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1 **Title Page**

2 **Title: Anamnestic Humoral Correlates of Immunity Across SARS-CoV-2 Variants of**
3 **Concern**

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24 Keywords: Vaccines, COVID-19, breakthrough, SARS-CoV-2, Omicron, Delta, antibodies

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27 **Abstract**

28 While immune correlates against SARS-CoV-2 are typically defined at peak
29 immunogenicity following vaccination, immunologic responses that expand selectively
30 during the anamnestic response following infection can provide mechanistic and detailed
31 insights into the immune mechanisms of protection. Moreover, whether anamnestic
32 correlates are conserved across VoCs, including the Delta and more distant Omicron
33 variant of concern (VoC), remains unclear. To define the anamnestic correlates of
34 immunity, across VOCs, we deeply profiled the humoral immune response in individuals
35 recently infected with either the Delta or Omicron VoC. While limited acute N-terminal
36 domain and RBD-specific immune expansion was observed following breakthrough, a
37 significant immunodominant expansion of opsinophagocytic Spike-specific antibody
38 responses focused largely on the conserved S2-domain of SARS-CoV-2 was observed 1
39 week after breakthrough infection. This S2-specific functional humoral response
40 continued to evolve over 2-3 weeks following both Delta and Omicron breakthrough
41 infection, targeting multiple VoCs and common coronaviruses. These responses were
42 focused largely on the fusion peptide 2 and heptad repeat 1, both associated with
43 enhanced rates of viral clearance. Taken together, our results point to a critical role of
44 highly conserved, functional S2-specific responses in the control of SARS-CoV-2
45 infection, across VOCs, and thus humoral response linked to virus attenuation can guide
46 next-generation generation vaccine boosting approaches to confer broad protection
47 against future SARS-CoV-2 VoCs.

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48 **Introduction**
49
50 Despite the remarkable vaccine efficacy observed in phase 3 SARS-CoV-2
51 vaccine trials, the waning of vaccine-conferred immunity and the emergence of
52 neutralizing antibody-resistant variants of concern (VoCs), such as the Delta (B.1.612)
53 and Omicron (B.1.529), led to a rapid increase in transmission events globally (*1-4*). Yet,
54 severe disease and death did not increase concomitantly suggesting that additional post-
55 transmission blocking immune responses contribute to control and clearance of infection
56 once it has occurred (*5*). However, the precise immunologic correlates of immunity,
57 following breakthrough infections remain incompletely defined. Moreover, whether these
58 correlates differ across VOCs, that exhibit striking differences in sequence, is unclear.
59 Neutralizing antibody responses were tightly linked to protective immunity in early
60 mRNA vaccine phase 3 trials, at a time when the dominant circulating strain was largely
61 matched to the vaccine antigen-insert sequence (*6-8*). However, with the introduction of
62 more neutralization-resistant VoCs, the predictive power of neutralization diminished (*2*).
63 Despite VoC evasion of neutralization (*9-11*), both T-cells (*12*) and binding antibodies
64 (*13*) were proposed as alternate immune mechanisms that could mediate post-treatment
65 control and clearance of infection. Post-challenge correlates analyses in non-human
66 primate vaccine models pointed to a rapid humoral anamnestic response, linked to the
67 rapid expansion of antibody-secreting cells within the respiratory tract, giving rise to
68 robust renewed pools of antibodies that may contribute to control and clearance of
69 infection (*14*). Yet, whether these antibodies contribute to attenuation of disease via the
70 simple neutralization and blockade of further spread or via the recruitment of the antiviral
71 activity of the local immune system, via Fc-effector functions, is unknown. Moreover,
72 whether the specificity and functional activity of the anamnestic response that evolves
73 following VoC infections are conserved may point to common or distinct mechanisms of
74 attenuation of disease.
75 Thus, while vaccine-induced immune correlates of protection are most often
76 focused on the identification of immunologic responses at peak-immunogenicity, these
77 immunologic markers may have limited consequence as mechanistic correlates of
78 immunity, as these responses may wane at the time of environmental exposure.
79 Conversely, immunologic signatures following exposure may provide critical insights into
80 the anamnestic response that is key to control and clearance of the infection. Thus, here
81 we deeply profiled the humoral immune response in a cohort of individuals with a recent,
82 documented Delta or Omicron SARS-CoV-2 infection. Systems Serology profiling
83 revealed a rapid expansion of Fc-receptor binding and opsinophagocytic humoral immune
84 responses across the VoC breakthroughs with a consistent preference for expansion for
85 the S2 subdomain of Spike, focused on the fusion peptide 2 and heptad repeat 1, that
86 tracked with enhanced viral clearance. These data point to a critical role for S2-specific
87 immunity as a key correlate of immunity across VoCs breakthrough infections.

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Results

Breakthrough COVID-19 Elicits Spike Sub-domain Humoral Responses

The perpetual emergence of new SARS-CoV-2 variants of concern have led to repeated waves of viral breakthrough infections, even in recently vaccinated individuals (*5*). However, vaccine-induced immunity continues to provide protection against severe disease and death, as evidenced by the severe disease caused by both Delta and Omicron preferentially in unvaccinated populations (*4, 15-17*). Emerging data point to the potential importance of the anamnestic response as a key contributor to the resolution of infection (*18*). Given the association between non-neutralizing antibody effector profiles and natural resolution of severe disease (*19*), we performed systems serology on sera from individuals who had completed their vaccine series and had subsequent documented Delta (n = 37) or Omicron (n = 23) VoC breakthrough, both 1 week and 2-3 weeks post-infection (means = 6.3 ± 2.8, and 18.8 ± 2.9 days, respectively) aimed at defining the specific humoral properties associated with the resolution of infection. All individuals had received the primary 2 dose series of an mRNA vaccine (Pfizer/BNT162b2 = 24, and Moderna mRNA-1273 = 16). Delta and Omicron breakthroughs ranged from 5 – 357 days from vaccination and 0 – 12 days from symptom onset. Given the significant antigenic distance between Delta and Omicron within the Spike protein, we also sought to define whether anamnestic correlates were consistent across VoCs (*20*).

The rapid expansion of Spike-specific, receptor-binding domain (RBD) and N-terminal domain (NTD) specific immune responses have been proposed as potential acute anamnestic correlates of immunity following vaccine breakthrough. However, no expansion was observed in IgG responses to the full-length Spike-specific, NTD, or RBD within the first week following breakthrough infection. Moreover, *de novo* IgM, IgG3, and IgA responses to full Spike were also not observed (**Figure 1A-C**). Conversely, IgM responses expanded to the conserved S2 domain of the Spike antigen within the first week following both Delta and Omicron breakthroughs. Interestingly, S2-specific IgA also expanded following Delta (**Figure 1D**), but not Omicron breakthrough. These data point to an unexpected, and selective expansion of *de novo* humoral immune responses to the highly conserved S2 domain of the Spike antigen as a key correlate of immunity following vaccine breakthrough infection.

After 2-3 weeks, a broader expansion was observed in the full Spike-specific IgG1 response compared to uninfected vaccinees in both Delta and Omicron breakthrough infection. Spike-specific IgG3 and IgA responses increased compared to the initial post-breakthrough timepoint (**Figure 1A**) but were not higher than levels observed in controls. A similar IgG1 RBD-specific expansion was observed across both Delta and Omicron cases after 2-3 weeks of infection (**Figure 1B**). Limited expansion was observed in NTD across isotypes and was exclusively observed in Delta breakthroughs (**Figure 1C**), likely associated with the greater conservation in NTD across the vaccine insert and the Delta NTD sequence. Conversely, 2-3 weeks post-breakthrough, a highly significant expansion was observed in the S2-specific response in IgG1, IgG3, IgM, and IgA1, over time as well as compared to uninfected controls (**Figure 1D**). These data point to a highly selective and preferential continued anamnestic maturation of the S2-specific response following both Delta and Omicron breakthrough infections in vaccinees.

