

1 **Immunological imprinting shapes the specificity of**
2 **human antibody responses against SARS-CoV-2**
3 **variants**

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28 **Summary**

29 The spike glycoprotein of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)
30 continues to accumulate substitutions, leading to breakthrough infections of vaccinated
31 individuals and prompting the development of updated booster vaccines. Here, we determined
32 the specificity and functionality of antibody and B cell responses following exposure to BA.5 and
33 XBB variants in individuals who received ancestral SARS-CoV-2 mRNA vaccines. BA.5
34 exposures elicited antibody responses that primarily targeted epitopes conserved between the
35 BA.5 and ancestral spike, with poor reactivity to the XBB.1.5 variant. XBB exposures also
36 elicited antibody responses that targeted epitopes conserved between the XBB.1.5 and
37 ancestral spike. However, unlike BA.5, a single XBB exposure elicited low levels of XBB.1.5-
38 specific antibodies and B cells in some individuals. Pre-existing cross-reactive B cells and
39 antibodies were correlated with stronger overall responses to XBB but weaker XBB-specific
40 responses, suggesting that baseline immunity influences the activation of variant-specific
41 SARS-CoV-2 responses.

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45 **Keywords**

46 SARS-CoV-2, mRNA vaccines, Omicron BA.5, XBB.1.5, antibodies, B cells, original antigenic
47 sin, immune imprinting, immune evasion, viral evolution

48

49 **Highlights**

50

51 • Variant breakthrough infections boost ancestral cross-reactive antibodies and B
52 cells

53

54 • First and second BA.5 exposures fail to elicit variant-specific antibodies and B
55 cells

56

57 • XBB infections and monovalent vaccinations elicit XBB.1.5-specific responses in
58 some individuals

59

60 • XBB.1.5-specific responses correlate with low levels of pre-existing humoral
61 immunity

62 Introduction

63 SARS-CoV-2 first emerged in 2019, prompting the rapid development of mRNA-LNP vaccines
64 that elicit potent neutralizing antibodies targeting the viral spike glycoprotein (Corbett et al.,
65 Hsieh et al., Jackson et al., 2020). The virus has since acquired many spike substitutions that
66 prevent the binding of antibodies elicited by vaccinations and infections. Notably, the BA.1
67 Omicron variant that began spreading widely in late 2020 possessed ~32 spike amino acid
68 substitutions compared to the ancestral SARS-CoV-2 strain (Mannar et al., 2022), leading to an
69 increase in ‘breakthrough’ infections of vaccinated individuals (Accorsi et al., Altarawneh et al.,
70 Cao et al., Kuhlmann et al., 2022; Levin et al., 2021; Pulliam et al., 2022).
71
72 Omicron breakthrough infections induce efficient anamnestic immune responses that recruit
73 memory T and B cells cross-reactive to the ancestral strain (Addetia et al., 2023; Kared et al.,
74 Koutsakos et al., 2022; Painter et al., 2023; Park et al., Wang et al., 2022; Weber et al., Yisimayi
75 et al., 2023). Many unanswered questions remain on how initial SARS-CoV-2 encounters affect
76 the specificity of antibodies elicited against variant viral strains. For example, bivalent boosters
77 containing BA.5 spike elicit B cell and antibody responses cross-reactive to the ancestral SARS-
78 CoV-2 strain in individuals who received ancestral mRNA-LNP vaccines (Addetia et al., 2023),
79 but this ‘immunological imprinting’ effect may be less pronounced in individuals who initially
80 received inactivated vaccines (Yisimayi et al., 2023). It remains unclear if repeat exposures with
81 SARS-CoV-2 variants can overcome memory B cell biases established by initial SARS-CoV-2
82 encounters (Addetia et al., Alsoussi et al., Schiepers et al., Yisimayi et al., 2023). Further, it is
83 unknown if variants with larger antigenic distances are better able to stimulate *de novo*
84 responses while recalling fewer memory B cells in individuals who received ancestral mRNA-
85 LNP vaccines.
86

87 Here, we elucidated the specificity of antibody and B cell responses elicited by BA.5 and XBB
88 exposures in individuals previously vaccinated with mRNA-LNPs expressing the ancestral
89 SARS-CoV-2 spike. We compared immune responses in individuals with different amounts of
90 cross-reactive B cells and antibodies at time of variant exposure. We used antigen-specific flow
91 cytometric analyses to interrogate spike-specific B cell responses and performed enzyme-linked
92 immunosorbent assays (ELISAs), neutralization assays, and absorption assays to characterize
93 SARS-CoV-2 reactive antibodies.

94

95 **Results**

96 **BA.5 breakthrough infections elicit antibodies that cross-react to ancestral SARS-CoV-2**

97 To better understand the specificity and functionality of antibodies elicited by breakthrough
98 infections, we characterized antibodies in sera collected from individuals (n=8) who received 3
99 doses of mRNA-LNP vaccines expressing the ancestral spike and were subsequently infected
100 with a BA.5 Omicron variant in 2022 (breakthrough infections occurred on average 291 days
101 since last vaccination; Painter et al., 2023) (**Figure 1A**). Sera were collected at baseline ~0-5
102 days (T1) and ~45 days (T2) after BA.5 breakthrough infection. We first quantified serum
103 antibodies reactive to full length spike proteins from the ancestral virus, the breakthrough variant
104 (BA.5), and a variant from 2023 that did not yet exist at the time of sample collection (XBB.1.5).
105 Antibodies reactive to all 3 spike proteins increased >2 fold after BA.5 breakthrough infection
106 (**Figure 1B**). Similar increases were observed when we measured antibodies reactive to the
107 receptor binding domain (RBD) of the spike protein from the ancestral, BA.5, and XBB.1.5
108 viruses, with the largest fold increase to BA.5 RBD (**Figure 1C**). Prior to breakthrough infection,
109 most participants had high neutralizing antibodies against ancestral SARS-CoV-2, but low or
110 undetectable levels of neutralizing antibodies against BA.5 and XBB.1.5 (**Figure 1D**).
111 Neutralizing antibodies against ancestral SARS-CoV-2 and the BA.5 variant were boosted upon
112 BA.5 variant breakthrough infection in most participants, whereas neutralizing antibodies

113 against XBB.1.5 were minimally boosted and remained low after infection (**Figure 1D**). This led
114 to an observed increase in antibody neutralization potency (the neutralizing antibody titer
115 divided by total spike targeting antibody titer) for ancestral SARS-CoV-2 and BA.5, but not
116 XBB.1.5 (**Figure 1E**). Thus, BA.5 breakthrough infections elicited neutralizing antibodies against
117 ancestral SARS-CoV-2 and BA.5, but these antibodies poorly neutralized the XBB.1.5 variant
118 that did not yet exist at the time of infection.

119
120 Given that we observed simultaneous increases in neutralizing antibodies reactive to the
121 ancestral SARS-CoV-2 virus and BA.5 variant, we hypothesized that BA.5 breakthrough
122 infections elicit neutralizing antibodies that recognize epitopes conserved between these
123 viruses. To address this, we performed ELISAs and neutralization assays with sera that were
124 previously absorbed with carboxyl-magnetic beads coupled with different spike proteins. In
125 these assays (Anderson et al., 2022; Arevalo et al., 2020), antibodies that bind to the spike-
126 coupled beads are removed and unbound antibodies are then assessed for reactivity against
127 different antigens (**Figure S1A**). As expected, beads coated with the ancestral SARS-CoV-2
128 spike were able to remove >99% of antibodies reactive to the ancestral SARS-CoV-2 full length
129 spike and RBD, while beads coated with either BA.5 or XBB.1.5 spikes reduced but did not
130 entirely remove these antibodies (**Figure 1F-G**). Consistent with this observation, sera absorbed
131 with beads coated with the ancestral SARS-CoV-2 spike could no longer neutralize ancestral
132 SARS-CoV-2, while sera absorbed with beads coated with either the BA.5 or XBB.1.5 spike still
133 contained antibodies capable of neutralizing the ancestral strain (**Figure 1H**). Conversely, beads
134 coated with either the ancestral SARS-CoV-2 spike or BA.5 spike efficiently removed antibodies
135 that bound (**Figure 1I-J**) and neutralized (**Figure 1K**) BA.5 virus, suggesting that nearly all
136 BA.5-reactive antibodies elicited by BA.5 breakthrough infections cross-react to the ancestral
137 SARS-CoV-2 spike. Throughout these studies, it became apparent that some of these
138 ancestral/BA.5 cross-reactive antibodies recognize epitopes that are altered in XBB.1.5, since

139 serum pre-incubation with beads coated with the XBB.1.5 spike did not eliminate binding (~20%
140 remaining, **Figure 1I-J**) or neutralization (~40% remaining, **Figure 1K**) of BA.5. These data,
141 combined with overall low XBB.1.5 titers (**Figure 1D, S1B-D**) suggest that BA.5 breakthrough
142 infections elicit antibodies that cross-react with the ancestral SARS-CoV-2 and BA.5 spikes, and
143 that substitutions in the XBB.1.5 variant prevent binding of a fraction of these antibodies,
144 especially neutralizing antibodies.

