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Germinal center dynamics in the mediastinal lymph node distinguish time-dependent and variant-specific immunity during SARS-CoV-2 secondary exposure

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29 **Abstract**

30 SARS-CoV-2 secondary infections remain a persistent challenge even though individuals have previous
31 immunity from natural infection or vaccination. Yet, the cellular mechanisms that determine whether a
32 secondary exposure is rapidly controlled by pre-existing immunity and results in sterilizing protection or
33 progresses to productive infection and disease remain poorly understood. Using homologous and
34 heterologous secondary-challenge experimental strategies in C57BL/6J mice, we show that protection
35 against a homologous SARS-CoV-2 Alpha secondary challenge in previously infected mice is time-
36 limited, remaining durable through 56 days post-primary infection, with breakthrough infection on day
37 84 post-primary infection. This loss of protection was not associated with waning neutralizing antibody
38 titers but instead correlated with progressive contraction of T follicular helper cells and germinal center
39 B cells in the mediastinal lymph node. In contrast, heterologous Beta secondary exposure in Alpha-
40 previously infected mice consistently failed to mount a robust germinal center response despite
41 mobilizing comparable or greater numbers of immune cells than homologous re-challenge. Instead,
42 RNA-seq and flow cytometry analyses revealed that this response was associated with an inflammatory
43 extrafollicular B cell response characterized by reduced *Bcl6* and *Aicda* expression and concomitant
44 robust upregulation of *Il6*. Taken together, these findings identify the germinal center recall response in
45 the mediastinal lymph node as an important cellular correlate of SARS-CoV-2 secondary immunity and
46 carry direct implications for the timing of booster immunization and the design of cross-variant vaccines.

47

48 **Introduction**

49 The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019 triggered
50 the coronavirus disease 2019 (COVID-19) pandemic. Approximately 7 million confirmed deaths have
51 been reported to the World Health Organization, and an estimated 14.9 million excess deaths occurred in
52 2020 and 2021 alone¹. SARS-CoV-2 spreads primarily through respiratory droplets and aerosols and
53 causes disease ranging from asymptomatic infection to fatal respiratory failure. Despite highly effective
54 vaccines, the virus continues to circulate, and reinfection remains common even in people with prior
55 immunity, driven both by the waning of that immunity and by the emergence of antigenically novel
56 variants that evade it¹⁻⁴. Neutralizing antibodies, which bind the spike glycoprotein on the virion surface
57 and block viral entry, are considered the principal mediators of sterilizing immunity to viral infection^{5,6}.
58 However, antibody titers alone do not fully account for the observed differences in the outcomes of a
59 secondary exposure, suggesting that additional cellular immune mechanisms may contribute to
60 protection^{7,8}. How humoral and cell-mediated immune mechanisms operate during secondary exposure
61 to various SARS-CoV-2 variants, and whether they differ depending on the time elapsed since primary
62 infection, is not well understood.

63 Upon antigen encounter, B cells can enter the germinal center (GC), a specialized microstructure within
64 secondary lymphoid organs where they undergo clonal expansion, somatic hypermutation, and affinity-
65 based selection and maturation. Together, these processes produce high-affinity antibodies and establish
66 long-lived immune memory⁹. Central to this process in the GC are T follicular helper (T_{FH}) cells
67 (specialized CD4⁺ T helper cells), which provide critical support for GC B cell survival, proliferation,
68 and differentiation into long-lived plasma cells or memory B cells (MBCs)^{9,10}. Both GC B cells and T_{FH}
69 cells are dependent on B cell lymphoma 6 (BCL6), a master transcriptional regulator that drives and
70 maintains their identity within the GC¹¹, and activation-induced cytidine deaminase (AID, encoded by

71 the *Aicda* gene), an enzyme essential for somatic hypermutation and class switch recombination⁹. BCL6-
72 expressing T_{FH} cells and organized GCs are lost from the thoracic lymph nodes of patients with fatal
73 COVID-19¹². Conversely, direct sampling of the draining lymph node in humans has shown that mRNA
74 vaccination induces GC responses that persist for months and that are not apparent from measurements
75 of circulating antibody alone¹³. Despite the essential role of GCs in antibody maturation and their central
76 role in the humoral response to SARS-CoV-2¹⁴, the dynamics of the GC response following secondary
77 exposure to homologous or heterologous SARS-CoV-2 remain poorly understood.

78 We previously examined secondary coronavirus exposures in the Syrian hamster (*Mesocricetus auratus*)
79 model and found that GC activation patterns and induction of *Bcl6* and *Aicda* genes in the mediastinal
80 lymph node differed markedly depending on the antigenic distance between primary and secondary
81 coronavirus infection¹⁵. Here, we used C57BL/6J mice, which offer a broader array of immunological
82 reagents, to directly characterize GC B cell and T_{FH} cell dynamics during homologous and heterologous
83 SARS-CoV-2 secondary exposures across multiple time points. Mice primed with SARS-CoV-2 Alpha
84 were secondarily challenged with either Alpha (homologous) or Beta (heterologous) at 21, 56, 84, or 365
85 days post-primary inoculation, with primary-only Alpha and Beta groups included as reference controls.
86 Virological, histopathological, serological (microneutralization), targeted and transcriptome-wide
87 mediastinal lymph node (MLN) gene expression, and multiparameter flow cytometric analyses of the
88 spleen, MLN, and lung were performed to characterize recall immune responses. We identified a
89 productive GC recall response in the draining lymph node as a key cellular correlate of secondary
90 protection, independent of circulating neutralizing antibody titers. These results have implications for
91 optimizing booster vaccination timing and the design of cross-variant SARS-CoV-2 vaccines.

92 **Results**

93 **Homologous secondary exposure to SARS-CoV-2 Alpha reveals time-dependent loss of sterilizing** 94 **protection**

95 To investigate the durability of immune protection following primary SARS-CoV-2 infection, we
96 established a homologous secondary exposure model in C57BL/6J mice (Figure 1A; Table 1). C57BL/6J
97 mice were inoculated intranasally with SARS-CoV-2 Alpha ($10^{4.15}$ TCID₅₀) on day 0 and rechallenged
98 with the same virus on day 21 (D21 Alpha 2°), 56 (D56 Alpha 2°), or 84 (D84 Alpha 2°) post-primary
99 inoculation (ppi). A primary-only group (Alpha 1°) served as the reference (Figure 1A; Table 1). Animals
100 were sampled on days 0, 2, 6, 9, and 14 ppi or post-secondary inoculation (psi). No group lost weight
101 after the homologous secondary challenge, although weight gain was attenuated in D84 Alpha 2° animals
102 (Figure 1B). In Alpha 1° animals, infectious virus peaked on day 2 ppi at $10^{1.8}$ and $10^{2.0}$ TCID₅₀/mL in
103 the right lung and nasal turbinates, respectively, and was cleared by day 6 (Figure 1C). Neither D21 nor
104 D56 Alpha 2° animals had recoverable infectious virus in either compartment at any psi time point. In
105 contrast, D84 Alpha 2° animals had detectable virus on day 2 psi ($10^{1.7}$ TCID₅₀/mL lung; $10^{2.6}$ TCID₅₀/mL
106 nasal turbinates) and carried the highest genomic viral RNA (vRNA) burden, most markedly in the nasal
107 turbinates (Supplementary Figure 1A).