Binding antibodies alone do not mediate immunologic clearance, thus we next probed whether the anamnestic expansion of antibodies also possessed the ability to bind

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134 to Fc-receptors (FcR), key to leveraging non-neutralizing innate immune effector
135 functions (*21, 22*). Interestingly, after 7 days of infection, FcR binding antibodies did not
136 emerge to most regions of the Spike antigen. However, a significant Spike-specific
137 FcγR3B binding response was detected in Delta breakthroughs (**Figure 1E**), and a trend
138 towards significance was noted to the S2-domain in both Delta and Omicron breakthrough
139 cases (**Figure 1H**).
140 However, 2-3 weeks following breakthrough, Spike-specific antibodies able to bind
141 to FcRs expanded in both individuals that experienced a Delta and Omicron breakthrough
142 (**Figure 1E**). An expansion of activating, opsinophagocytic FcγR2A and inhibitory FcγR2B
143 binding RBD-specific antibodies was observed in both Delta and Omicron breakthroughs
144 (**Figure 1F**). A more limited expansion of inhibitory FcγR2B and neutrophil-specific
145 FcγR3B binding NTD-specific antibodies was observed (**Figure 1G**). Conversely, S2-
146 specific FcR binding expanded highly significantly across all FcRs (**Figure 1H**).
147 Collectively, these data point to an expansion of FcR binding to several Spike
148 subdomains, including a selective expansion across isotype, subclass, and FcR binding
149 to the highly conserved S2-domain of the Spike antigen (Supplementary Figure 1).
150

151 *Recognition of S2 is Expanded in Breakthrough Cases*

152 To next directly compare the extent of the anamnestic expansion across Spike
153 domains across the breakthrough cases, we examined the fold increase in IgG1 levels
154 across subdomains of Spike, VoCs, and common human coronaviruses (HCoVs). While
155 humoral immune responses increased to all domains in Delta breakthroughs a clear and
156 more significant expansion was observed in S2-specific IgG1 titers (**Figure 2A**). A similar
157 highly significant expansion was observed in S2-specific IgG1 responses in Omicron
158 breakthroughs; however a more limited expansion was observed in RBD and NTD-
159 specific responses, likely due to greater sequence disparity between the original vaccine
160 antigen and Omicron within these domains (**Figure 2B**). These data point to S2 as the
161 immunodominant anamnestic target of the humoral immune response following Delta and
162 Omicron breakthrough.
163 Comparison of the breadth of the fold anamnestic expansion across Spike VoCs
164 pointed to an overall similar expansion of response to all VoCs in Delta breakthrough
165 cases (**Figure 2C**), with the exception of the Alpha variant Spike-specific response that
166 expanded preferentially. Conversely, all VoC Spike-specific responses expanded
167 significantly in Omicron breakthrough infection (**Figure 2D**), likely due to the highly
168 divergent nature of the Omicron spike that drove enhanced B cell recruitment and affinity
169 maturation. Moreover, analysis of responses to two common human coronaviruses
170 (HCoVs), α (229E) and β (OC-43), revealed a selective expansion of cross-β-CoV
171 immunity in Delta and Omicron breakthrough cases, likely due to the closer phylogenetic
172 relation of β-CoV to SARS-CoV-2, particularly in the S2-domain (*23*) (**Figure 2E-F**).
173 Moreover, analysis of the overall humoral coordination within the SARS-CoV-2 responses
174 revealed similar expansion profiles across Delta and Omicron breakthroughs
175 (Supplementary Figure 2). Thus, overall, breakthroughs of Delta or Omicron VoCs are
176 associated with similar anamnestic immunity, marked by a novel expansion of cross-VoC
177 and β-CoV immunity, likely directed at the conserved S2 region of SARS-CoV-2.
178

179 *S2 is the Subdomain that Drives Humoral Expansion Post-breakthrough*

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180 To next define a minimal multivariate signature of Delta or Omicron breakthrough
181 infection among vaccinated individuals, we performed partial least squares discriminant
182 analysis (PLS-DA) on antibody responses collected in breakthrough cases 2-3 weeks
183 following infection. Significant heterogeneity was observed in multivariate antibody
184 profiles across Delta (**Figure 3A**) and Omicron (**Figure 3B**) breakthrough cases
185 compared to uninfected vaccinated controls. In fact, vaccinated controls demonstrated a
186 highly homogeneous antibody profile, with controls consistently clustering in a small area
187 of the multivariate space in both comparisons (**Figure 3A-B**). Conversely, both Delta
188 breakthrough (**Figure 3A**) and Omicron breakthrough (**Figure 3B**) segregated nearly
189 completely from uninfected control profiles based on Fc-profiling data. To gain further
190 insights into the specific features that were most distinct across breakthrough and
191 uninfected vaccine profiles, a variable importance plot was generated, highlighting the
192 minimal features that were required to resolve antibody profiles (**Figure 3C**). Strikingly,
193 of the NTD-specific antibody responses, only NTD-specific IgA responses were
194 preferentially enriched among both Delta and Omicron breakthroughs. Conversely,
195 distinct RBD-specific FcR binding responses, but not isotype titers, were highly
196 discriminatory of breakthrough cases compared to vaccinated controls, marked by higher
197 RBD-specific FcγR3B and FcγR2A in Omicron breakthroughs and FcγR3A and FcγR2B
198 responses selectively expanded in Delta breakthroughs.
199 Instead, both S2-specific isotype titers and FcR binding antibodies were
200 preferentially expanded across Delta and Omicron breakthrough cases compared to
201 uninfected vaccinees. Specifically, a coordinated expansion of neutrophil-specific
202 FcγR3B and opsinophagocytic FcγR2A binding antibodies was selectively expanded in
203 both Delta and Omicron breakthrough profiles. Additionally, S2-specific IgM and IgA
204 responses expanded preferentially in Omicron breakthroughs, and IgG3, FcγR3A, and
205 FcγR2B levels were selectively observed in Delta breakthrough cases. Moreover, a near-
206 identical pattern of S2 expansion was observed in a second, independent cohort across
207 Delta and Omicron breakthroughs after 14 days (Supplementary Figure 3). These data
208 collectively, highlight the broader selective expansion of functional FcR binding
209 responses, largely focused on S2, across both Delta and Omicron breakthrough
210 infections.
211
212 *Breakthrough infection drives a functional S2-specific humoral immune expansion*
213 Whether the expansion of S2-specific immunity is simply a biomarker of exposure
214 to the virus or a mechanistic correlate of immunity is unclear. However, binding alone is
215 insufficient to drive antiviral control or clearance, thus we next aimed to determine the
216 functional capacity of the breakthrough anamnestic response. Critically, among the
217 antibody effector functions, we previously observed a preferential expansion of Spike-
218 specific opsinophagocytic activity in natural survivors of severe disease (*22*) and thus we
219 profiled the Spike- and S2-specific antibody-dependent monocyte and neutrophil
220 phagocytic profile. Limited expansion of Spike-specific antibody-dependent cellular
221 monocyte phagocytic (ADCP) activity was observed across the Delta and Omicron
222 breakthrough cases (**Figure 4A**). Conversely, we observed a limited S2-specific ADCP
223 expansion within the first week of breakthrough infection in Delta breakthroughs, but a
224 highly significant expansion of S2-specific ADCP 2-3 weeks following breakthrough .
225 Conversely, S2-specific ADCP was already significantly expanded in Omicron-infections