145

146 **A second BA.5 exposure elicits antibodies that cross-react to ancestral SARS-CoV-2**

147 We next analyzed sera from an additional 9 individuals who received 3 doses of an mRNA-LNP
148 vaccine expressing the ancestral SARS-CoV-2 spike who were later exposed twice to BA.5
149 through sequential infections and vaccinations (**Figure 2A**). These individuals received a dose
150 of bivalent ancestral/BA.5 spike mRNA-LNP vaccine either before (n=4) or after (n=5)
151 experiencing a BA.5 breakthrough infection. Sera were collected early (~0-5 days, T1) and ~45
152 days (T2) after the second BA.5 exposure, which occurred on average 76 days (27-156) after
153 the first BA.5 exposure. Antibodies reactive to the ancestral, BA.5, and XBB.1.5 spike proteins
154 were high prior to the second BA.5 exposure and were minimally boosted (**Figure 2B-C**). Most
155 individuals had high baseline neutralizing antibodies against both ancestral SARS-CoV-2 and
156 BA.5 and these antibodies were minimally boosted upon second BA.5 exposure (**Figure 2D**).
157 Neutralizing antibody titers against the XBB.1.5 variant were low in most individuals both before
158 and after the second BA.5 exposure (**Figure 2D**). We observed a modest decrease in serum
159 neutralization potency for all variants tested after secondary BA.5 exposure (**Figure 2E**).
160 Antibody responses were similar regardless of whether the first or second BA.5 exposure was
161 via infection or vaccination (**Figure S2A-C**).

162

163 We performed additional absorption assays and found that, similar to antibodies elicited by
164 single BA.5 breakthrough infections, BA.5-reactive antibodies from individuals exposed twice

165 with BA.5 bound to the ancestral SARS-CoV-2 spike, but only partially recognized the XBB.1.5
166 spike (**Figure 2F-K, S2D-F**). Thus, antibodies elicited by a second BA.5 spike exposure did not
167 target BA.5-specific epitopes, but instead recognized epitopes conserved in the ancestral spike
168 that became mutated in the XBB.1.5 variant.

169

170 **Cross-reactive B cell responses dominate first and second BA.5 exposures in individuals** 171 **previously vaccinated with mRNA-LNP vaccines expressing the ancestral spike**

172 We hypothesized that cross-reactive antibodies in our study were likely produced by B cells
173 generated after the initial exposures to the ancestral spike and subsequently recalled in
174 response to first and second BA.5 exposures. To measure levels of cross-reactive B cells
175 elicited by first and second BA.5 exposures, we performed a flow cytometric B-cell antigen
176 probe assay using PBMCs collected from participants at timepoints corresponding to our
177 serological assays (**Fig. 3A, S3**). We quantified B cells that recognized the RBD of ancestral,
178 BA.5, and XBB.1.5 spike proteins. Following first BA.5 exposures, the proportion of ancestral
179 RBD-binding B cells that also bound BA.5 RBD increased in most individuals, and these
180 responses did not greatly increase following a second BA.5 exposure (**Fig. 3B**). In further
181 accordance with our serological data, XBB.1.5 cross-reactivity was only observed in a fraction of
182 BA.5-binding B cells at all timepoints, and XBB.1.5 cross-reactivity did not increase following
183 BA.5 infection (**Fig. 3C-D**). We did not observe robust variant-specific B cell responses, as
184 extremely low frequencies of B cells bound BA.5 or XBB.1.5 RBD without binding ancestral
185 RBD (BA.5-specific and XBB.1.5-specific, respectively), and the frequency of BA.5-only and
186 XBB.1.5-only B cells did not increase following BA.5 exposure (**Fig. 3E-F**). Together, these data
187 demonstrate that ancestral cross-reactive B cells are stimulated upon BA.5 exposure, leading to
188 a heavily cross-reactive antibody response with no detectable production of antibodies that
189 target novel epitopes on the RBD of the BA.5 spike.

190

191 **BA.5 exposures elicit antibodies targeting conserved RBD residues mutated in XBB.1.5**

192 Our data suggest that BA.5 exposures boost antibodies that target spike residues that are
193 conserved between ancestral and BA.5 variants but are mutated in XBB.1.5. To map the
194 specificity of these antibodies, we completed additional absorption assays with BA.5 spike
195 proteins that were engineered to possess different RBD amino acid substitutions. We absorbed
196 serum samples with BA.5 spike mutants that had one or two XBB.1.5 RBD amino acid
197 substitutions at residues that are shared between ancestral and BA.5 spikes but differ in
198 XBB.1.5 (spike amino acid residues 346, 368, 445/446, 460, and 490) (**Figure 4A**). We also
199 absorbed serum with 7 additional BA.5 spike proteins with different combinations of XBB.1.5
200 RBD mutations, including a mutant that possessed all RBD substitutions that are shared
201 between ancestral and BA.5 spikes but differ in XBB.1.5 spike (protein M7, **Figure 4B**).
202 Absorption with BA.5 spikes with each single substitution decreased the ability of serum
203 antibodies to bind wildtype BA.5, suggesting that a fraction of polyclonal antibodies targeted
204 each mutated site (**Figure 4C**). Absorption with BA.5 proteins with multiple mutations further
205 decreased the binding of BA.5-reactive antibodies (**Figure 4C**). Similar results were obtained
206 when we repeated experiments with sera isolated from ancestral vaccinated individuals after
207 two BA.5 exposures (**Figure 4D**). Sera absorbed with the M7 protein containing all XBB.1.5
208 RBD substitutions efficiently neutralized BA.5, indicating that a large fraction of BA.5
209 neutralizing antibodies targeted epitopes that are conserved between the ancestral and BA.5
210 RBDs but not the XBB.1.5 RBD (**Figure 4E, F, S4**). Collectively, these data support the
211 hypothesis that BA.5 exposures elicit antibodies that target epitopes conserved in the ancestral
212 spike RBD that later became mutated in the XBB.1.5 variant.

213

214 **XBB exposures elicit antibodies cross-reactive to the ancestral spike in individuals**
215 **previously vaccinated with mRNA-LNP vaccines expressing the ancestral spike**

216 XBB, the recombinant variant of BA.2.10.1 and BA.2.75 sublineages containing ~43 spike
217 mutations, and its subvariants, namely XBB.1.5, have dominated infections since late 2022 and
218 resulted in a shift to a new monovalent booster vaccine in September 2023 (CDC,
219 Selvavinayagam et al., Wang et al., 2023; WHO 2022). We longitudinally sampled individuals
220 who were exposed to XBB.1.5 via the monovalent mRNA-LNP booster (n = 12) or by XBB-
221 subvariant breakthrough infections (symptom onset between 1/2/2022 and 10/1/2022, n = 10)
222 and assessed the specificity and functionality of antibody and B cell responses (**Figure 5A**). We
223 observed a robust increase in antibodies that bound and neutralized the ancestral and XBB.1.5
224 variant 15 and 45 days after XBB.1.5 monovalent mRNA-LNP vaccination or XBB.1.5 infection
225 (**Figure 5B-D**). While increases in antibody titers to both viruses were significant, the fold-
226 increase in XBB.1.5 neutralizing titers was ~5-fold higher than that of ancestral strain, and
227 neutralization potency of XBB.1.5 antibodies increased slightly following XBB.1.5 exposure,
228 whereas that of ancestral did not (**Figure 5D-E**). We found that XBB.1.5 monovalent mRNA-
229 LNP vaccination rapidly boosted XBB.1.5 neutralizing antibodies that peaked 15 days after
230 vaccination, whereas antibodies elicited by XBB.1.5 infections steadily rose for 45 days after
231 exposure, consistent with earlier reports of slower kinetics of immune responses elicited by
232 breakthrough infections (Koutsakos et al., 2022; Painter et al., 2023) (**Figure S5A-C**). Although
233 early kinetics differed, antibodies elicited by XBB.1.5 infections and vaccinations were at similar
234 levels by 45 days after exposure (**Figure S5A-C**).

235
236 We performed absorption assays using sera collected 45 days after XBB.1.5 exposures to
237 identify antibody specificities elicited by XBB infections and monovalent vaccinations. Beads
238 coated with the XBB.1.5 spike poorly absorbed antibodies that bound (**Figure 5F**) and
239 neutralized (**Figure 5G**) the ancestral SARS-CoV-2 strain. Conversely, beads coated with the
240 ancestral spike absorbed most XBB.1.5-reactive and neutralizing antibodies (**Figure 5H-I, S5E**),
241 suggesting that the majority of antibodies elicited by XBB exposures bound to epitopes

242 conserved in the ancestral spike. While most individuals produced XBB.1.5-reactive antibodies
243 that targeted epitopes conserved in the ancestral spike, we identified some individuals who
244 produced XBB.1.5-specific responses that were only partially absorbed by beads coated with
245 the ancestral spike (**Figure 5H-I, S5E**). These data suggest that XBB.1.5, which is more
246 antigenically distant compared to BA.5, is capable of eliciting variant-specific responses in a
247 subset of individuals who were previously vaccinated with mRNA-LNP expressing the ancestral
248 spike.