108 Lung histopathology was scored across five parameters (Figure 1D; Supplementary Figure 1B). Changes
109 were minimal in D21 Alpha 2° animals; D56 Alpha 2° animals had a significant rise in the inflammation
110 score at day 6 psi that subsequently resolved; and D84 Alpha 2° animals had the greatest changes, with
111 significant differences in both inflammation and blood vessel scores. No homologous rechallenge group
112 scored above zero for bronchiolar pathology at any time point. Taken together, these data define a
113 reproducible window of sterilizing immunity to homologous Alpha that is intact at day 56 but lost by day
114 84.

115 **Day84 breakthrough infection occurs despite neutralizing antibody titers and germinal-center**
116 **gene expression comparable to protected secondary exposures**

117 Because circulating neutralizing antibody is the principal correlate of sterilizing immunity to respiratory
118 viruses^{5,16–18}, we first asked whether it explained the D84 breakthrough (Figure 2A). We evaluated
119 SARS-CoV-2 Alpha neutralizing antibody endpoint titers in plasma from all animal groups (Figure 2A).
120 Alpha 1° animals seroconverted, with titers detectable by day 9 and a geometric mean titer (GMT) of
121 25.2 on day 14 ppi. All three secondary groups mounted robust and statistically indistinguishable
122 secondary antibody responses peaking at day 14 psi (D21, GMT 640; D56, 508; D84, 508), suggesting
123 that neutralizing antibody does not account for the time-dependent loss of protection observed in D84
124 animals.

125 We next profiled T cell and B cell/GC gene expression in the MLN by PrimePCR (Figures 2B, 2C).
126 Alpha 1° animals upregulated *Cd3e*, *Cd4*, *Cd8a*, *Cxcr3*, and *Il12b*, each peaking at day 14 ppi (7.6- to
127 10.5-fold over day 0), and strongly induced *Bcl6* and *Aicda* (25.3- and 31.5-fold at day 14 ppi), consistent
128 with a de novo primary GC response. All three secondary groups instead downregulated T-cell genes
129 relative to their day 0 psi baselines. Their B cell panels were dominated by inflammatory and plasma cell
130 mediators rather than by GC regulators: D21 Alpha 2° animals induced *Il6* (32.5-fold, day 14 psi) with
131 only marginal *Bcl6* and *Aicda* (1.8- and 1.7-fold), and D56 Alpha 2° animals mounted the strongest *Il4*
132 response of any group (90.0-fold increase at day 14 psi) alongside *Il6* and *Prdm1* (19.3- and 3.6-fold),
133 consistent with plasma cell differentiation. Importantly, *Bcl6* and *Aicda* remained low across all three
134 secondary groups (maxima 4.0- and 4.6-fold, against 25.3- and 31.5-fold in primary infection) and did
135 not distinguish the protected D21 and D56 animals from the breakthrough D84 group. Like neutralizing
136 titer, the targeted MLN GC transcript panel does not explain the day 84 breakthrough.

Progressive loss of T_{FH} and GC B cell responses in the mediastinal lymph node correlates with breakthrough infection during homologous SARS-CoV-2 secondary exposure

To resolve the cellular basis of this timing-dependent protection observed in D21 and D56 animals, but lost by D84, we quantified T and B cell subsets by flow cytometry in spleen, MLN, and lung, using Alpha 1° animals as the reference (gating strategies, Supplementary Figures 2A and 3A). We focused our analyses on day 2 psi, the point at which breakthrough viral infection was detected in D84 but not in D21 or D56 animals. The full kinetics are shown in Supplementary Figures 3 and 4.

CD4⁺ T cell numbers at day 2 psi were highest in D21 and D56 Alpha 2° animals in all three tissues and lowest in Alpha 1° and D84 Alpha 2° animals (Figure 2D, upper row; Supplementary Figure 3A). The effect was most pronounced in the draining MLN, where mean counts fell from 9.8×10^4 (D21) to 3.8×10^4 (D56) to 1.2×10^4 (D84). EM T cells followed the same trend, peaking in D21 animals (1.2×10^7 spleen; 4.1×10^5 MLN; 4.4×10^3 lung; Figure 2D, middle row). The T_{FH} compartment separated the groups most clearly (Figure 2D, lower row). D21 animals reached 6.6×10^6 , 1.2×10^4 , and 3.8×10^4 cells in spleen, MLN, and lung, respectively. However, D84 animals reached only 4.4×10^5 , 1.8×10^3 , and 1.2×10^3 , with the deficit concentrated in the MLN and lung, suggesting a diminished capacity to rapidly re-establish follicular helper activity in secondary lymphoid organs and at the site of infection following antigen re-encounter.

The B cell arm mirrored this trend (Figure 2E; Supplementary Figures 4A-C). Total B cell numbers were preserved in the spleen across all intervals (1.7 - 1.9×10^7) but were markedly lower in the D84 MLN (6.4×10^3) than in either D21 (1.2×10^4) or D56 (4.7×10^4) animals (Figure 2E, upper row; Supplementary Figures 4A). GC B cells were likewise lowest in D84 animals in every tissue: 1.9×10^6 (D84) against 2.65×10^6 (D21) and 5.6×10^6 (D56) in spleen, 3.3×10^4 against 6.2×10^4 and 5.4×10^4 in lung, and 1.7×10^3 against 4.2×10^3 and 8.4×10^3 in MLN (Figure 2E, middle row; Supplementary Figures 4B).

160 MBCs showed the same tissue profiles (Figure 2E, lower row; Supplementary Figures 4C). D21 animals
161 maintained the highest MBCs in the MLN (1.4×10^3) and lung (1.2×10^4), whereas D84 animals had
162 almost none in the MLN (4.9×10^1) despite retaining large MBCs in the splenic pool (1.6×10^6). Taken
163 together, these data reveal that breakthrough virus replication in D84 Alpha 2° animals coincided with a
164 progressive failure to re-establish the T_{FH}-GC recall response within the draining MLN, and not with a
165 systemic loss of memory cells.