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226 by day 7 and continued to expand over the next few weeks following breakthrough
227 infection.
228 Antibody-mediated neutrophil phagocytosis (ADNP) has been linked to natural
229 resolution of infection, convalescent plasma therapeutic activity, and vaccine-mediated
230 immunity (*19, 24*). Interestingly, Spike-specific ADNP activity was not expanded over the
231 first week of breakthrough infection in Delta breakthroughs, but did expand highly
232 significantly over the following 2-3 weeks following infection. However, Spike-specific
233 ADNP expanded more rapidly after breakthrough Omicron infection. Interestingly, S2-
234 specific ADNP was not observed in either Delta or Omicron cases 1 week after
235 breakthrough, but S2-specific ADNP responses expanded significantly over the next 2
236 weeks following infection (**Figure 4B**). Neutralization expansion of Spike increased but
237 was correlated exclusively with the RBD domain (*25*). That neutralization did not correlate
238 with S2, even though titers were bolstered, further highlights the non-neutralizing activity
239 of S2-specific IgG1 post-breakthrough (Supplementary Figure 4). Thus, these data point
240 to a simultaneous expansion of both neutralizing and opsinophagocytic antibodies, with
241 a slightly earlier expansion of opsinophagocytic responses that may be key to the early
242 capture, blockade, and elimination of the virus upon transmission.
243
244 *S2-expansion differences across mRNA platforms*
245 Emerging data point to differences in Pfizer/BNT162b2 and Moderna/mRNA-1273
246 vaccine real world efficacy (*26*) and in immune Fc-profiles likely attributable to differences
247 in dose, vaccine intervals, lipid-nanoparticle differences, and potential differences in
248 mRNA chemistry (*27*). To determine if the 2 mRNA platforms induced a similar
249 anamnestic response, breakthrough cases were split across individuals that received
250 either of the mRNA vaccine platforms (**Figure 5**). Recipients of the Pfizer/BNT162b2
251 vaccine experienced a more rapid rise in Spike-specific IgG titers following breakthrough
252 infection, although Pfizer/BNT162b2 and mRNA-1273 vaccinees reached similar Spike-
253 specific titers 2-3 weeks after breakthrough infection (**Figure 5A**). Conversely,
254 Pfizer/BNT162b2 vaccinees experienced a more significant and sustained increase in S2-
255 specific immunity following breakthrough infection compared to Moderna/mRNA-1273
256 recipients (**Figure 5B**). However, despite this differential quantitative expansion,
257 enhanced titer-corrected opsinophagocytic activity was observed following mRNA-1273
258 vaccination (**Figure 5C**-**D**), pointing to a distinct shift in responses across the 2 vaccine
259 platforms, with a robust quantitative increase in S2-titers following Pfizer/BNT162b2 but
260 more functional per-S2-specific antibody response following Moderna/mRNA-1273
261 vaccination.
262
263 *Selective expansion of fusion peptide and heptad repeat 1 specific immunity*
264 To finally define whether S2-specific responses targeted particular regions of S2,
265 we finally mapped the expanding breakthrough response across peptides spanning the
266 S2 domain of the Spike antigen. Using peptides spanning regions across the S2-segment
267 of the SARS-CoV-2 Spike antigen, a highly focused expansion of antibodies was noted
268 to the second fusion peptide (FP2) and the heptad repeat 1 (HR1) (**Figure 6A**) across
269 both Delta and Omicron breakthrough cases 7 days after breakthrough. While the
270 response evolved to target additional regions of the S2 antigen in both groups over the
271 first month following breakthrough infection, the FP2- and HR1-specific responses

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272 remained most highly immunodominant. At a more granular level, FP2 and HR1 IgG titers
273 were already significantly higher than observed in controls after the first week of infection
274 across VoC breakthrough groups (**Figure 6B-C**). Moreover, these responses remained
275 persistently higher over the following 2-3 weeks. Lastly, we probed whether responses to
276 particular subregions within S2 were correlated with virus clearance. Comparison of
277 SARS-CoV-2 RNA decay slopes with S2-peptide-specific IgG expansions over the course
278 of the first 3 weeks of breakthrough infection pointed to a highly significant inverse
279 correlation between the slope of the evolution of FP2-specific responses and viral
280 clearance (**Figure 6D**). Additionally, S2-peptide-specific responses were also significantly
281 associated with viral clearance, pointing to a potentially critical role of multiple S2-specific
282 responses in the elimination of viral replication. Collectively, these data point to a common
283 immunodominant S2 anamnestic response to FP2, and the heptad repeats that may form
284 the basis of a rapid functional humoral response required to capture, contain, and
285 eliminate VoCs until T cells are able to traffic, expand, and eliminate remaining virus to
286 ultimately clear the disease.

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287 **Discussion**

288 Correlates of immunity represent immunological biomarkers that are statistically 289 enriched in individuals that exhibit protective immunity in vaccine trials (*19, 28-32*). 290 Correlates may be mechanistically involved in protective immunity, but can also represent 291 surrogates of other immunological mechanisms key to anti-pathogen control. Most 292 correlates of immunity are defined at the time of peak immunogenicity, aimed at defining 293 immune responses able to predict clinical outcomes. However, within most trials, 294 infections occur over prolonged periods of time, and vaccine-induced immune responses 295 may have waned from peak immunogenicity. Thus, the immunological markers 296 associated with protection over time may differ from those observed at peak 297 immunogenicity. However, the collection of samples prior to infection over time in large 298 Phase 3 trials is cost-prohibitive. Instead, the analysis of responses that selectively 299 expand soon after infection can offer additional insights into the mechanism(s) by which 300 vaccine-induced immunity reacts to control a challenge.

301 Phase 3 peak immunogenicity correlates analyses pointed to a strong association 302 between vaccine-induced Spike-specific neutralizing and binding antibody titers with 303 protection across the mRNA platforms (*6, 8*). Here we profiled the post-infection 304 immunological profiles that evolved in breakthrough infections, with a unique interest in 305 defining whether the kinetics of breakthrough correlates of immunity were consistent 306 across variants of concern. While an expansion of full-length Spike IgG responses was 307 observed in both Delta and Omicron breakthrough infections, limited expansion was 308 observed in NTD-specific titers across both groups. Instead, the majority of the Spike-309 specific expansion was related to a unique anamnestic expansion of early S2 FP2- and 310 HR1-specific IgM antibodies able to leverage monocyte phagocytosis, followed by a more 311 mature S2 FP2- and HR1-specific IgG FcR binding neutrophil recruiting response 312 observed both in Delta and Omicron breakthroughs. Thus, despite the immunodominant 313 vaccine-induced response to the RBD, these data point to a critical and unexpected role 314 of S2-specific functional humoral immunity as critical anamnestic correlates of immunity 315 across VoCs.

316 Early studies of immune correlates of natural resolution of COVID-19 pointed to a 317 selective expansion of S2-specific functional humoral immunity in survivors of natural 318 severe COVID-19 infection. S2-specific antibodies were noted in survivors of severe 319 COVID-19 from the time of intensive care unique admission (*22, 31*). Moreover, expanded 320 S2-specific humoral immune responses were also noted in children (*32*), individuals that 321 developed milder forms of COVID-19, as well as in individuals that developed 322 asymptomatic infections (*31*). Interestingly, these natural S2-specific humoral immune 323 responses may have emerged from pre-existing common-coronavirus specific humoral 324 immune responses, that also expanded, marked by preferential FcR-binding, among 325 individuals with asymptomatic infection. Common β-coronaviruses are largely conserved 326 in their S2 domains. S2-specific monoclonals exhibit cross-coronavirus reactivity and *in* 327 *vivo* protection in an Fc-dependent manner (*33*), arguing that these less potent 328 neutralizing antibodies target a highly conserved region of the SARS-CoV-2 Spike may 329 depend on non-neutralizing mechanisms of action. Given the robust association between 330 pre-existing common-coronavirus immunity and attenuated COVID-19 (*32*), these data 331 point to a critical role for cross-reactive S2-specific humoral immunity in both infection and 332 vaccine-induced immune responses.