249
250 Next, we characterized antibodies in sera from 3 individuals who were infected with an XBB-
251 variant and subsequently received the XBB.1.5 monovalent booster (average 139 days after
252 infection, **Figure 5J**). Unlike a second BA.5 exposure, a second XBB exposure greatly
253 increased XBB.1.5 neutralizing antibody titers and potency (**Figure 5K, S5F-H**). Using
254 absorption assays, we also observed increases in both XBB.1.5-specific binding and
255 neutralizing antibodies following the second XBB exposure (**Figure 5L-N, S5I-K**). Similar to
256 single XBB exposures, the majority of neutralizing antibodies elicited by two XBB exposures
257 targeted epitopes in the ancestral spike, with XBB.1.5-specific antibodies constituting only 17%
258 of total XBB.1.5 neutralizing antibodies. Nonetheless, these experiments demonstrate that XBB
259 can provoke variant-specific responses in a subset of individuals who previously received
260 mRNA-LNP vaccines expressing the ancestral spike, and that these responses can be further
261 boosted by sequential XBB exposures.

262
263 **Cross-reactive B cell responses dominate following XBB exposures in individuals**
264 **previously vaccinated with mRNA-LNP vaccines expressing the ancestral spike**

265 To further explore the relationships between cross-reactive and variant-specific responses
266 elicited by XBB, we analyzed antigen-specific B cells elicited after XBB exposures. We
267 observed a significant increase in ancestral and XBB.1.5-reactive B cells by 15 days post-

268 exposure, followed by a slight contraction by day 45 (**Fig 6A-B**). The percent of ancestral RBD-
269 binding B cells that were cross-reactive with XBB.1.5 increased significantly by day 7 and
270 remained above baseline at day 45 (**Fig. 6C**). The frequencies of XBB.1.5-specific B cells (cells
271 that did not cross-react with ancestral RBD) were low but significantly expanded between days
272 0 and 7 before contracting by day 45 (**Fig. 6D, Fig. S6A**). There was substantial heterogeneity
273 in the magnitude of XBB.1.5-specific B cell expansion, suggesting that variant-specific
274 responses may be restrained in some individuals (**Fig. 6D**). Despite expanding significantly
275 during acute XBB exposures, the proportion of XBB.1.5 RBD-binding B cells that were specific
276 for XBB.1.5 did not increase, consistent with the cross-reactive response remaining dominant
277 compared to variant-specific responses (**Fig. 6E**).

278
279 CD71 is expressed following B cell activation, with reactivated memory B cells expressing
280 higher CD71 than stimulated naïve B cells (Auladell et al., 2019). CD71 was rapidly upregulated
281 after XBB exposure and was significantly elevated on XBB.1.5 cross-reactive and XBB.1.5-
282 specific B cells at day 7 and day 15 compared to bulk B cells or B cells that bound the ancestral
283 RBD but not the XBB.1.5 RBD (**Fig. 6F-G, S6B**). XBB.1.5-specific B cells expressed lower
284 CD71 than XBB.1.5 cross-reactive B cells, consistent with differences between recall responses
285 from memory B cells and *de novo* responses arising from naïve B cells (Auladell et al., 2019).

286 XBB.1.5 cross-reactive B cells were class-switched and expressed IgG, whereas most XBB.1.5-
287 specific B cells were not class-switched to IgA or IgG (**Fig. 6H**), suggesting that they were likely
288 derived from *de novo* responses. In addition to rapid CD71 upregulation, XBB.1.5-binding
289 plasmablasts, including XBB.1.5-specific plasmablasts, increased dramatically by day 7 after
290 XBB.1.5 exposure (**Fig 6I-J**). Taken together, these data suggest that XBB elicits a potent B cell
291 response that is largely cross-reactive with the ancestral spike, with some individuals generating
292 variant-specific B cells.

293

294 Finally, we wanted to determine what cellular or serological metrics were associated with the
295 production of XBB.1.5-specific responses. Elevated baseline antibody titers, ancestral and
296 XBB.1.5 RBD-binding B cell frequencies, and XBB.1.5 cross-reactive B cell frequencies were all
297 associated with diminished XBB.1.5-specific B cell responses (**Fig. 6K, S6C-D**). Moreover, high
298 pre-existing antibody titers against XBB.1.5 spike were associated with decreased XBB.1.5-
299 specific neutralizing antibody titers (**Fig. 6L**). As expected, there was a positive correlation
300 between XBB.1.5-specific B cells at day 7 and peak XBB.1.5-specific neutralizing antibody titers
301 (**Fig. 6L**). XBB.1.5-specific antibodies were also correlated with XBB.1.5-specific B cell
302 expansion and the percent of XBB.1.5-binding B cells that were XBB.1.5-specific (**Fig. S6E**).
303 Together, these analyses support a model in which pre-existing B cells and antibodies influence
304 the development of variant-specific responses, where individuals with lower pre-existing
305 antibody titers and fewer cross-reactive memory B cells are better able to activate naïve
306 XBB.1.5-specific B cells and produce XBB.1.5-specific antibodies. However, baseline cross-
307 reactive B cells are strongly correlated with peak XBB.1.5 neutralizing antibody titers, whereas
308 XBB.1.5-specific B cell responses have no association with overall XBB.1.5 neutralizing
309 antibody titers after infection or vaccination (**Fig. 6M**). This is consistent with the observation
310 that the majority of XBB.1.5 neutralizing antibodies cross-react to the ancestral spike. Overall,
311 these analyses highlight the diversity of the humoral response to XBB and define several key
312 factors contributing to the development of variant-specific B cells and antibodies.

313

314 **Discussion**

315 Through longitudinal sampling and intensive serological and cellular analyses, our studies
316 demonstrate that B cells elicited by ancestral SARS-CoV-2 mRNA vaccines are efficiently
317 recalled by SARS-CoV-2 variants, leading to the rapid production of neutralizing antibodies
318 against epitopes conserved between ancestral and variant strains. We found that the BA.5 spike
319 elicits antibodies that recognize epitopes conserved between the ancestral and BA.5 spikes, but

320 fail to bind to the more antigenically distant XBB.1.5 spike. Similarly, the majority of antibodies
321 elicited by XBB infection or monovalent vaccination targeted epitopes conserved between the
322 ancestral and XBB.1.5 spikes. Both BA.5 and XBB elicited responses that were dominated by
323 memory B cells primed by prior ancestral mRNA-LNP vaccinations; however, unlike BA.5, we
324 found that XBB elicited low levels of variant-specific B cell and antibody responses in some
325 individuals.

326
327 Our data are consistent with reports showing antibody responses to SARS-CoV-2 variants
328 typically cross-react to the ancestral spike in individuals who initially encountered ancestral
329 SARS-CoV-2 antigens (Addetia et al., Chalkias et al., 2023; Kared et al., Koutsakos et al.,
330 2022; Painter et al., 2023; Park et al., 2022; Tortorici et al., 2023; Wang et al., 2022; Wang et al.,
331 Weber et al., Yisimayi et al., 2023). Clinical studies have shown that BA.5 and XBB.1.5 variant
332 booster vaccines are highly effective at reducing deaths and hospitalizations caused by
333 circulating variants (Carr et al., Hoffmann et al., Lin et al., Lin et al., Shresta et al., Tan et al., Tan
334 et al., Wang et al., 2023). It is likely that antibodies targeting epitopes conserved with the
335 ancestral SARS-CoV-2 spike contribute to variant booster vaccine protection. It is important to
336 highlight that cross-reactive B cells elicited by earlier ancestral SARS-CoV-2 mRNA-LNP
337 vaccinations are strongly correlated with the induction of high levels of XBB.1.5 neutralizing
338 antibodies after monovalent XBB.1.5 vaccination. Therefore, while ‘immune imprinting’ by
339 ancestral SARS-CoV-2 mRNA-LNP vaccines clearly influence the specificity of antibodies
340 elicited by variant infections and vaccinations, these prior vaccinations are also highly beneficial
341 for establishing memory B cells that can be rapidly recruited to produce neutralizing antibodies
342 against SARS-CoV-2 variants.

343
344 Further studies should evaluate if SARS-CoV-2 antigens with greater antigenic distances are
345 able to efficiently elicit variant-specific *de novo* antibody responses in individuals who were

346 previously vaccinated with ancestral mRNA-LNP vaccines. In our studies, XBB exposures
347 promoted the development of low-level variant-specific XBB.1.5-specific antibody responses in
348 some individuals. These variant-specific responses were associated with lower baseline SARS-
349 CoV-2 XBB.1.5-specific antibody titers and B cell frequencies, suggesting that factors such as
350 epitope masking and feedback inhibition may potentially limit the recruitment of naïve B cells
351 that target novel epitopes on variant spike proteins (Bergström et al., 2017; Schaefer-Babajew
352 et al., 2023; Tas et al., 2022). It will also be important to quantify the level of somatic
353 hypermutation in B cells that produce antibodies that bind specifically to variants. While it is
354 likely that variant-specific antibodies in our studies are derived from *de novo* activated B cells, it
355 is also possible that these antibodies are produced from memory B cells that have lost binding
356 to ancestral spike through somatic hypermutation.