166 **Transcriptome-wide profiling of the draining lymph node separates GC from plasma cell and**
167 **atypical B cell programs across homologous secondary exposure intervals.**

168 To define the molecular basis of interval-dependent recall, we performed transcriptomic profiling of the
169 MLN on day 2 psi (days 23, 58, and 86 ppi), the earliest time point at which interval-dependent
170 differences emerged, together with primary-only Alpha 1° animals at matched pre-secondary time points
171 and naïve controls. . Two days after primary Alpha inoculation, the MLN had already committed to a GC
172 program, inducing *Bcl6*, *Mef2b*, *Irf4*, and *Foxo1* together with *Il21*, whereas GC effector and surface
173 genes, including *Aicda*, *Cxcl13*, *Mki67*, and *Cxcr5*, remained below naïve levels (Supplementary Figure
174 5A).

175 We next examined how each homologous rechallenge interval resolved the GC transcriptional program
176 (Figure 3A; Supplementary Figure 5B). The three intervals occupied distinct transcriptional states on day
177 2 psi. D21 Alpha 2° animals, which exhibited the strongest protection, showed the highest expression of
178 GC and T_{FH}-associated genes, including *Bcl6*, *Sh2d1a*, *Cxcr4*, *Mki67*, and *Cd27*, consistent with active
179 GC formation and B cell proliferation. In contrast, D84 Alpha 2° animals displayed the lowest expression
180 of these GC-associated genes, while showing increased expression of genes associated with plasma cell
181 differentiation (*Prdm1*, *Xbp1*, *Mzb1*, *Sdc1*, and *Jchain*), atypical B cells (*Itgax*, *Tbx21*, and *Fcrl5*), and
182 mature or resting B cells (*Ms4a1*, *Ighd*, *Fcer2a*, *Cr2*, and *Cd22*) (Figure 3A; Supplementary Figure 5B).

183 Genome-wide differential expression analysis using D84 Alpha 2° as the reference identified 199 genes
184 uniquely upregulated in D84 relative to D21 Alpha 2°, 77 relative to D56 Alpha 2°, and 135 relative to
185 naive controls, with only 7 to 19 shared between any two contrasts (Figure 3B). Thus, the greatest number
186 of interval-specific transcriptional changes was observed between the protected D21 Alpha 2° and
187 breakthrough D84 Alpha 2° groups. Consistent with this analysis, MA (log-ratio vs. average intensity)
188 plot showed that significant genes spanned the full range of mean normalized expression (Figure 3C).
189 Functional enrichment analysis revealed that genes upregulated in D84 relative to D21 were enriched for
190 adaptive immune response and immunoglobulin complex terms (Figure 3D), whereas the D84 versus
191 D56 and D84 versus naive contrasts were dominated by metabolic, transport, cytoskeletal, RNA
192 processing, and cell cycle terms (Supplementary Figures 5C, 5D). Taken together, these data indicate
193 that the breakthrough observed at the D84 interval was characterized by a diminished GC transcriptional
194 program and increased expression of plasma cell, atypical B cell, and resting B cell-associated programs.

195 **Heterologous SARS-CoV-2 Beta secondary exposure results in productive infection without**
196 **preferential recall of Alpha or Beta-specific neutralizing antibodies**

197 We next asked whether pre-existing immunity developed toward the Alpha variant of SARS-CoV-2 could
198 protect against Beta variant infection, where the Beta variant carries the receptor-binding domain
199 substitutions K417N/T, E484K, and N501Y associated with partial escape from established SARS-CoV-
200 2 immunity^{19–21}. Mice primed with Alpha ($10^{4.15}$ TCID₅₀) were secondarily challenged with Beta (10^5
201 TCID₅₀) on day 21 (D21 Beta 2°), 56 (D56 Beta 2°), or 84 (D84 Beta 2°) ppi, matching the intervals used
202 for homologous secondary challenges (Figure 4A). To analyze the secondary responses against a
203 baseline, we first profiled primary Beta (Beta 1°) infection in naive C57BL/6J mice (Supplementary
204 Figure 6). Beta 1° animals gained weight steadily, passing 110% of baseline by day 12 (Supplementary
205 Figure 6A). Infectious virus in the lung and nasal turbinate peaked at day 2 ppi, reaching an average of

206 $10^{5.2}$ TCID₅₀/mL and $10^{4.5}$ TCID₅₀/mL in the right lung and nasal turbinate, respectively, and then cleared
207 (Supplementary Figure 6B, lower panels). Genomic vRNA analysis revealed a similar early peak, but it
208 declined and lingered after the clearance of the viral infection (Supplementary Figure 6B, upper panels).
209 Histological analyses indicated that the lungs of Beta 1° infected animals had low percentages of the
210 lung affected, with low corresponding scores for inflammation, blood vessels, bronchioles, and overall,
211 with no significant day-to-day differences (Supplementary Figures 6C, 6D). The adaptive response,
212 however, was intact in the Beta primary infected animals. Neutralizing antibody titers increased over the
213 two weeks, reaching GMT 53 by day 14 ppi (Supplementary Figure 6E). In the MLN, the T-cell-
214 associated genes *CD3*, *CD4*, *CD8*, *CXCR3*, and *IL-12* were upregulated, reaching peak expression on
215 day 14 ppi at ~11, 9, 13, 12, and 12-fold increases over baseline, respectively. Similarly, the GC
216 regulators *BCL6* and *AID* also peaked on day 14 ppi, exhibiting a 31- and 38-fold increase over baseline,
217 respectively (Supplementary Figures 6F, 6G).

218 The cellular response developed gradually, and the timing of the maximum responses differed by tissue
219 (Supplementary Figures 6H, 6I). CD4⁺ T cells peaked on day 6 in the MLN and lung, averaging 1.9×10^5
220 and 7.1×10^4 cells, and on day 9 in the spleen, averaging 1.4×10^7 cells (Supplementary Figure 6H,
221 upper row). EM T cells expanded earliest in the spleen, reaching an average of 5.8×10^5 cells on day 6,
222 and later in the MLN and lung, on day 9 reaching 3.4×10^3 and 5.05×10^3 cells, respectively
223 (Supplementary Figure 6H, middle row). T_{FH} cells showed the same pattern, with an early day-2 peak in
224 the MLN (4.2×10^3 cells), a late day-9 peak in the lung (1.1×10^4 cells), and a day-14 rebound in the
225 spleen (4.7×10^6 cells) after an early dip (Supplementary Figure 6H, lower row). Total B, MBC, and GC
226 B cell numbers all increased across the spleen, MLN, and lung, with GC B cells rising progressively to
227 their highest levels at day 14 in every tissue, averaging 1.6×10^6 , 1.8×10^3 , and 5.0×10^4 cells in the
228 spleen, MLN, and lung, respectively (Supplementary Figure 6I).