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333 The S2 includes the fusion machinery required for viral entry (*34*), requiring precise
334 packaging and movement upon Spike binding to the host angiotensin-2 (ACE2) receptor.
335 Thus, unlike other regions of the Spike antigen, the S2 is less mutable and has shown
336 conservation across distinct VOCs, with only ~2 and 12 mutations in Delta and Omicron
337 respectively (depending on sublineage), representing the most highly conserved region
338 of the SARS-CoV-2 Spike antigen. However, the S2-directed response is less dominant
339 following mRNA vaccination, likely related to the 2 proline stabilization introduced in the
340 protein to stabilize the antigen during vaccination (*35, 36*). This stabilization holds S1 and
341 S2 in a pre-fusion state, likely required to drive robust immunity to the primary target of
342 neutralizing antibodies, the RBD. However, this stabilization also may make S2 less
343 accessible to the immune response. Infection with the virus generates copious amounts
344 of Spike in its inherently destabilized form. This allows immune surveillance networks to
345 sample both S1 and S2 domains in pre- and post-fusion forms, triggering an anamnestic
346 response. The differences in the anamnestic immunodominance of S1 and S2 may relate
347 to the fact that S1 is presented largely as soluble protein, whereas S2 may be presented
348 in a particulate form, due to the C-terminal transmembrane anchoring domain of the
349 protein. Conversely, S2-responses may gain a competitive advantage due to the high
350 degree of conservation across VoCs, able to recruit pre-existing B cells, whereas
351 previously programmed RBD- and NTD-specific B cells may struggle to bind to the
352 incoming VoC due to significant antigenic variation (*1, 2, 4*). However, despite the
353 enhanced sequence conservation between the vaccine strain and Delta compared to
354 Omicron, S2-specific immunity expanded in both Delta and Omicron breakthroughs,
355 suggesting that S2-specific anamnestic correlates may be key to protection across VoCs.
356 Differences in dose, vaccination interval, lipid-nanoparticle composition, and
357 mRNA chemistry all contribute to differences in antibody subclass/isotype and Fc-
358 receptor binding profiles across mRNA vaccines (*27*). Here we observed differences in
359 the anamnestic response following Pfizer/BNT162b2 and Moderna/mRNA-1273
360 immunization, linked to a higher magnitude expansion of anamnestic immunity in
361 Pfizer/BNT162b2 vaccinees and a functional expansion in Moderna/mRNA1273
362 vaccinees, although both breakthrough profiles resulted in an expansion of S2-specific
363 immunity. These differences may be related to real-world efficacy differences across the
364 platforms, with reduced breakthrough infections observed in Moderna/mRNA1273
365 vaccinees, potentially related to higher IgA titers and functional humoral immunity (REF)
366 that may provide a more robust barrier against infection at the mucosal barrier. However,
367 the expansion of S2-specific immunity following both vaccines, focused on FP2 and HR1,
368 may be related to their accessible positions on the Spike antigen (*37*-*39*). Thus, strategies
369 to boost functional humoral immunity to these critical sights may represent a promising
370 future strategy to maintain long-term protection against severe disease and death against
371 current and future VoCs. Whether these S2-specific responses can work in concert with
372 T cells that also target conserved regions of the SARS-CoV-2 Spike remains unclear (*12*);
373 however, these responses point to important, unexpected targets of the immune response
374 that may be key to the durable protection against disease severity. Moreover, these
375 regions that show higher conservation between VoCs, and β-coronaviruses in general,
376 that are expanded post-infection could be viewed as rational vaccination booster targets.

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377 **Methods**
378

379 *Study Approval*

380 Approval for study for breakthrough COVID-19 at Massachusetts General Brigham was
381 approved under protocol 2021P000812 by the Mass General Brigham IRB. For this
382 cohort, symptomatic COVID-19 patients seen for outpatient care were recruited based
383 on a positive SARS-CoV-2 test (**Table S1**). Sequencing from anterior nasal swabs was
384 performed for VoC identification. The use of healthy donor blood for cellular functional
385 assays is approved under protocol 2021P002628 by the Mass General Institutional IRB.

386 The Hospitalized or Ambulatory Adults with Respiratory Viral Infections (HAARVI) study
387 was approved by the University of Washington Human Subjects Division Institutional
388 Review Board (STUDY00000959).

389 *Antigens*

390 All antigens and peptides used in this study are listed in **Table S2** and **Table S3**. The
391 protein antigens were received in lyophilized powder form and resuspended in water to
392 a final concentration of 0.5 mg/mL. Peptides were received in solution and, if necessary,
393 were buffer exchanged using Zeba-Spin columns (ThermoFisher, USA).

394 *Immunoglobulin isotype and Fc receptor binding*

395 Sera was collected from participants at two time points post-COVID-19 diagnosis, with
396 the first time point being 6.3 ± 2.8 days post-observed start date, and 18.8 ± 2.9 days
397 post-observed start date. The two groups were clustered together for analyses and
398 classified as < 1 Week and 2-3 Weeks.
399

400 Systems serology for antigen-specific recognition was done using custom multiplex
401 magnetic Luminex beads (Luminex Corp, TX, USA) as previously as previously described
402 (*21*). Antigens and peptides were coupled to beads through carbodiimide-NHS ester-
403 coupling chemistry. The antigen- or peptide-coupled beads were incubated with heat-
404 inactivated serum (1:100 for IgG2, IgG3, IgG4, IgM, and IgA1, 1:250 for IgG1, and 1:750
405 for Fcγ-receptor binding) overnight at 4°C in 384 well plates (Greiner Bio-One, Germany).
406 Secondary antibodies were PE-conjugated and incubated with samples at room
407 temperature for 1 hour at a 1:100 dilution in sterile-filtered Assay Buffer (1X PBS, pH =
408 7.4, 0.1 % BSA, 0.05 % Tween – 20). For Fcγ-receptors, PE-streptavidin (Agilent
409 Technologies, CA, USA) at a 1:1000 dilution.
410

411 For flow cytometry analysis, the IQue Screener PLUS cytometer (IntelliCyt) was used
412 using customized gating for each bead region. Fluorescence in the BL2 channel was
413 quantified and exported into .csv files and subsequently analyzed (see below).
414

415 *Viral loads quantitation*

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416 SARS-CoV-2 RNA was extracted and quantified as previously described (*20, 40*). VoC
417 identification through sequencing was done using a previously validated method (*25*).
418 Peak viral loads for each patient were quantified and the mean peak viral loads between
419 VoC was calculated.