357
358 Future studies should also determine if mRNA-LNP and inactivated virus vaccines elicit different
359 'immunological imprints' that affect the specificity and magnitude of antibody responses elicited
360 by variant infections and vaccinations. Comparative analyses have previously focused on
361 general immunogenicity and effectiveness against severe disease, showing higher antibody
362 titers and better overall protection in mRNA-LNP vaccinated individuals compared to inactivated
363 virus vaccinated individuals (Lim et al., 2021, Premikha et al., 2019), Yisimayi and colleagues
364 recently demonstrated that individuals who previously received inactivated SARS-CoV-2
365 vaccine produced robust variant-specific responses after subsequent Omicron exposures
366 (Yisimayi et al., 2023). Taken with our data and others, this suggests that inactivated virus
367 vaccines potentially establish weaker 'immunological imprints' compared to mRNA-LNP
368 vaccines.

369
370 The human immune landscape against SARS-CoV-2 is becoming more heterogenous as
371 variants emerge and infection and vaccination histories become diverse among different

372 individuals. Most humans have been ‘immunologically imprinted’ with antigens from the
373 ancestral SARS-CoV-2 strain, but that will inevitably change as time progresses. Most children
374 born today will be first introduced to SARS-CoV-2 antigens in the form of a variant infection or
375 variant vaccination, leading to the formation of different memory B cell populations compared to
376 individuals first exposed to ancestral SARS-CoV-2 antigens. Though the impact of birth year
377 imprinting on the evolution of future SARS-CoV-2 variants is unknown, there is evidence of
378 influenza epidemic virus susceptibilities being shaped by childhood exposures (Arevalo et al.,
379 2020; Gostic et al., 2016; Gostic et al., 2019). Overall, an improved understanding of how
380 SARS-CoV-2 immune history influences antibody specificity to new variants will be critical for
381 rationally designing future vaccines and mitigating disease burden.

382

383 **Limitations of this study**

384 While most individuals in these cohorts followed a similar 3-dose vaccination with mRNA-LNP
385 expressing the ancestral spike followed by BA.5 breakthrough infection, some individuals
386 experienced infections with prior variants, as shown in **Supplemental Table 1**. The XBB cohort
387 included individuals with a more diverse exposure history. We did not follow a single longitudinal
388 cohort of individuals following first and second BA.5 exposures and through subsequent
389 XBB.1.5 vaccinations, which introduces variance between all cohorts. Our antibody functionality
390 assays were limited to pseudovirus neutralization assays, which have been shown to be highly
391 correlative with live virus neutralization assays. For our BA.5 cohorts, we only measured
392 functionality of antibodies at two timepoints and specificity at a single timepoint; it is possible
393 epitope targeting continues to change over time after antigen exposure. Additional analyses of
394 late timepoints will be important to include in future studies to understand how antibody
395 specificities change over time.

396

397

398 **Acknowledgements**

399 We would like to thank the study participants for their generosity in making the study possible.
400 We also thank the Penn Cytomics and Cell Sorting Resource Laboratory for access to
401 instruments, members of the Hensley and Wherry labs for helpful discussions and feedback,
402 and the Immune Health team. This project has been funded in part with Federal funds from the
403 National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of
404 Health and Human Services, under Contract No. 75N93021C00015 (S.E.H. and E.J.W.) and
405 Grant Nos. U19AI082630 (S.E.H. and E.J.W.), AI105343 (E.J.W.), AI108545 (E.J.W.), AI155577
406 (E.J.W.), AI149680 (E.J.W.); and the Parker Institute for Cancer Immunotherapy (to EJW).
407 S.E.H. holds an Investigators in the Pathogenesis of Infectious Disease Awards from the
408 Burroughs Wellcome Fund. D.B.R. was supported by an MD fellowship of the Boehringer
409 Ingelheim Fonds.

410

411 **Author Contributions**

412 Conceptualization, T.S.J., M.M.P., E.J.W., and S.E.H.; Methodology, T.S.J., S.H.L., and M.M.P.;
413 Investigation, T.S.J., S.H.L., R.K.A., N.R.D., D.B.R., and M.M.P.; Formal Analysis, T.S.J. and
414 M.M.P.; Visualization, T.S.J. and M.M.P.; Writing – Original Draft, T.S.J. and S.E.H., Writing –
415 Review & Editing, T.S.J., S.H.L., M.M.P., E.J.W., D.C.D., and S.E.H.; Supervision, D.C.D.,
416 E.J.W., and S.E.H.; Funding Acquisition, E.J.W. and S.E.H.; Resources, E.J.W. and S.E.H.

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418 **Declaration of Interests**

419 E.J.W. is a member of the Parker Institute for Cancer Immunotherapy. E.J.W. is an advisor for
420 Arsenal Biosciences, Coherus, Danger Bio, IpiNovyx, Janssen, New Limit, Marengo, Pluto
421 Immunotherapeutics Related Sciences, Santa Ana Bio, and Synthekine. E.J.W. is a founder of
422 and holds stock in Coherus, Danger Bio, and Arsenal Biosciences.

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424 **Inclusion and Diversity**

425 We support inclusive, diverse, and equitable conduct of research.

Figure Legends

Figure 1. BA.5 breakthrough infection elicits cross-reactive antibodies evaded by XBB.1.5.

A) Schematic of participants in this study who were vaccinated 3x with the SARS-CoV-2 ancestral mRNA-LNP vaccine who then had a BA.5 breakthrough infection. B)-C) Antigen-specific IgG ELISAs were performed using sera from timepoints indicated in panel A) against ancestral, BA.5, and XBB.1.5 full-length spike (B) and RBD (C) proteins. Endpoint titers are reported as reciprocal serum dilutions. D) SARS-CoV-2 pseudotype neutralization assays were performed using sera obtained at timepoints indicated in panel A) against ancestral SARS-CoV-2, BA.5, and XBB.1.5 pseudoviruses. Values reported are focus reduction neutralization test (FRNT) 50, or reciprocal serum dilution at which <50% viral input foci are observed. E) Neutralization potency was calculated by dividing FRNT50 values by spike IgG titer. F-G) Antigen-specific IgG ELISAs were performed using T2 absorbed sera and ELISA plates coated with the ancestral spike (F) or ancestral RBD (G). H) SARS-CoV-2 pseudotype neutralization assays were performed using T2 absorbed serum and ancestral SARS-CoV-2 pseudovirus. I-J) Antigen-specific IgG ELISAs were performed using T2 absorbed and ELISA plates coated with BA.5 spike (I) and BA.5 RBD (J). K) Neutralization assays were performed using T2 absorbed serum and BA.5 pseudotyped virus. For all, individual points are average of $n = 2$ technical replicates. Red/black bars indicate geometric mean. Wilcoxon signed-rank test with benjamini-hochberg correction for multiple testing. All comparisons to timepoint 1 or mock absorption. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Secondary BA.5 exposure elicits cross-reactive antibodies that bind weakly to XBB.1.5.

A) Schematic of participants in this study who were vaccinated 3x with the SARS-CoV-2 ancestral mRNA-LNP vaccine who then had two BA.5 exposures. B)-C) Antigen-specific IgG ELISAs were performed using sera from timepoints indicated in panel A) against ancestral,

BA.5, and XBB.1.5 full-length spike (B) and RBD (C). Endpoint titers are reported as reciprocal dilutions. D) SARS-CoV-2 pseudotype neutralization assays were performed using sera obtained at timepoints indicated in panel A) against ancestral SARS-CoV-2, BA.5, and XBB.1.5 pseudoviruses. Values reported are focus reduction neutralization test (FRNT) 50, or reciprocal serum dilution at which <50% viral input foci are observed. E) Neutralization potency was calculated by dividing FRNT50 values by spike IgG titer. F)-G) Antigen-specific IgG ELISAs were performed using T2 absorbed sera and ELISA plates coated with the ancestral spike (F) or ancestral RBD (G). H) SARS-CoV-2 pseudotype neutralization assays were performed using T2 absorbed serum and ancestral SARS-CoV-2 pseudovirus. I-J) Antigen-specific IgG ELISAs were performed using T2 absorbed sera and ELISA plates coated with BA.5 spike (I) and BA.5 RBD (J). K) Neutralization assays were performed using T2 absorbed serum and BA.5 pseudotyped virus. For all, Individual points are average of n = 2 technical replicates. Red/black bars indicate geometric mean. Wilcoxon signed-rank test with benjamini-hochberg correction for multiple testing. All comparisons to timepoint 1 or mock absorption. * p<0.05, ** p<0.01.

Figure 3. Cross-reactive B cells are recruited in response to primary and secondary BA.5 exposures in vaccinated individuals. A) Representative flow-cytometry plot showing gating strategy for probe positive memory B cells. B) The percent of ancestral RBD-binding B cells that cross-bind to BA.5 RBD before and after first (left) and second (right) exposure to BA.5. C) The percent of ancestral RBD and BA.5 RBD cross-binding B cells that also cross-bind to XBB.1.5 RBD before and after first (left) and second (right) exposure to BA.5. D) The percent of ancestral RBD-binding B cells that cross-bind BA.5 and XBB.1.5 RBD before and after first (left) and second (right) exposure to BA.5. E-F) The frequency of BA.5 RBD-binding (E) and XBB.1.5 RBD-binding (F) B cells that do not cross-react with ancestral RBD before and after first (left) and second (right) exposure to BA.5.