229 The heterologous secondary challenge groups had different profiles compared to the homologous
230 secondary challenges. All Beta 2° animals lost weight following secondary challenge, in contrast to
231 time-matched homologous Alpha 2° counterparts (Figure 4B). Consistent with the clinical findings, live
232 virus was recovered from both the lung and the nasal turbinate of all three Beta 2° groups on day 2 psi,
233 indicating that Alpha priming did not deliver sterilizing immunity against Beta challenge. Interestingly,
234 lung viral load increased as time increased from primary infection, where those receiving a secondary
235 challenge on day 21, 56, and 84 had titers of $10^{3.8}$, $10^{5.2}$, and $10^{5.8}$ TCID₅₀/mL, respectively. The nasal
236 turbinate viral titers had a similar trend: $10^{0.9}$ (D21) to $10^{2.4}$ (D56) to $10^{4.1}$ (D84) TCID₅₀/mL (Figure 4C).
237 Genomic vRNA remained detectable across multiple psi time points (Figure 4D). On day 2 psi, lung
238 vRNA reached $10^{6.4}$, $10^{8.6}$, and $10^{9.9}$ equivalent TCID₅₀/mL in the D21, D56, and D84 Beta 2° groups,
239 respectively, exceeding the levels observed in the corresponding time-matched Alpha 2° groups at each
240 time point.

241 We next measured Alpha and Beta-specific neutralizing antibody titers throughout the heterologous
242 secondary exposure (Figure 4E). In all three Beta 2° groups, neutralizing antibody titers increased
243 following secondary challenge, reaching highest levels at day 9 psi. At this peak, Alpha and Beta-specific
244 titers were comparable within each group, with no significant differences at any psi time point (D21 Beta
245 2°, GMT 1016 and 1280; D56 Beta 2°, 1016 and 1016; D84 Beta 2°, 1016 and 508), indicating no
246 preferential recall of either Alpha- or Beta-specific neutralizing antibodies. Taken together, primary
247 Alpha immunity neither blocked Beta infection nor prevented Beta-associated weight loss, and the
248 neutralizing response it recalled did not distinguish the priming Alpha variant from the challenge Beta
249 variant.

250

251 **Heterologous SARS-CoV-2 Beta secondary exposure produces bronchiolar lung pathology that is**
252 **absent during homologous Alpha rechallenge**

253 To determine whether the timing and antigenic identity of secondary exposure influenced lung pathology,
254 we evaluated H&E-stained lung sections from all homologous and heterologous rechallenge groups
255 across five histopathological categories (Figure 5A). Both homologous Alpha 2° and heterologous Beta
256 2° animals exhibited interstitial and perivascular inflammation. However, bronchiolar pathology,
257 characterized by epithelial vacuolar degeneration, necrosis, and hyperplasia, was observed exclusively
258 in the Beta 2° groups (Figure 5A). The percentage of affected lung tissue, overall inflammation scores,
259 and perivascular inflammation scores did not differ significantly between homologous and heterologous
260 challenge groups. In contrast, bronchiolar pathology scores were significantly higher following
261 heterologous Beta secondary exposure, reaching 2.0/4 in the D56 and 2.3/4 in the D84 Beta 2° groups
262 on day 2 psi and 1.7/4 in the D21 Beta 2° group on day 6 psi, whereas homologous Alpha 2° animals
263 consistently scored 0/4 throughout the study. Notably, bronchiolar lesions were absent following
264 homologous Alpha rechallenge and remained minimal during primary Beta infection (Supplementary
265 Figures 1B and 6D), indicating that this profile was associated with heterologous secondary exposure
266 rather than with Beta infection alone. Collectively, these findings identify bronchiolar pathology as the
267 distinguishing pulmonary lesion associated with heterologous Beta-secondary exposure.

268 **Heterologous Beta secondary exposure redistributes T_{FH} and effector T-cell responses to**
269 **respiratory tissues and uncouples T_{FH} expansion from coordinated GC B-cell responses**

270 To define the cellular response to heterologous Beta secondary challenge, we compared Beta 2° animals
271 with their time-matched Alpha 2° counterparts by MLN gene expression (Figure 6A) and flow cytometry
272 (Figure 6B; Supplementary Figure 6A). As in homologous rechallenge, T cell genes were broadly
273 downregulated relative to day 0 psi baselines, with *Cxcr5* and *Cxcr3* most consistently suppressed (nadir

0.1-fold at day 14 in D21 and D84 Beta 2° animals). Each interval retained a distinct residual signature. D21 Beta 2° animals induced only *Il12b* (3.8-fold, day 14), D56 animals mounted a sustained *IL-2* response (8.0-fold at day 2, 9.7-fold at day 14), and D84 animals had a sharp but transient day 2 induction of *CD3* (12.0-fold), *Il2* (13.8-fold), and *Cd8a* (4.8-fold), with *Cxcr5* remaining suppressed (0.3-fold). Flow cytometric analysis revealed tissue-specific differences in T and B cell responses (Figure 6B, 7B; Supplementary Figures 7A, 7B). Splenic CD4⁺ T cells were significantly higher in homologous animals at the D21 and D56 intervals (peaks 6.8×10^7 versus 1.4×10^7 , and 4.0×10^7 versus 1.2×10^7) with no difference at D84, and splenic EM T and T_{FH} cells were likewise higher in D21 Alpha 2° animals (Figure 6B; Supplementary Figure 7A). In the MLN and lung, the direction reversed: Beta 2° animals accumulated significantly more CD4⁺, EM T, and T_{FH} cells at multiple intervals, with the largest differences in the lung.