420 *Evaluation of antibody-mediated functions*

421 Antibody-dependent cellular phagocytosis (ADCP) by monocytes and neutrophil
422 phagocytosis (ADNP) were quantified using a validated, flow cytometry-based bead
423 phagocytic assay. Fluorescently labeled microspheres were coupled to antigens through
424 biotinylation and conjugation to neutravidin beads. Diluted and heat-inactivated serum
425 samples were incubated with the antigen-coupled neutravidin beads to create a pre-
426 immune complex. The solution was then incubated with THP-1 monocytes (ATCC,
427 Manassas, USA) or primary-derived neutrophils (*21*). For ADNP, cells were stained with
428 anti-CD66b Pac blue antibody to calculate the percentage of CD66b+ neutrophils. Cells
429 were fixed with 4% paraformaldehyde. Microsphere uptake was quantified by the
430 percentage of microsphere-positive cells x MFI of microsphere-positive cells.

431 *Statistical Analyses*

432 Data visualizations and analysis were done using R Studio V 1.4.1103 or GraphPad
433 Prism. Box and whisker plots were generated using ggplot showing the mean and
434 standard deviation for each group as factors. An initial ANOVA was performed to
435 identify significant groupings. A Wilcoxon-rank sum test and pairwise T-tests were used
436 to determine grouping between two groups. For all analyses * stands for $p < 0.05$, and
437 ** stands for $p < 0.01$. All codes and scripts are available upon request and no original
438 code was created for this manuscript.

439
440

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Figure Legends

Figure 1. Recognition and expansion of Spike and Spike subdomains post-breakthrough. (A) Full-length Spike (D614G) was assayed for antibody recognition in non-breakthrough, vaccinated controls (white, column 1), vaccinated Delta <1 Week (yellow, column 2) or 2-3 Weeks post-breakthrough (orange, column 3), and vaccinated Omicron <1 week (blue, column 4) or 2-3 Weeks post-breakthrough (dark blue, column 5). (B) Same as A, but for the receptor-binding domain (RBD). (C) Same as B, but for the N-terminal domain (NTD). (D) Same as B, but for the S2 domain. (E) Same as A, but for Fcγ-receptor (FcγR) recognition of full-length Spike. (F) Same as E, but for the RBD. (G) Same as F, but for the NTD. (H) Same as F, but for the S2 domain. * = $p < 0.05$, and ** = $p < 0.01$ for all panels.

Figure 2. Conserved regions of Spike are selectively expanded in breakthrough cases. (A) Fold changes in IgG1 binding of the subdomains of Spike (NTD in black, RBD in blue, and S2 in red) for vaccinated, Delta breakthrough cases at <1 week or 2-3 Weeks post-breakthrough. (B) Same as A, but for vaccinated Omicron breakthrough cases. (C) Fold changes in IgG1 binding of the full-length Spikes from VoC in Delta breakthrough cases. (D) Same as C, but for Omicron breakthrough cases. (E) Fold changes in IgG1 binding of the Spike of common CoV Spikes in Delta breakthrough infections at < 1 Week or 2-3 Weeks post-breakthrough. SARS-CoV-2 nucleocapsid (N) is used as a control for infection. (F) Same as C, but for Omicron breakthroughs.
* = $p < 0.05$, and ** = $p < 0.01$ for all panels.

Figure 3. Expansion of S2 recognition is a marker for breakthrough COVID-19. (A) Partial least squares determinant analysis (PLS-DA) clustering of vaccinated, controls (black), and vaccinated Delta breakthrough (orange) immunological signatures. Shown are the clusters at 2-3 Weeks post-breakthrough. (B) Same as A, but for vaccinated Omicron breakthrough (blue) immunological signatures. (C) Ranked sum of identified features in the PLS-DA by subdomain. Shown is the distance on LV1, which was the major axis of separation by both Delta and Omicron breakthroughs at 2-3 Weeks post-breakthrough.

Figure 4. Expansion of S2 recognition is functionally linked to antibody-mediated opsinophagocytic activity. (A) Antibody-mediated cellular phagocytosis (ADCP) by monocytes was quantified using sera from vaccinated, non-breakthrough controls (column 1, white), vaccinated Delta breakthroughs at < 1 Week (yellow, column 2) or 2-3 Weeks post-breakthrough (orange, column 3), and vaccinated Omicron breakthroughs at < 1 Week (blue, column 4) or 2-3 Weeks post breakthrough (dark blue, column 5) for full-length Spike (left) and for the S2 domain alone (right). (B) Same as A, but for antibody-dependent neutrophil phagocytosis (ADNP) isolated from healthy donors. * = $p < 0.05$, and ** = $p < 0.01$ for all panels.

Figure 5. Breakthrough COVID-19 in mRNA vaccinated individuals results in distinct S2 functional expansions. (A) Fold changes in full-length Spike IgG1 in breakthrough COVID-19 cases in individuals vaccinated with the BNT162b2 (blue) or the mRNA1273 (red) mRNA vaccines at <1 Week or 2-3 Weeks post-breakthrough. (B) Fold

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487 changes in S2-specific IgG1 in breakthrough COVID-19 cases in BNT162b2 (blue) or the
488 mRNA1273 (red) mRNA vaccine recipients at <1 Week or 2-3 Weeks post-breakthrough.
489 (C) ADCP functional units for individuals vaccinated with BNT162b2 (blue) or the
490 mRNA1273 (red) mRNA vaccine against full-length Spike or S2. Functional units were
491 quantified by taking the endocytic score divided by the MFI of IgG1 towards the antigen.
492 (D) Same as C, but for ADN* = $p < 0.05$, and ** = $p < 0.01$ for all panels.
493
494 **Figure 6. Immunodominant expansion of S2 is focused to the fusion peptide and**
495 **heptad repeat region 1 for VoC breakthroughs**. (A) Peptides spanning subregions of
496 S2 were assayed for IgG1 expansion post-breakthrough by Delta (left two columns) and
497 Omicron (right two columns) during the <1 Week and 2-3 Weeks post-breakthrough
498 responses; heatmap legend is shown on the right. (B) The fusion peptide 2 (FP2) was
499 assayed for IgG1 recognition in non-breakthrough, vaccinated controls (white, column
500 1), vaccinated Delta <1 Week (yellow, column 2) or 2-3 Weeks post-breakthrough
501 (orange, column 3), and vaccinated Omicron <1 week (blue, column 4) or 2-3 Weeks
502 post-breakthrough (dark blue, column 5). (C) Same as B, but for heptad repeat 1
503 subregion 6 (HR1-6). (D) Correlations between viral loads and IgG1 S2 peptide
504 recognition for 2-3 Weeks post-breakthrough samples. * = $p < 0.05$.
505
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507 **Acknowledgments**

508 We thank Mark and Lisa Schwartz, Terry and Susan Ragon, and the SAMANA Kay
509 MGH Research Scholars award for their support. GA also receives funding from the
510 Massachusetts Consortium on Pathogen Readiness (MassCPR), the Gates Global
511 Health Vaccine Accelerator Platform, and the NIH (3R37AI080289-11S1, R01AI146785,
512 U19AI42790-01, U19AI135995-02, U19AI42790-01, P01AI1650721, U01CA260476 –
513 01, CIVIC75N93019C00052).

514

515 **Disclosure**

516 Galit Alter is a founder/equity holder in Seroymx Systems and Leyden Labs. GA has
517 served as a scientific advisor for Sanofi Vaccines. GA has collaborative agreements
518 with GSK, Merck, Abbvie, Sanofi, Medicago, BioNtech, Moderna, BMS, Novavax, SK
519 Biosciences, Gilead, and Sanaria.