Figure 4. Antibodies elicited by successive ancestral SARS-CoV-2 and BA.5 exposures target conserved residues that are mutated in the XBB.1.5 RBD. A) Ancestral RBD structure with residues that are different in BA.5 are highlighted in purple, and residues conserved between ancestral and BA.5 but different in XBB.1.5 are highlighted in teal (PDB 6M0J). B) List of recombinant mutations created for this study using the BA.5 spike backbone. C) IgG ELISA using T2 breakthrough sera after absorption with single mutants and those listed in panel B. D) IgG ELISA using T2 sera from individuals exposed twice with BA.5 after absorption with single mutants and those listed in panel B. E,F) Neutralization assays were performed using T2 absorbed serum from single (E) and two (F) BA.5 exposures using BA.5 pseudotype virus. For all, individual points are average of $n = 2$ technical replicates. Black bars indicate geometric mean. Wilcoxon signed-rank test with benjamini-hochberg correction for multiple testing. All comparisons to mock absorption. * $p < 0.05$, ** $p < 0.01$.

Figure 5. Primary and secondary XBB exposures elicit mostly antibodies that cross-react to the ancestral SARS-CoV-2 spike. A) Schematic of participants in this study who were exposed to XBB. B)-C) Antigen-specific IgG ELISAs were performed using sera from timepoints indicated in panel A) against ancestral and XBB.1.5 full-length spike (B) and RBD (C). Endpoint titers are reported as reciprocal dilutions. D) Neutralization assays were performed using serum obtained at timepoints indicated in panel A) against ancestral and XBB.1.5 SARS-CoV-2 pseudoviruses. Values reported are focus reduction neutralization test (FRNT) 50, or reciprocal serum dilution at which $< 50\%$ viral input foci are observed. E) Neutralization potency was calculated by dividing FRNT50 values by spike IgG titer. F),H) Antigen-specific IgG ELISAs were performed using sera (collected 45 days after XBB1.5 exposure) after absorption with ancestral or XBB.1.5 spike proteins. G),I) SARS-CoV-2 pseudotype neutralization assays were performed using sera (collected 45 days after XBB.1.5 exposure) after absorption with ancestral or XBB.1.5 spike proteins. J) Schematic of participants in this study who were exposed twice to XBB. K) SARS-

CoV-2 pseudotype neutralization assays were performed using sera obtained at timepoints indicated in panel J) using ancestral and XBB.1.5 pseudoviruses. L),M) Antigen-specific IgG ELISAs and N) SARS-CoV-2 neutralization assays were performed using sera (collected 45 days after each XBB.1.5 exposure) after absorption with ancestral or XBB1.5 spike proteins. For all, Individual points are average of $n = 2$ technical replicates. Red/black bars indicate geometric mean. Wilcoxon rank-sum test with benjamini-hochberg correction for multiple testing. All comparisons to timepoint 1 or mock absorption. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Cross-reactive B cells dominate immune responses elicited by XBB, but variant-specific responses are observed in individuals with low pre-existing immunity. A-B) The percent of total B cells that bind A) ancestral RBD and B) XBB.1.5 RBD before and after exposure to XBB.1.5. C) The percent of ancestral RBD-binding B cells that cross-bind to XBB.1.5 RBD before and after exposure to XBB.1.5. D) The fold expansion of B cells that bind XBB.1.5 RBD but not ancestral RBD (XBB.1.5-specific) following XBB.1.5 exposure. E) The percent of total XBB.1.5 RBD-binding B cells that do not bind ancestral RBD. F) Representative flow cytometry plots depicting longitudinal changes in CD71 expression on different RBD-binding B cell populations from a single individual. G) Summary data of median CD71 expression on the indicated RBD-binding B cell populations at 0, 7, 15, and 45 days post-XBB exposure. H) Summary data of the isotype distribution of RBD-binding B cells at 0, 7, 15, and 45 days post-XBB exposure. I-J) The percent of total B cells that are CD38+CD27+ plasmablasts and I) bind XBB.1.5 RBD or J) bind XBB.1.5 RBD and do not bind ancestral RBD (XBB.1.5-specific). K-M) Correlations within B cell and antibody responses. K) Correlation of day 7 fold change in XBB.1.5-specific B cells as in panel D with day 0 percent of total B cells that bind ancestral RBD (left); day 0 percent of total B cells that bind XBB.1.5 RBD (center); day 0 XBB.1.5 spike-binding antibodies by ELISA (right). L) Correlations of ancestral spike absorbed XBB.1.5 neutralizing antibody titers at day 45 (as in Fig. 5N) with day 0 XBB.1.5 spike-binding antibodies quantified

by ELISA (left), and the percent of total B cells that bind XBB.1.5 RBD but not ancestral RBD at day 7 (right). M) Correlation of overall day 45 XBB.1.5 neutralizing antibody titers (unabsorbed) with the day 0 percent of ancestral RBD+ B cells that cross-bind XBB.1.5 RBD; the day 15 percentage of total B cells that bind XBB.1.5 RBD, the day 15 percentage of total B cells that bind XBB.1.5 RBD but do not bind ancestral RBD (XB.1.5-specific), and the day 15 fold change in XBB-specific B cells as in D. Points represent individual subjects and thin lines indicate individual subjects sampled longitudinally. Horizontal bars represent means. Statistics were calculated using two-sided Wilcoxon test with Benjamini-Hochberg correction for multiple comparisons. Statistics without brackets are in comparison to day 0. Correlation statistics were calculated using Spearman rank correlation and are shown with Pearson trend lines for visualization.

Methods

Human Subject Recruitment and Sampling

Human subjects were recruited for this study for longitudinal sampling before and after either mRNA booster vaccination or SARS-CoV-2 breakthrough infection (IRB#851465). Full cohort and demographic information are provided in **Supplemental Table 1**. Additional healthy donor PBMC samples were collected with approval from the University of Pennsylvania Institutional Review Board (IRB# 845061). For many individuals, baseline samples were acquired prior to SARS-CoV-2 infection or vaccination but at least 2 months after their most recent infection or vaccine dose. These samples are labeled as day 0 throughout the manuscript. Samples were collected on days 7, 15, and 45 based on the date of booster vaccination or the reported date of first symptom onset (IRB#851465). Prior SARS-CoV-2 infection status was determined by self-reporting. All participants were otherwise healthy, with no self-reported history of chronic health conditions, and none were hospitalized during SARS-CoV-2 infection. Peripheral blood samples (30-100mL) and clinical questionnaire data were collected at each study visit.

Peripheral blood sample processing

Following standard phlebotomy procedures, 30-100mL of venous blood was collected into sodium heparin tubes. Plasma was separated by centrifugation at 1800xg for 15 minutes, aliquoted, stored at -80°C , and heat inactivated for 30 minutes at 56°C prior to binding and neutralizing antibody analyses. After removing plasma, the remaining fractions were diluted with RPMI + 1% FBS + 2mM L-Glutamine + 100 U Penicillin/Streptomycin (R1) to achieve a final volume double that of the original whole blood. The diluted blood was then layered over 15mL lymphoprep gradients (STEMCELL Technologies) in SEPMATE tubes (STEMCELL Technologies) and spun at 1200g for 10 minutes. The peripheral blood mononuclear cell (PBMC) fraction was harvested from SEPMATE tubes, washed once with R1 and pelleted, and

cell pellets were resuspended in ACK lysis buffer (Thermo Fisher). After 5 minutes of red blood cell lysis at room temperature, the lysis was quenched by adding R1. PBMCs were then filtered through a 70µm cell strainer and counted using a Countess automated cell counter (Thermo Fisher). Aliquots containing 10×10^6 PBMCs were cryopreserved in 90% FBS 10% DMSO and stored at -80°C for later flow cytometric analyses.

Recombinant Protein Production and Expression

SARS-CoV-2 full-length spike or RBD plasmids were transfected into 293F cells at 1×10^6 cells/mL at a $1 \mu\text{g} : 1 \text{mL}$ ratio. Cells were incubated for 6 days (full-length spike) or 4 days (RBD) at 37°C and 150 RPM before purification. To purify, cultures were centrifuged at $3200 \times g$ for 6 minutes to clarify supernatant. Ni-NTA resin (Qiagen) was resuspended and 5mL was transferred into a 50mL conical for each flask being purified. Volume was brought to 50mL with sterile PBS and conical tubes were centrifuged for 10 minutes at $3200 \times g$. After centrifugation, excess PBS was removed, and resin was added to clarified supernatant. Resin-supernatant mixtures were incubated for 2 hours at 4°C and 220 RPM. After incubation, resin-supernatant was added to gravity columns (Bio-rad), then washed 4X with wash buffer (50 mM NaHCO_3 , 300 mM NaCl, 20 mM imidazole, pH 8) and eluted in a total volume of 8 mL elution buffer (50 mM NaHCO_3 , 300 mM NaCl, 300 mM imidazole, pH 8). Eluted protein was concentrated and PBS buffer exchanged using 10kDa (RBD) or 30kDa (full-length spike) centrifugal filters (Sigma-Aldrich). Protein concentrations were measured by BCA assay (ThermoFisher).

