespan of mice. Thus, we performed this proof-of-concept study using outdated variants to assess the potential of EABR-modified boosters. We greatly appreciate the reviewer's understanding and acknowledge this limitation of our study, which is highlighted in the added "Limitations of the study" section in the revised version of the manuscript (lines 570-598).

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) The acronym RBD in the title should be spelled out.

We thank the reviewer for raising this point. We made this change in the revised version of the paper.

(2) Lines 167-168 describe no differences between the cohorts at day 244. It should also be stated that for all timepoints, there are no significant differences.

We modified the revised manuscript according to the reviewer's suggestion (line 170).

Reviewer #2 (Recommendations for the authors):

(1) Given the focus on developing broad vaccines for future coronavirus outbreaks, it would be particularly informative to test whether the EABR antigens elicit broadened/heightened responses against other (beta)coronaviruses. If enough serum is left, it would seem straightforward to conduct neutralization assays against non-SARSCoV-2 coronaviruses.

We thank the reviewer for this valid suggestion. Unfortunately, the extensive analysis of the serum samples, including spike and RBD ELISAs and neutralization assays against multiple variants, deep mutational scanning, and depletion assays, used up the serum samples for most mice. We agree that it would be interesting to investigate whether bivalent EABR boosters elicit pan-sarbecovirus responses in future studies.

(2) In the bar plots for antibody titer changes, shown as log₁₀ fold change, it is quite hard to interpret the difference between bars (e.g., what is the fold change difference between each bar in the same time point?). A table of mean {plus minus} SD values would be helpful.

That's a great suggestion. We added a table (Table S1) presenting all the geometric mean neutralization titers for all timepoints and variants in the revised version of the manuscript.

(3) The development of heterotrimers as potential antigens is very interesting, but it seems out of place in the current manuscript. This should likely be in a separate, standalone manuscript.

We thank the reviewer for commenting on the heterotrimer part of our manuscript. The presented work was not intended to advance the development of heterotrimers as potential antigens. Instead, our findings demonstrate that bivalent spike mRNA vaccines readily generate heterotrimers, which could promote intra-spike crosslinking and potentially impact antibody epitope targeting profiles as suggested by the deep mutational scanning data for the bivalent S-EABR mRNA booster (Fig. 4; Fig. S7-8). We think this is an important consideration that warrants further investigation with regards to the development of future bivalent or multivalent vaccines.

(4) As a minor note, the sequences of the variants used or accession numbers should be provided in the Methods, since different groups have used different mutations for variants.

We added the accession numbers for the vaccine strains used in this study (lines 604605).

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