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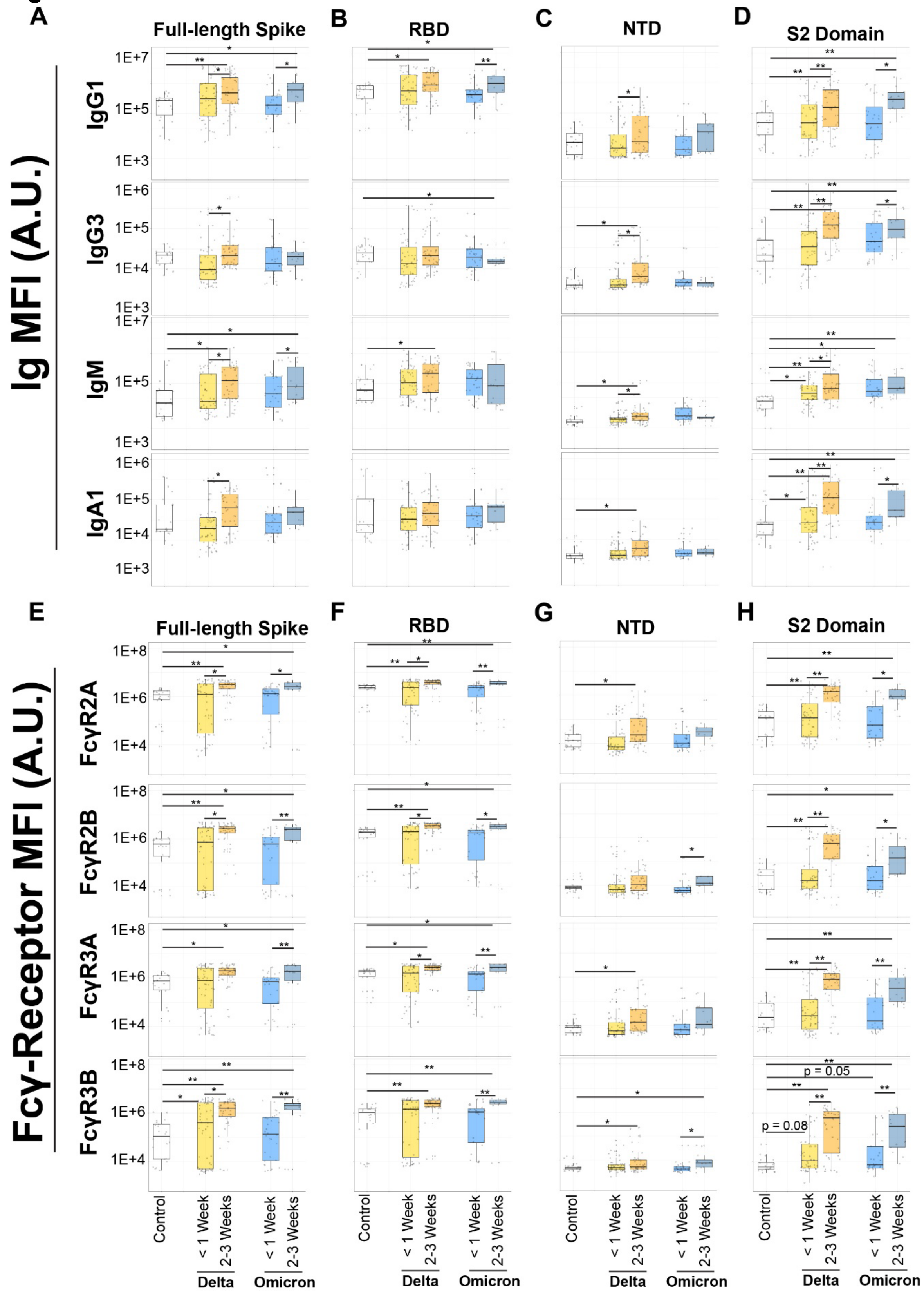
521 **Figure 1**



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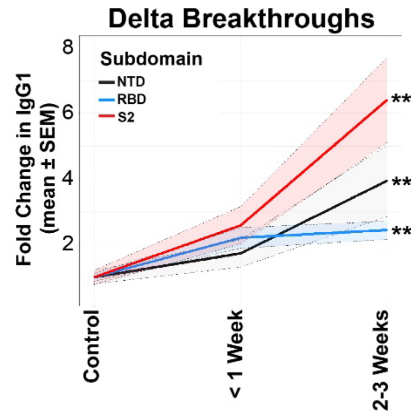
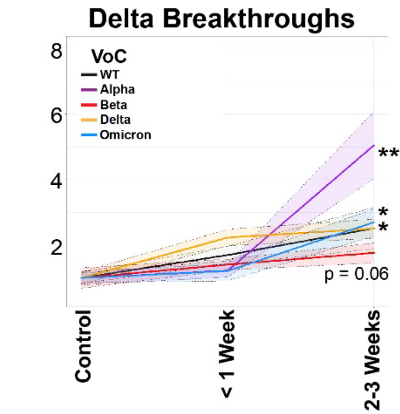
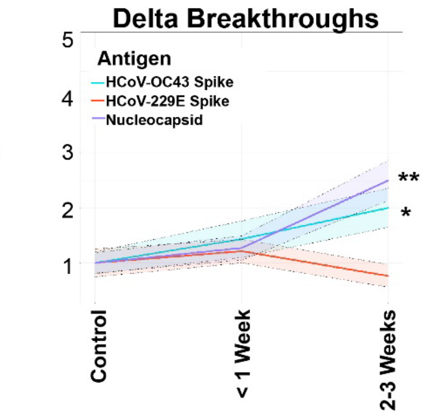
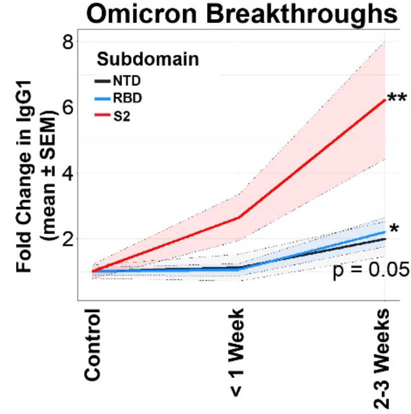
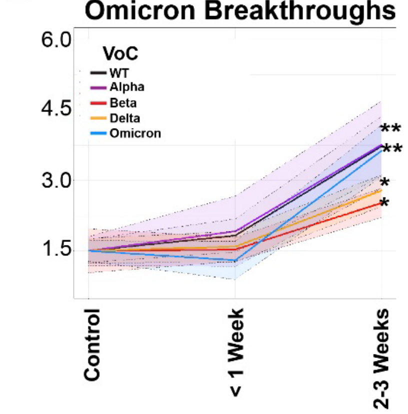
524 **Figure 2**

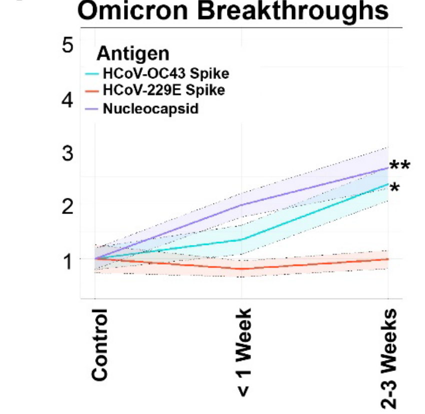












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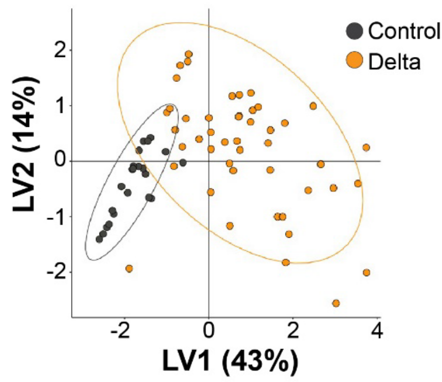
527 **Figure 3**