We then asked whether the T_{FH} cells accumulating in the MLN and lung supported a GC response (Figure 7). All three Beta 2° groups suppressed *Bcl6* and *Aicda* early. In D21 Beta 2° animals, the suppression was sustained across the time course (0.1- and 0.0-fold by day 14) and was accompanied by the highest *Il6* induction recorded in this study (61.7-, 48.5-, and 69.1-fold on days 2, 6, and 14), together with *Il4* (27.4-fold, day 2) and *Il21* (5.7-fold, day 6). D56 and D84 Beta 2° animals partially recovered *BCL6* and *Aicda* by day 14 (3.9- and 4.5-fold for D56; 3.4- and 5.8-fold for D84 *Bcl6* and *Aicda*, respectively) and induced correspondingly less *Il6* (22.2- and 5.5-fold at day 2, D56 and D84, respectively); D56 animals were additionally marked by *Prdm1* induction (15.6-fold, day 6) and sustained *Il4* across the time course. Across the heterologous groups, the magnitude of *Il6* induction tracked inversely with the recovery of germinal centre transcription (Figure 7A). Flow cytometry showed a similar inverse relationship (Figure 7B). MBC counts were comparable between challenge types in the spleen and MLN at D21 but significantly higher in Beta 2° animals in the MLN at D56 and D84, and in the lung across intervals. GC B cells, by contrast, were tissue dependent. Alpha 2° animals held significantly more GC B cells than

time-matched Beta 2° animals in the spleen and MLN at both the D21 and D56 intervals (at D56: spleen, Alpha 2° 7.1×10^6 versus Beta 2° 4.0×10^5 ; MLN, Alpha 2° 8.4×10^3 versus Beta 2° 3.3×10^3), whereas Beta 2° animals held significantly more in the lung (at D56: Beta 2° 5.4×10^5 versus Alpha 2° 5.4×10^4). By D84, MLN GC B cells had fallen to similarly low levels in both groups (Alpha 2° 2.5×10^3 versus Beta 2° 3.5×10^3). Together, these findings demonstrate that heterologous Beta secondary exposure reshapes rather than uniformly suppresses secondary immunity. Although Beta 2° animals accumulated higher T_{FH} and effector T-cell populations within the lung and MLN, these responses were not accompanied by coordinated GC B-cell expansion. Instead, heterologous secondary responses promoted inflammatory and plasma cell-associated B-cell transcription, increased MBC formation, and produced tissue-specific alterations in GC B-cell responses.

Long-term homologous and heterologous secondary responses differ in B-cell mobilization but converge on GC B-cell frequency

Long-term protection following primary infection is expected to wane, but whether the capacity to mount a secondary GC response persists after protective immunity has declined remains unknown. This distinction is important because durable immunological memory may persist even after measurable protection has been lost, potentially allowing a subsequent exposure to rapidly re-engage B-cell responses. We therefore asked whether secondary GC responses could still be recalled one year after primary infection, and whether this capacity differed between homologous and heterologous re-exposure. Alpha-primed mice were consequently challenged on day 365 ppi with either Alpha (D365 Alpha 2°) or Beta (D365 Beta 2°) and sampled at days 0, 2, 6, 9, and 14 psi (Figure 8). Because absolute cell numbers had contracted to their lowest levels at this interval, we report frequencies relative to total live cells or the relevant parent population rather than absolute counts. EM T cells and T_{FH} cells were expressed as a proportion of CD4⁺ T cells, whereas GC B cells were expressed relative to the total B-cell population.

CD4⁺ T cell frequencies contracted sharply after secondary challenge in both groups and then recovered on tissue-specific schedules (Figure 8A, upper row). In the spleen, both groups fell from a shared day 0 baseline of 16.6% to between 3.5 and 6.3% through day 9, after which D365 Alpha 2° animals recovered to 14.9% by day 14 while D365 Beta 2° animals reached only 5.9% (Figure 8A, upper row, left panel). The MLN differed in timing rather than magnitude, with D365 Alpha 2° peaking early (11.8% on day 2 against 6.1% in D365 Beta 2°) and D365 Beta 2° peaking later (11.6% on day 6 against 5.6%). Both groups returned to near-baseline frequencies by day 14 (15.3 and 12.8%) (Figure 8A, upper row, middle panel). In the lung, D365 Alpha 2° frequencies declined progressively to 1.5% on day 9 and 0.9% on day 14, whereas D365 Beta 2° animals retained CD4⁺ T cells at 8.1 and 4.4% on those days (Figure 8A, upper row, right panel), consistent with the preferential accumulation of CD4⁺ T cells in respiratory tissue after heterologous secondary challenge observed at the earlier intervals (Supplementary Figure 7A). EM T and T_{FH} cells were undetectable in spleen and MLN in both groups at every time point, with the only measurable lung signal reflecting a single animal (Figure 8A, middle and lower rows). No significant differences between homologous and heterologous secondary challenge were detected in any tissue for either subset.

Against this shared T cell collapse, the B cell compartments diverged in size but not in the GC compartment (Figure 8B). D365 Alpha 2° animals expanded splenic B cell frequencies from 43.7% to 67.4% by day 9 and MLN frequencies from 36.1% to 58.3%, whereas D365 Beta 2° animals declined from 17.6 to 24.7% and 15.0 to 30.0%, respectively. The lung percentages differed significantly only on day 9 (23.8% versus 6.1%). GC B cell frequencies, however, were comparable between the groups in all three tissues, peaking at 16.1% (spleen) and 14.8% (MLN) in homologous animals on day 2 and at 26.4% in the heterologous lung on day 14. Collectively, these findings demonstrate that one year after primary infection, homologous and heterologous secondary recall responses differed in the magnitude of B-cell mobilization but not in the proportion of B cells occupying the germinal centre compartment.

346 Discussion

347 A second infection with SARS-CoV-2 is common in individuals with pre-existing immunity, but the
348 cellular mechanisms that distinguish contained from productive secondary infection remain incompletely
349 defined. Using homologous and heterologous secondary exposure in C57BL/6J mice, we identify a time-
350 dependent loss of protection against homologous Alpha secondary challenge, with protection retained at
351 D21 and D56 but lost by D84. This transition was associated with contraction of T_{FH} and GC B-cell
352 compartments in the draining MLN rather than waning circulating neutralizing antibody titres. By
353 contrast, heterologous Beta exposure resulted in breakthrough infection across all tested intervals. Beta-
354 exposed animals mobilized comparable or greater numbers of T and B cells yet suppressed *Bcl6* and
355 *Aicda* and induced *Il6*, consistent with reduced GC commitment and a shift toward an inflammatory B-
356 cell differentiation program associated with extrafollicular responses^{22–26}. Importantly, neutralizing
357 antibody titres alone were insufficient to explain protection in the homologous groups. Despite similar
358 titres at secondary challenge across the D21, D56, and D84 groups, only D84 animals supported
359 productive viral replication. These findings are consistent with reports that circulating antibody is an
360 imperfect correlate of sterilizing protection once cellular memory remains partially intact^{5,8}.