Carboxyl-Magnetic Bead Coupling and Serum Absorptions

Recombinant spike proteins were coupled to carboxyl magnetic beads (Ray-biotech) at a ratio of $35 \mu\text{g}$ antigen to $100 \mu\text{L}$ beads. The bead-antigen mixture was vortexed then incubated at 4°C with rocking for 2 hours. The unbound fraction was removed by placing beads to a magnetic stand, and beads were quenched by incubating in 50 mM Tris, pH 7.4 for 15 minutes

at RT with rocking. After 15 minutes, beads were placed back on the magnet to remove the quenching buffer. Beads were washed 4x with wash buffer (DPBS supplemented with 0.1% BSA and 0.05% Tween-20). For each wash, 1 bead volume of wash buffer was added to the beads, vortexed, then placed back on the magnet. Beads were finally resuspended in 1 bead-volume of wash buffer before storage at 4°C.

For absorptions, sera were first diluted 1:35.7 with sterile DPBS and then brought to 1:50 dilution with the addition of recombinant protein-coupled beads at a final bead:sera volume ratio of 1:2. The bead-sera mixture was vortexed, then shaken at 1100 RPM for 1 hour at RT. Bead-sera mixtures were then placed on a magnet for separation and unabsorbed fractions were removed and transferred to a clean tube.

Antigen-specific ELISA

Antigen-specific ELISAs were performed as previously described (Anderson et al., 2021). Briefly, high-binding plates (ThermoFisher) were coated with 2 µg/mL of recombinant protein or with DPBS to control for background antibody binding at 4°C overnight. The next day, plates were washed 3x with PBS containing 0.1% Tween 20 (PBS-T) and blocked for 1 hour with 200µL blocking buffer (DPBS supplemented with 3% milk powder and 0.1% tween-20). Plates were then washed 3x with PBS-T, and 50µL serum diluted in dilution buffer (DPBS supplemented with 1% milk and 0.1% Tween-20) were added. After 2 hours of incubation, plates were washed 3x with PBS-T and 50 µL goat anti-human IgG HRP conjugate (Jackson) was added to each well and allowed to incubate for 1 hour. Plates were washed 3x with PBS-T, then 50 µL SureBlue 3,3',5,5'-tetramethylbenzidine substrate (SeraCare) was added to each well. The reaction was allowed to incubate in the dark for 5 minutes before addition of 25 µL 250 mM hydrochloric acid stop solution. Plates were shaken to distribute solution and read at OD450nm immediately using a Spectramax plate reader (Molecular Devices). Serum antibody titers were

obtained from a standard curve of serially diluted pooled serum (starting dilution 1:100).

Standard curves were included on all plates for plate-to-plate variation. PBS controls were included for each sample dilution plate to account for nonspecific binding. Antibody titers for each sample were measured in two technical replicates performed on separate days.

SARS-CoV-2 Pseudovirus Production

SARS-CoV-2 pseudotyped vesicular stomatitis virus (VSV) were produced as previously described (Anderson et al., 2021; Goel et al., 2021). Briefly, 293T cells were seeded at 3.5×10^6 cells in collagen-coated 10 cm tissue culture dish, incubated for 24 hours, and transfected using calcium phosphate with 25 μ g of SARS-CoV-2 variant plasmids encoding a codon optimized SARS-CoV-2 spike gene with an 18-residue truncation in the cytoplasmic tail. After 24 hours, the SARS-CoV-2 Spike-expressing cells were infected for 2-4 hours with VSV-G pseudotyped VSV Δ G-RFP at an MOI of ~2-4. Virus-containing media was removed, and the cells were re-fed with media. VSV Δ G-RFP SARS-CoV-2 pseudotypes were harvested 24-28 hours after infection, clarified by centrifugation twice at 6000xg, then aliquoted and stored at -80°C .

Quantification of SARS-CoV-2 pseudotype neutralizing antibody titers

Focus reduction neutralization titers were determined as previously described. Briefly, 96-well collagen-coated plates were seeded with 2.5×10^4 TMPRSS2 expressing VeroE6 cells per well the night prior. The next day, heat-inactivated sera or post-absorption sera were serially diluted 2-fold and mixed with 200-300 focus forming units per well of VSV Δ G-RFP SARS-CoV-2 pseudotype viruses. Media used for serum and virus dilution contained 600 ng/mL of an anti-VSV-G antibody 1E9F9 to ensure neutralization of any VSV-G carryover virus from pseudotype production. The serum-virus mixture was incubated for 1 hour at 37°C before being plated on VeroE6 TMPRSS2 cells. After 22 hours of incubation at 37°C , plates were washed with DPBS

and fixed with 4% paraformaldehyde. The spots were visualized and counted on an S6 FluoroSpot Analyzer (CTL). The focus reduction neutralization titer 50% (FRNT50) was determined as the greatest serum dilution at which focus count was reduced by at least 50% relative to control wells infected with only pseudotype viruses in the absence of human serum. FRNT50 titers for each sample were measured in two technical.

SARS-CoV-2-specific B cell flow cytometric analyses

Antigen-specific B cells were detected using biotinylated proteins in combination with different streptavidin (SA)–fluorophore conjugates as described (Goel et al., 2021). All reagents are listed in **Supplemental Table 2**. For each sample, biotinylated proteins were multimerized with fluorescently labeled streptavidin (SA) for 1.5 h at 4 °C at the following ratios, all of which are ~4:1 molar ratios calculated relative to the SA-only component irrespective of fluorophore: 200 ng full-length spike protein was mixed with 20 ng SA-BV421, 30 ng NTD was mixed with 12 ng SA-BV786, 25 ng WT RBD was mixed with 12.5 ng SA-PE, 25 ng XBB.1.5 RBD was mixed with 12.5 ng SA-BV711, 25 ng XBB.1.5 RBD was mixed with 12.5 ng SA-PE-Cy7, 25 ng BA.5 RBD was mixed with 12.5 ng SA-BUV615, 25 ng BA.5 was mixed with 12.5 ng SA-APC, 50 ng S2 was mixed with 12 ng SA-BUV737, and 50 ng nucleocapsid protein was mixed with 14 ng SA-BV605. In total, 12.5 ng SA-PE-Cy5 was used as a decoy probe without biotinylated protein to gate out cells that nonspecifically bound SA. All experimental steps were performed in a 50/50 mixture of PBS + 2% FBS and Brilliant buffer (BD Bioscience). Antigen probes were prepared individually and combined after multimerization with 5 µM free D-biotin (Avidity) to minimize potential cross-reactivity between probes. For staining, 2–10 × 10⁶ PBMCs per sample were thawed from cryopreservation and prepared in a 96-well round-bottom plate. Cells were first stained with Fc block (BioLegend, 1:200) and Ghost Violet 510 Viability Dye (Tonbo) for 15 min at 4 °C. Cells were then washed and stained for 1 h at 4 °C with 50 µl antigen probe master mix containing the above probes combined immediately before staining. Following this incubation period, cells were

washed again and stained with anti-CD27-BUV395, anti-CD3-BUV563, anti-CD38-BUV661, anti-IgD-BV480, anti-CD19-BV750, anti-IgA-FITC, anti-CD21-PE-CF594, anti-IgG-AF700 and anti-CD71-APC-H7 for 30 min at 4 °C. After surface stain, cells were washed and fixed in 1× Stabilizing Fixative (BD Biosciences) overnight at 4 °C.

Data Analysis

All data were analyzed using Graphpad Prism version 10.1.1 or R version 4.3.2 and visualized using R studio. PyMOL version 2.5.5 was used to visualize ancestral SARS-CoV-2 RBD.

Custom R scripts are available upon request.