A



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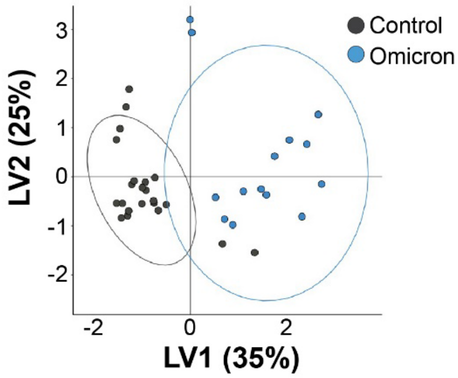

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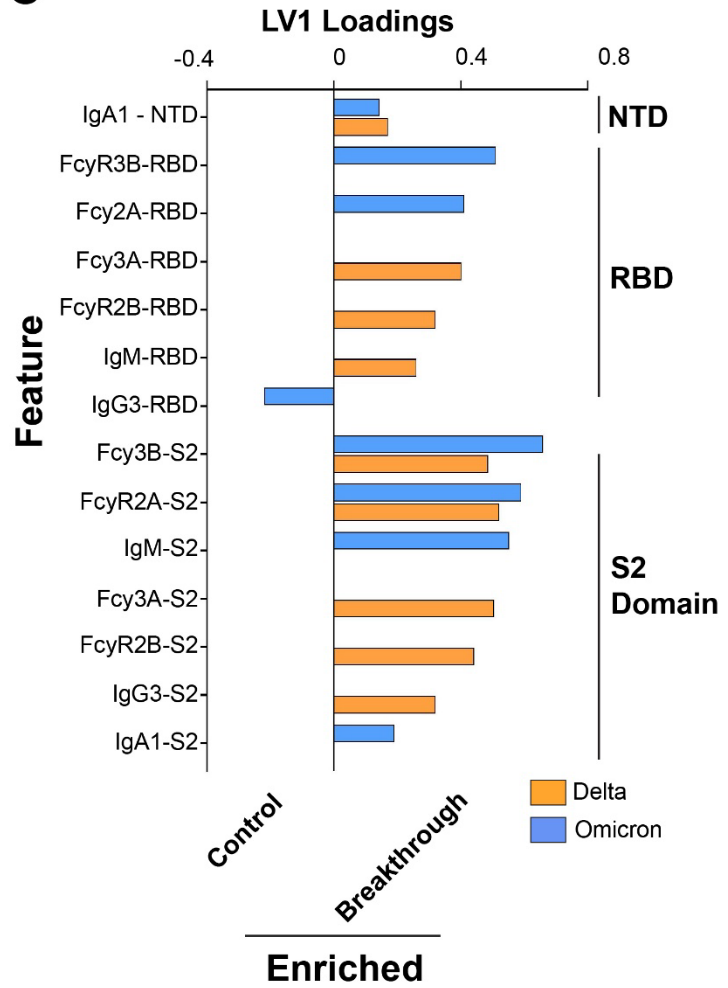

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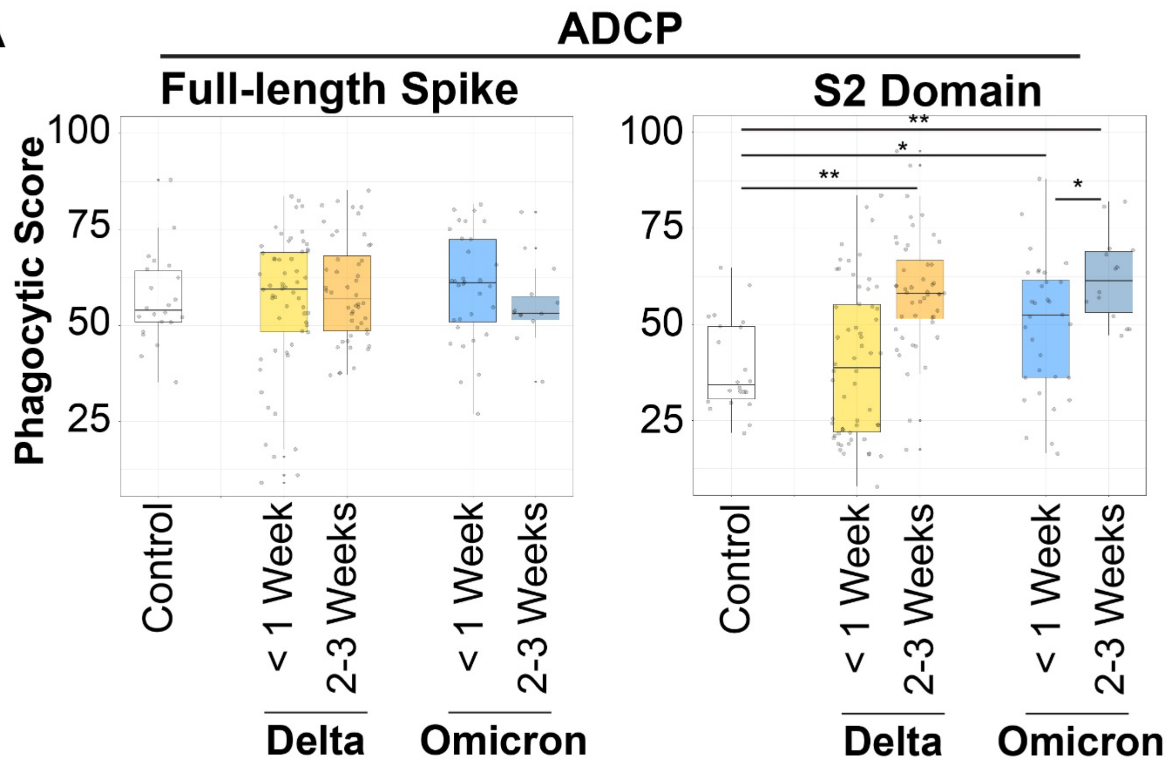
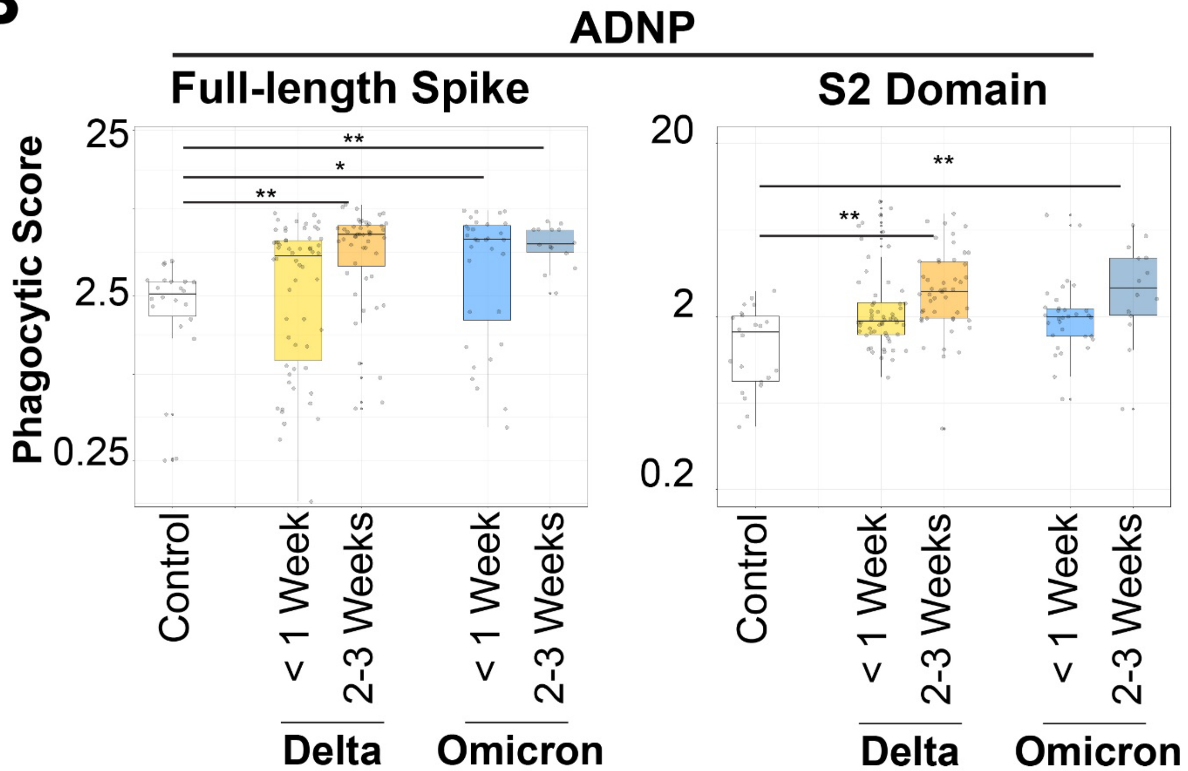
530 **Figure 4**