361 Targeted MLN gene expression analysis further showed that *Bcl6* and *Aicda* remained similarly low
362 across all secondary challenge groups, including the protected homologous groups. Thus, attenuation of
363 GC transcriptional markers relative to primary infection appears to be a general feature of secondary
364 recall or responses in this model rather than a specific feature of breakthrough infection. This is consistent
365 with the constrained dynamics of secondary GC responses, including limited MBC re-entry and altered
366 clonal selection compared with primary responses in prime-boost models^{27–30}. Instead, the measurements
367 that best distinguished the protected from breakthrough animals were cellular and transcriptome-wide.
368 By the time breakthrough virus became detectable (day 2 psi), D84 animals exhibited the lowest MLN

369 T_{FH} and GC B-cell responses together with a transcriptional shift away from GC-associated signatures
370 and toward plasma cell differentiation and atypical/resting B-cell signatures. These findings suggest that
371 delayed homologous secondary challenges increasingly engage pre-existing humoral memory at the
372 expense of productive GC reactivation, potentially sustaining circulating neutralizing antibody titres
373 while failing to restore the local immune architecture required for rapid viral control.

374 In the MLN, Beta 2° animals exhibited reduced *Bcl6* and *Aicda* expression from day 2 and induced *Il6*
375 to the highest fold changes recorded in this study (Figure 7A). The magnitude of *Il6* induction inversely
376 tracked with recovery of GC-associated transcription by day 14. *Il6*-associated inflammatory
377 environments and *Prdm1* induction have been linked to enhanced extrafollicular plasmablast
378 differentiation at the expense of GC commitment^{23–25}. Similar extrafollicular B-cell activation states have
379 been described in severe human COVID-19, where they correlate with early neutralizing antibody
380 production alongside adverse clinical outcomes³¹. The elevated MBC numbers observed in the absence
381 of corresponding GC expansion are compatible with altered MBC differentiation dynamics rather than
382 simply increased GC output^{32–34}. These findings parallel our previous observations in Syrian hamsters,
383 in which heterologous coronavirus challenge similarly elicited a qualitatively altered secondary immune
384 response dependent on the similarity of the first and second exposures¹⁵.

385 The GC deficit was tissue-specific rather than global. MLN GC B-cell numbers were lower in Beta 2°
386 than in time-matched Alpha 2° animals at the D21 and D56 intervals, whereas lung GC B-cell numbers
387 showed the opposite pattern (Figure 7B). These findings suggest that GC-like B-cell responses are
388 differentially regulated across tissues, with pulmonary responses potentially occurring independently of
389 the organized GC response in the draining MLN.

390 Extending the model to one year after priming revealed distinct patterns of long-term maintenance within
391 the T- and B-cell compartments. EM T-cell and T_{FH} frequencies declined to or below the limit of detection

in the spleen and MLN of both groups, with no difference between homologous and heterologous secondary challenge. Previous studies have shown that T_{FH} populations contract substantially following resolution of infection in the absence of sustained survival signals^{33,35}, and SARS-CoV-2-specific CD4⁺ T-cell memory has been reported to decline over several months after infection³⁶. D365 Alpha 2° animals expanded total B-cell frequencies in the spleen and MLN, whereas D365 Beta 2° animals did not, yet GC B-cell frequencies remained comparable between groups across all tissues examined. Thus, one year after priming, the homologous advantage appears to reside in enhanced B-cell recruitment rather than differential GC commitment. This differs from the response observed at D21 and D56, when homologous and heterologous challenge produced distinct GC outputs despite similar levels of immune cell mobilization. The progressive contraction of the memory compartment may therefore obscure the qualitative GC defect associated with heterologous challenge at later time points. Increasing evidence indicates that recall outcomes are shaped by qualitative properties of the residual MBC pool, including anatomical localization, affinity, and competition from pre-existing antibody^{28,29,37,38}. These factors provide a potential explanation for the divergence observed one year after priming, where differences in B-cell recruitment persisted despite comparable GC frequencies following secondary exposure.

Heterologous exposure was also the only condition that produced bronchiolar injury, albeit minor pathology. Homologous Alpha 2° and primary Beta 1° animals exhibited no and minimal detectable bronchiolar pathology, respectively, across all intervals and time points (Supplementary Figure 1B), whereas Beta 2° infection produced marked bronchiolar changes at multiple psi time points (Figure 5B). Beta 2° animals also lost weight while Beta 1° animals did not, despite comparable early replication. These observations may suggest that the Alpha-primed response to Beta antigen contributes to tissue injury rather than merely failing to prevent it. Similar enhancement of pathology after heterologous coronavirus exposure has been reported in K18 mice and Syrian hamsters^{39,40}.

415 Our study has limitations. The C57BL/6J model does not reproduce the full spectrum of human SARS-
416 CoV-2 respiratory disease; however, it does provide a preclinical model susceptible to antigenically
417 distinct viruses that enable us to dissect cellular mechanisms governing secondary immunity due to the
418 abundance of immunological reagents for mice. We did not measure mucosal antibody responses, which
419 may contribute to the sterilizing protection observed at the D21 and D56 intervals⁴¹. In addition, the
420 Alpha-to-Beta comparison represents a limited antigenic distance relative to later Omicron variants, and
421 whether increasing antigenic divergence further alters GC recall capacity remains an important
422 question^{4,42,43}. Finally, bulk RNA sequencing of whole MLN cannot resolve transcriptional programs at
423 the cellular level, and GC responses were defined by Fas and GL7 expression rather than spatial
424 confirmation. Future studies incorporating spatial approaches and cellular resolution transcriptomics will
425 be required to further define the anatomical and molecular organization of recall responses.

426 These findings have direct implications for booster timing and cross-variant vaccine design. A key
427 determinant of recall protection and secondary responses may be the capacity to re-establish a productive
428 GC response in the draining lymph node rather than circulating antibody titres alone, which remained
429 comparable despite loss of protection at D84. The extrafollicular bias observed following heterologous
430 exposure suggests that variant-updated vaccines may need to overcome immune imprinting and promote
431 productive GC engagement to achieve broader protection^{4,44,45}. Together, these findings redefine recall
432 protection and secondary responses as a function of cellular immune organization rather than antibody
433 abundance alone and identify GC recall capacity as a central consideration for future vaccine design.

434 **Methods**

435 **Ethics statement**

436 All animal work was performed in accordance with the guidelines of the Canadian Council on Animal
437 Care (CCAC) under Animal Use Protocol (AUP) number 20210033, approved by the University Animal
438 Care Committee (UACC) Animal Research Ethics Board at the University of Saskatchewan. All mouse
439 procedures were conducted under 5% isoflurane anesthesia. All work involving risk group 3 (RG3)
440 pathogens was performed in the Vaccine and Infectious Disease Organization's (VIDO) Containment
441 Level 3 (CL3) facility (InterVac), Saskatoon, Saskatchewan, Canada. Biosafety protocols were approved
442 by the University of Saskatchewan Biosafety Protocol Approval Committee (BPAC) and overseen by
443 VIDO's Biosafety Officer.