References

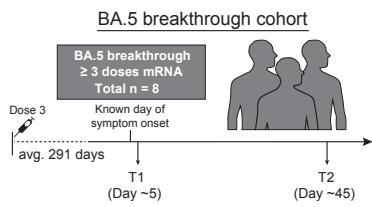
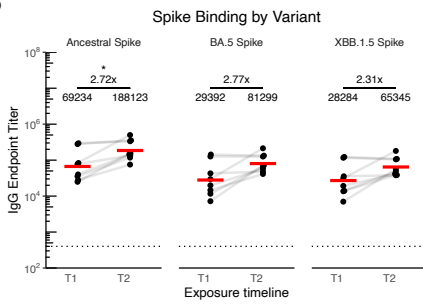
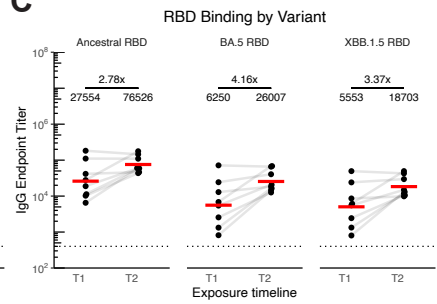
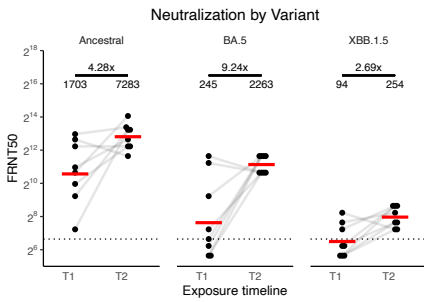
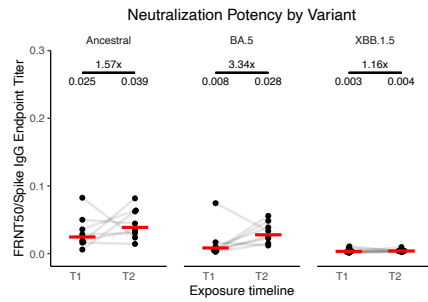
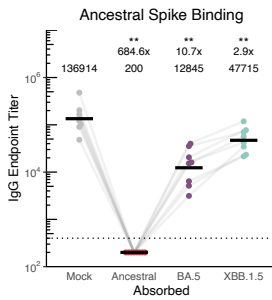
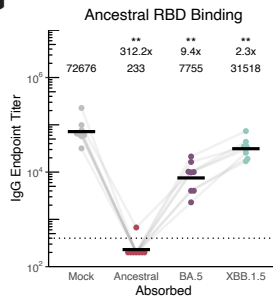
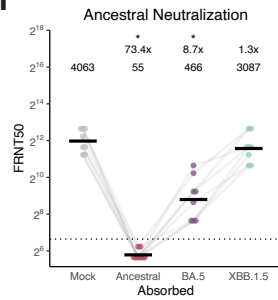
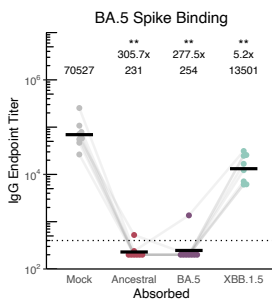
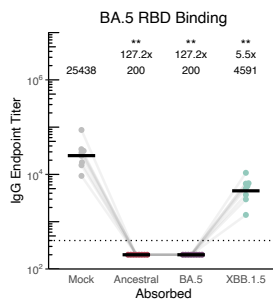
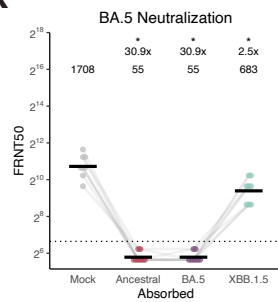
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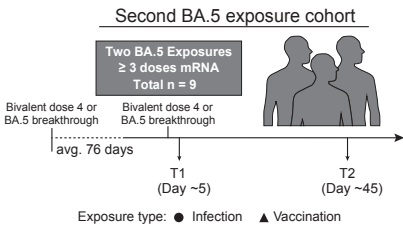
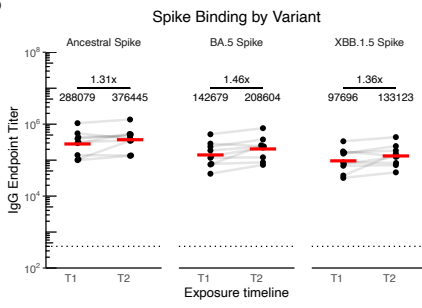
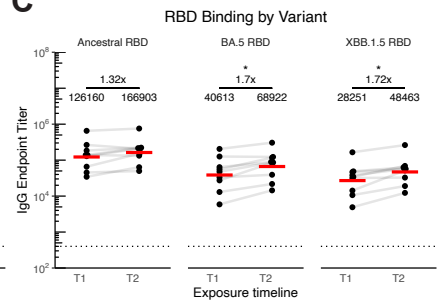
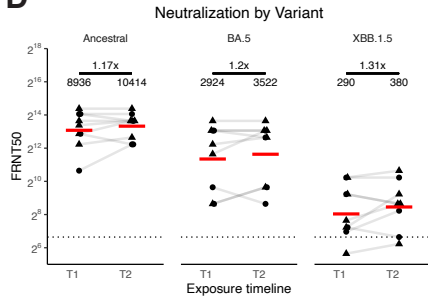
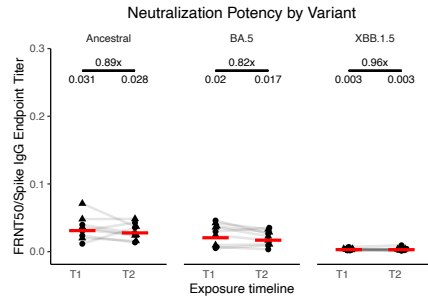
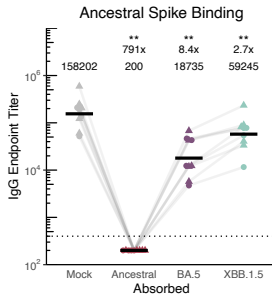
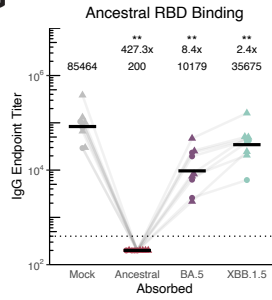
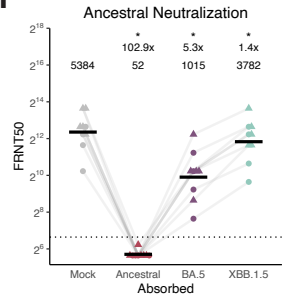
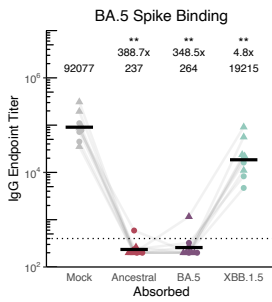
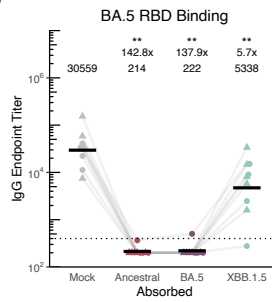
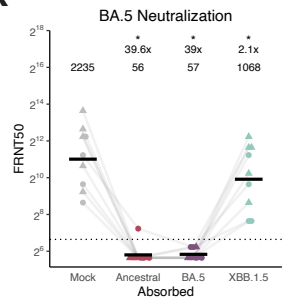
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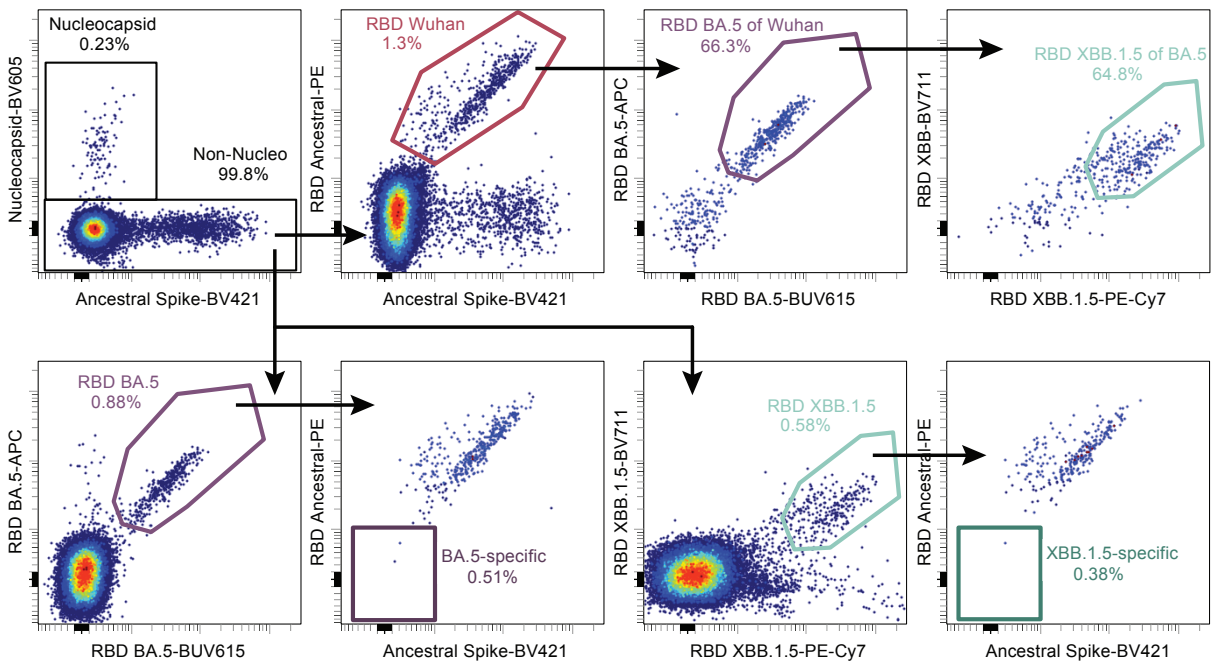
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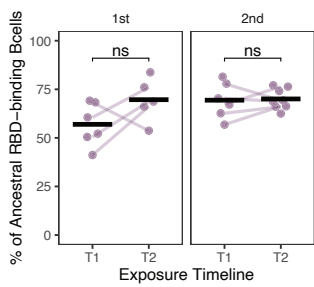
1A**B****C****D****E****F****G****H****I****J****K**

2A**B****C****D****E****F****G****H****I****J****K**

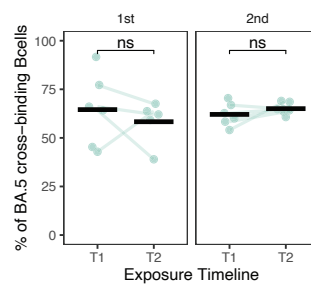
3A Memory B cells (CD3- CD19+ IgD- CD38-)