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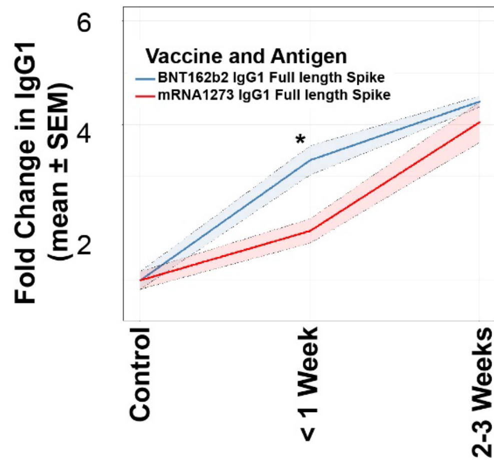
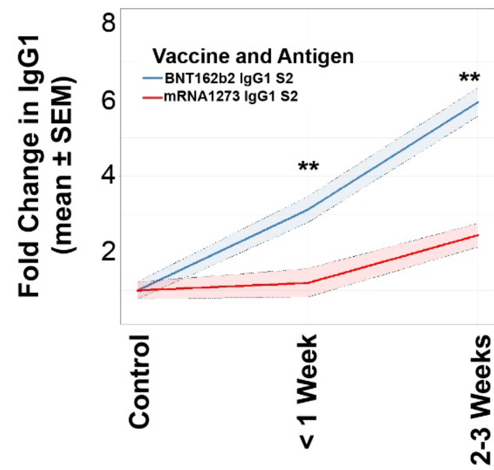
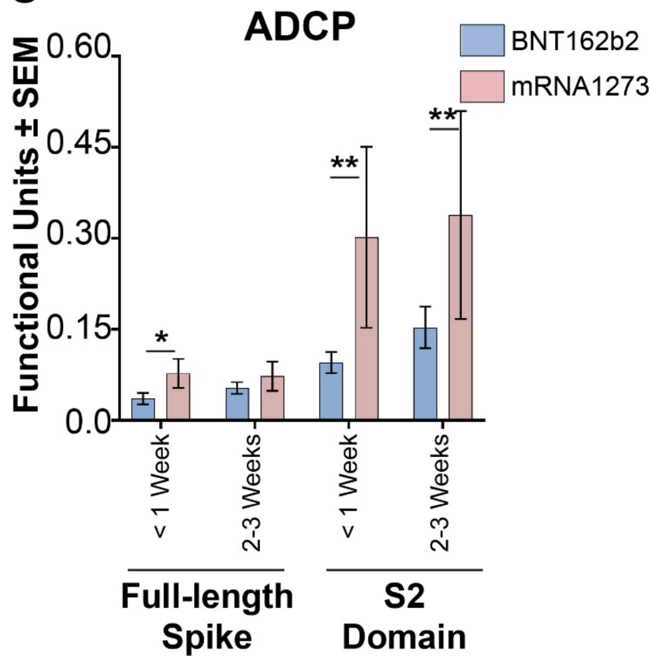
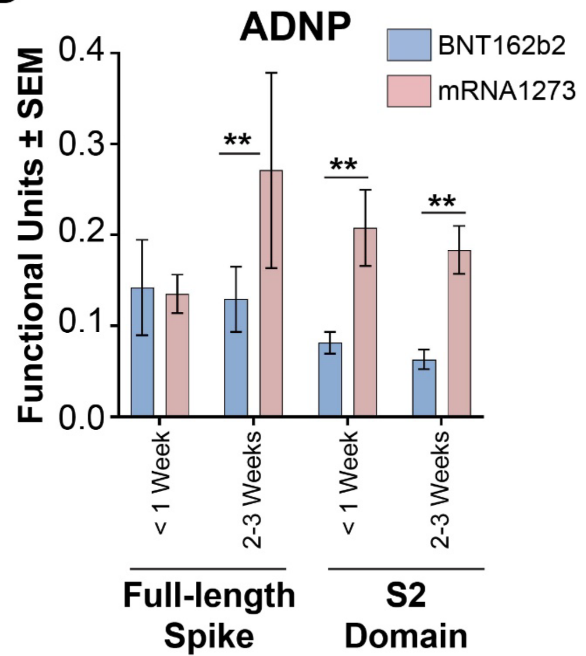
533 **Figure 5**









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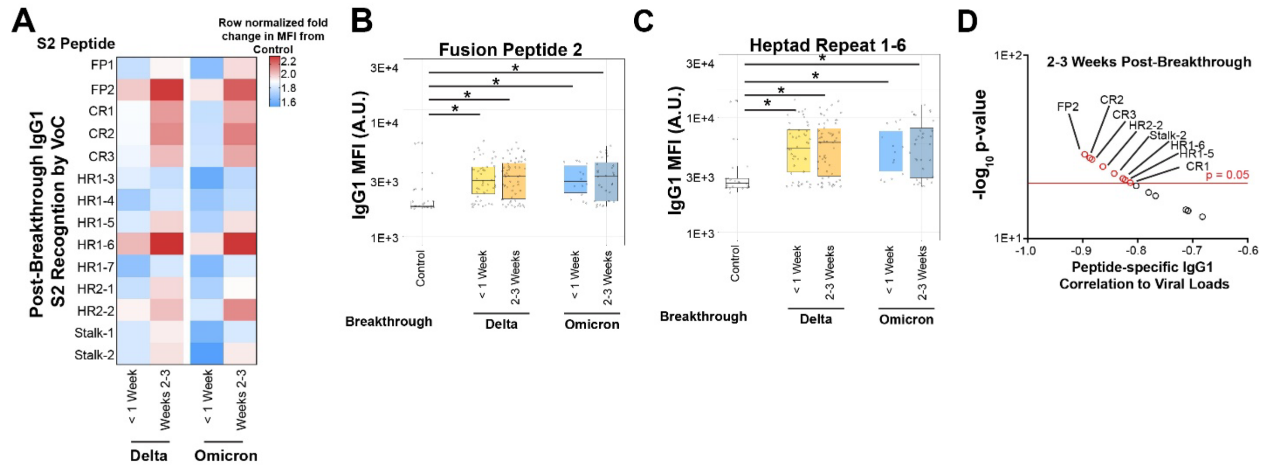
536 **Figure 6**

537



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