444 **SARS-CoV-2 variants**

445 SARS-CoV-2 Alpha (GenBank accession OQ781006) and SARS-CoV-2 Beta (NCBI BioProject
446 accession SAMN24618764) were obtained from clinical isolates collected at Alberta Health Services and
447 the Roy Romanow Provincial Laboratory, respectively. Both variants were propagated on Vero-76 cells
448 in viral DMEM (vDMEM), consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Cat.
449 #11965092) supplemented with 2% fetal calf serum (Wisent Bioproducts, Cat. #090-150), 100 U/mL
450 penicillin and 100 µg/mL streptomycin, and 2 µg/mL TPCK-trypsin. Viral stocks were titrated by TCID₅₀
451 assay on Vero-76 cells before use in animal experiments. All work with infectious SARS-CoV-2 was
452 performed within the VIDO CL3 facility.

453 **Animals, experimental design, and sample collection**

454 Female wildtype C57BL/6J mice (Jackson Laboratories, Bar Harbor, USA; Strain No. 000664), aged 6-
455 8 weeks at the time of primary inoculation, were used throughout. All inoculations were performed

456 intranasally under 5% isoflurane anesthesia in a volume of 50 μ L per animal. Ten experimental groups
457 were established as follows (Group numbers and descriptions in Table 1): Group 1 (Alpha 1 $^{\circ}$) received
458 a primary inoculation with SARS-CoV-2 Alpha at $10^{4.15}$ TCID₅₀ only; Group 2 (Beta 1 $^{\circ}$) received a
459 primary inoculation with SARS-CoV-2 Beta at 10^5 TCID₅₀ only. Groups 3-6 received a primary
460 inoculation with SARS-CoV-2 Alpha at $10^{4.15}$ TCID₅₀, followed by a homologous secondary inoculation
461 with SARS-CoV-2 Alpha at $10^{4.15}$ TCID₅₀ on day 21 (D21 Alpha 2 $^{\circ}$, Group 3), 56 (D56 Alpha 2 $^{\circ}$, Group
462 4), or 84 (D84 Alpha 2 $^{\circ}$, Group 5), or day 365 (D365 Alpha 2 $^{\circ}$, Group 6) ppi. Groups 7-10 received a
463 primary inoculation with SARS-CoV-2 Alpha at $10^{4.15}$ TCID₅₀, followed by a heterologous secondary
464 inoculation with SARS-CoV-2 Beta at 10^5 TCID₅₀ on day 21 (D21 Beta 2 $^{\circ}$, Group 7), 56 (D56 Beta 2 $^{\circ}$,
465 Group 8), 84 (D84 Beta 2 $^{\circ}$, Group 9), or day 365 (D365 Beta 2 $^{\circ}$, Group 10) ppi. For all secondary
466 exposure groups, samples were collected on days 0, 2, 6, 9, and 14 ppi, corresponding to the respective
467 days post primary inoculation indicated in Table 1. For primary-only control groups (Groups 1 and 2),
468 samples were collected on days 0, 2, 6, 9, and 14 ppi. At each time point, three animals per group were
469 humanely euthanized by isoflurane overdose and subjected to full necropsy for the collection of the right
470 lung, nasal turbinates, spleen, MLN, and blood. Four animals per group per time point were euthanized
471 for the collection of spleen, MLN, lung, and blood for flow cytometry analyses. Ten animals per group
472 were maintained through day 14 post-inoculation to monitor survival and body weight based on pre-
473 defined humane intervention criteria. Body weight was recorded daily and expressed as a percentage of
474 each animal's pre-inoculation baseline weight.

475 **Tissue homogenization and TCID₅₀ assay for live viral load**

476 Nasal turbinates and the right lung were collected at necropsy and immediately homogenized in serum-
477 free DMEM using a Qiagen TissueLyser II. Clarified homogenates were used for both TCID₅₀ and qRT-
478 PCR analyses. To quantify live infectious virus, 10-fold serial dilutions of clarified tissue homogenates

479 were prepared in vDMEM and inoculated in triplicate onto confluent Vero-76 cells in 96-well flat-bottom
480 plates. Plates were incubated for 1 hour at 37°C with 5% CO₂, after which inocula were removed and
481 replaced with fresh vDMEM. Plates were incubated for 5 days at 37°C with 5% CO₂, and cytopathic
482 effect (CPE) was monitored daily. CPE results recorded on day 5 post-inoculation were used to calculate
483 TCID₅₀/mL by the Reed-Muench method. The limit of detection was 10^{1.0} TCID₅₀/mL.

484 **Viral RNA quantification by qRT-PCR**

485 Tissue RNA was extracted from homogenized nasal turbinate and lung samples using the Qiagen RNeasy
486 Mini Kit (Cat. #74106; Qiagen, Toronto, Canada) according to the manufacturer's instructions. SARS-
487 CoV-2 genomic viral RNA (vRNA) was quantified by quantitative reverse transcription PCR (qRT-PCR)
488 using Qiagen QuantiFast RT probe master mix with primer and probe sets targeting the human
489 coronavirus envelope (E) gene, as previously described⁴⁶. Reactions were performed in duplicate or
490 triplicate on a StepOnePlus Real-Time PCR System in 96-well format (Thermo Fisher Scientific). Viral
491 RNA levels are expressed as equivalent TCID₅₀/mL, calculated by interpolation from a standard curve
492 generated from serial dilutions of a known-titer virus stock run in parallel.

493 **Microneutralization assay**

494 Blood was collected at necropsy into EDTA-coated tubes and centrifuged to yield plasma. Plasma
495 samples were heat-inactivated at 56°C for 30 minutes before use. Serial two-fold dilutions of heat-
496 inactivated plasma were prepared in low-serum vDMEM in round-bottom 96-well plates, beginning at a
497 1:10 starting dilution. SARS-CoV-2 Alpha or SARS-CoV-2 Beta virus was diluted to 100 TCID₅₀ per
498 well in vDMEM and added at a 1:1 ratio to diluted plasma (60 µL plasma dilution plus 60 µL diluted
499 viruses per well). Plasma-virus mixtures were incubated at 37°C for 1 hour and then transferred onto
500 confluent Vero-76 cells in 96-well plates. After a further 1-hour adsorption at 37°C, plasma-virus
501 mixtures were removed and replaced with fresh vDMEM. Plates were monitored daily for CPE for 5

502 days. The endpoint neutralization titer was defined as the reciprocal of the lowest plasma dilution that
503 completely suppressed CPE on day 5 post-inoculation. Results are expressed as endpoint titer (1/x)
504 geometric mean titer (GMT).