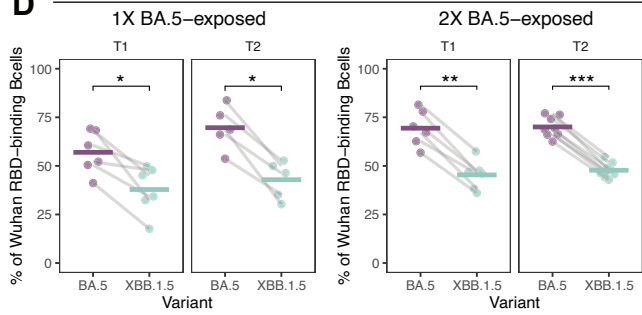
B %BA.5-binding of Ancestral



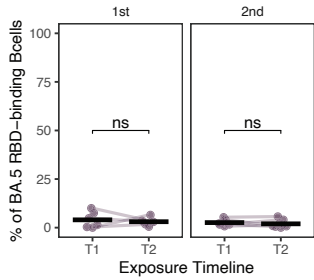
C %XBB.1.5-binding of BA.5



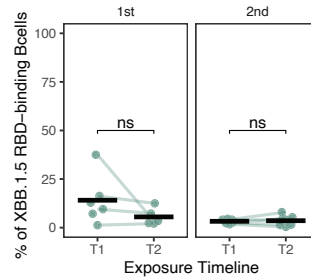
D Variant cross-binding

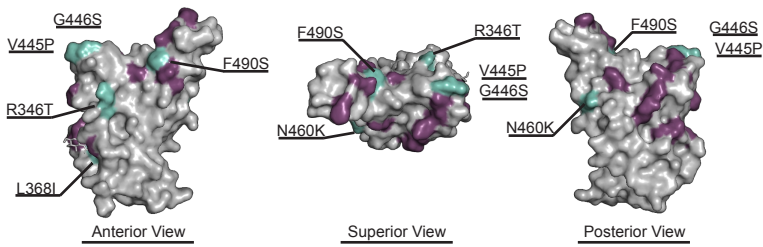


E %BA.5-specific

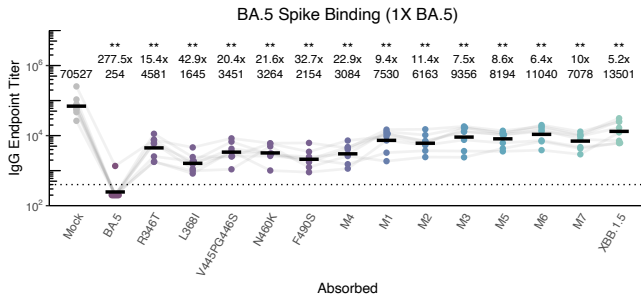
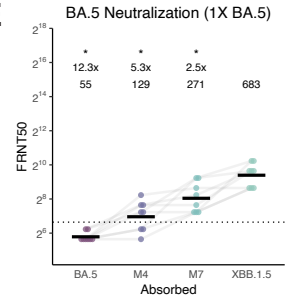
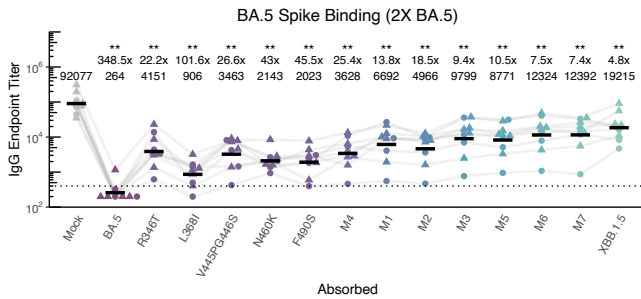
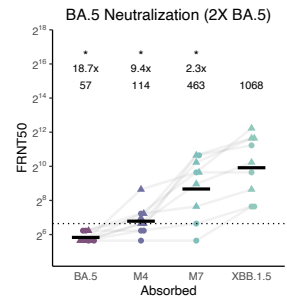


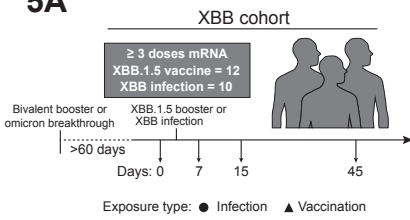
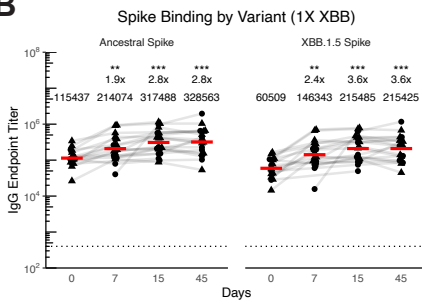
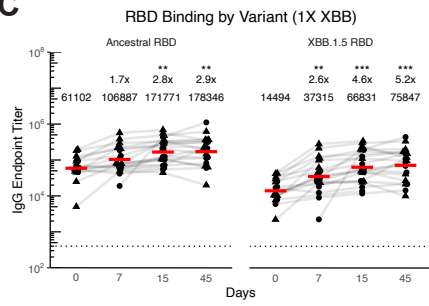
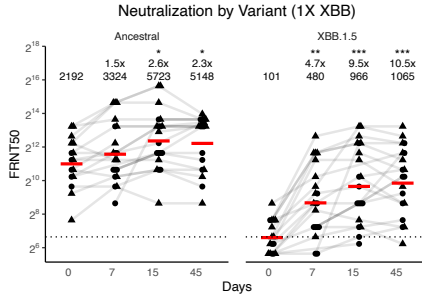
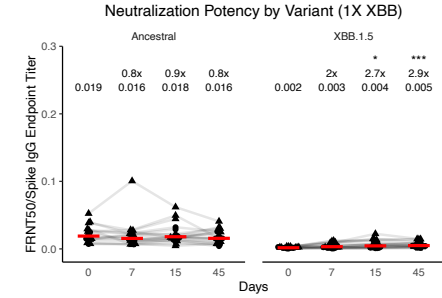
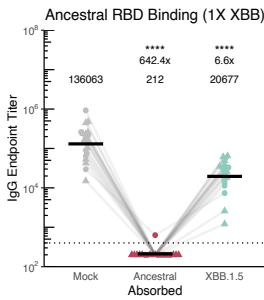
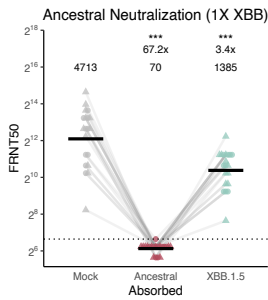
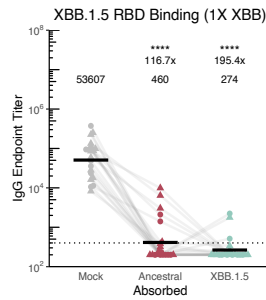
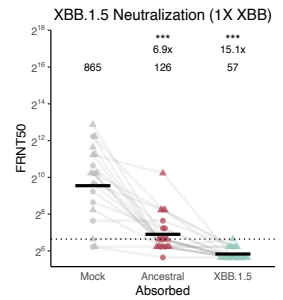
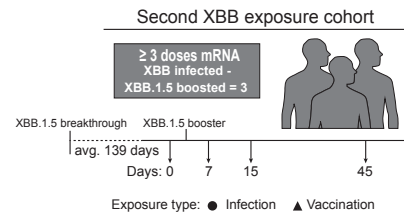
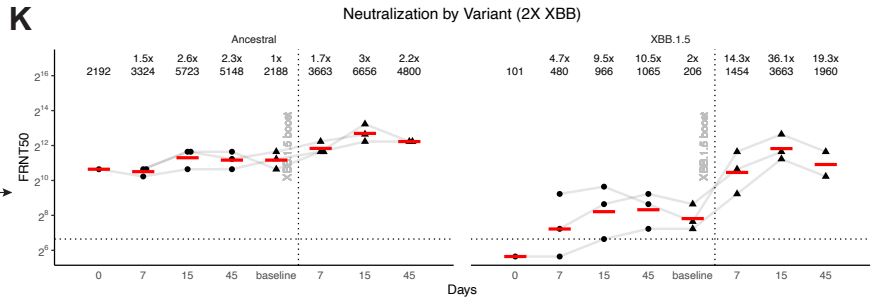
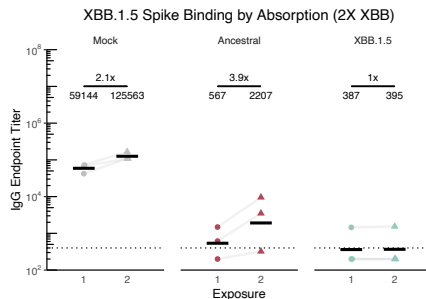
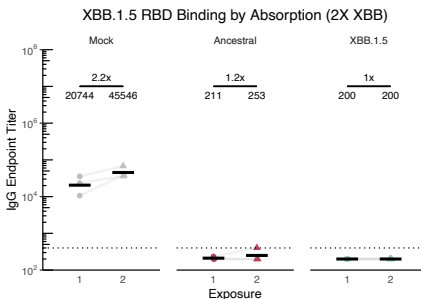
F %XBB.1.5-specific



4A**B**

Mutant ID	R346T	L386I	V445P	G446S	N460K	F490S
M1						
M2						
M3						
M4						
M5						
M6						
M7						

C**E****D****F**

5A**B****C****D****E****F****G****H****I****J****K****L****M****N**