505 **RNA extraction and host gene expression by PrimePCR**

506 MLN were collected at necropsy and immediately placed in RNAlater (Qiagen) for storage at -80°C until
507 processing. Tissue RNA was extracted using the Qiagen RNeasy Mini Kit as described above. cDNA
508 was synthesized from extracted RNA as previously described⁴⁷. Host gene expression was assessed using
509 BioRad PrimePCR assays (Bio-Rad Laboratories, Hercules, USA) designed against the following mouse
510 gene targets: *Cd3e*, *Cd4*, *Cd8a*, *Cxcr5*, *Cxcr3*, *Il2*, *Il12b*, *Cd19*, *Bcl6*, *Aicda*, *Prdm1*, *Il21*, *Il6*, *Tnf*, and
511 *Il4* (see Supplementary Table 1 for full assay details). All reactions were performed in triplicate on a
512 QuantStudio 6 Real-Time PCR System in 384-well format (Thermo Fisher Scientific) according to the
513 manufacturer's instructions. Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method, where
514 $\Delta\Delta Ct$ was derived by first normalizing the Ct of each target gene to the Ct of the housekeeping gene
515 beta-actin (*BACT*) within each sample ($\Delta Ct = Ct_{\text{target}} - Ct_{\text{BACT}}$), and then normalizing each sample's
516 ΔCt to the mean ΔCt of the respective group's day 0 psi baseline samples ($\Delta\Delta Ct = \Delta Ct_{\text{sample}} -$
517 $\Delta Ct_{\text{baseline}}$). Fold changes greater than 1 represent upregulation relative to baseline; fold changes less
518 than 1 represent downregulation. The colour scale was capped at 90-fold; no value exceeded this cap.
519 Values exceeding 15 and below 90 are represented in red in heatmap displays. A minimum of three
520 animals were analyzed per group per time point.

521 **Quality assessment, alignment, assembly, and estimation of differentially expressed genes**

522 Initially, the Fastp (v. 0.20.0) (<https://github.com/OpenGene/fastp>), an ultra-fast tool for quality control,
523 adapter trimming, and filtering of raw reads, was employed for the preprocessing and subsequently
524 cleaned reads were aligned to the mouse reference genome GRCm39 (GCA_000001635.9) using the

525 HISAT2 (v.2.1.0) (<https://github.com/DaehwanKimLab/hisat2>) package. In the next step, the SAMtools
526 package (v. 1.21) (<https://github.com/samtools/>) was utilized to convert the resulting SAM files into
527 BAM files to generate sorted alignment files, followed by calculating the mapping statistics. The
528 resulting sorted BAM files were considered for the transcript assembly and quantification using the
529 StringTie tool (v. 2.1.1) (<https://github.com/gpertea/stringtie>) with reference to the genome annotation
530 file of the mouse genome, *Mus_musculus.GRCm39.115.gtf.gz*, which was obtained from the Ensemble
531 Browser (<https://useast.ensembl.org/index.html>). Subsequently, sample-specific GTF files were
532 generated, followed by calculating the count matrix using the StringTie tool. In the next step, RNA-seq
533 differential gene expression analysis was done using the raw gene-level count matrix and a corresponding
534 metadata table describing the experimental conditions. Differential expressions were computed using the
535 PyDESeq2 implementation of the DESeq2 framework, which models count data using a negative
536 binomial distribution with size factor and normalization and dispersion estimation. Pairwise comparisons
537 were performed between all experimental groups (86, 58, 23, and control), resulting in six contrasts.
538 Unique and shared genes between each contrast were analyzed. For each contrast, log2 fold changes and
539 Wald test p-values were extracted, and multiple testing correction was applied using the Benjamini-
540 Hochberg procedure to control the false discovery rate. Genes with an adjusted p-value <0.05 and
541 absolute log2 fold change ≥ 2 were considered significantly differentially expressed. In addition, a
542 predefined panel of genes on interest was evaluated across all contrasts to assess their differential
543 expression profile. For this subset, log2fold change values from each pairwise comparison were extracted
544 and visualized as a heatmap. All the analyses were executed in Python (version 3.12) using the modules
545 PyDESeq2 (version 0.5.4), numpy (version 2.4), pandas (version 3.0.3), and seaborn (version 0.13.2).

546

547

548 **Gene ontology**

549 Calculated DEGs were considered for the Gene Ontology (GO) analysis using the g: profiler server
550 (<https://biit.cs.ut.ee/gprofiler/gost>), a web server devoted to providing a facility to perform the functional
551 enrichment analysis of hundreds of species. The official gene symbols of the calculated DEGs were
552 uploaded, and functional enrichment analysis was performed with the mouse genome in the background
553 and genes were mapped for the important GO categories, including biological process (BP), molecular
554 functions (MF), cellular components (CC), KEGG pathways, etc. The GO enrichment analysis was
555 performed with the default statistical parameters.

556 **Single-cell suspension preparation**

557 Spleen, MLN, and lungs were collected into FACS buffer (phosphate-buffered saline (PBS)
558 supplemented with 2% fetal bovine serum (FBS) and 2 mM sodium azide (NaN_3)). Before mechanical
559 dissociation, lung tissue was placed in a 2 mL microcentrifuge tube containing digestion buffer consisting
560 of 5 mg/mL Collagenase Type I and 1 mg/mL DNase I in PBS supplemented with 0.5% bovine serum
561 albumin (BSA) and incubated at 37°C for 15 minutes with intermittent agitation. Following digestion,
562 all tissues were mechanically dissociated by passage through 40 μm cell strainers using a sterile syringe
563 plunger and washed with wash medium consisting of RPMI-1640 supplemented with 10 mM HEPES
564 and 50 $\mu\text{g/mL}$ gentamicin. Cell suspensions were pelleted by centrifugation at $800 \times g$ for 4 minutes at
565 4°C, and the wash step was repeated once. Following additional washes, cells were resuspended in FACS
566 buffer and counted using a hemocytometer before plating in 96-well round-bottom plates for downstream
567 staining.

568 **Flow cytometry**

569 All staining steps were performed on ice and in the dark unless otherwise specified. Cell suspensions
570 were centrifuged at $800 \times g$ for 4 minutes at 4°C, and cells were resuspended in 100 μL Fc blocking

571 buffer (2% FBS in PBS) for 20 minutes on ice to block non-specific antibody binding. After
572 centrifugation, cells were resuspended in 50 μ L of live/dead viability stain and incubated on ice for 30
573 minutes in the dark. Cells were then washed and resuspended in 100 μ L of the appropriate surface
574 antibody cocktail (see Supplementary Table 2) and incubated on ice for 30 minutes in the dark. For T
575 cell staining, following live/dead discrimination and exclusion of myeloid cells and B cells using a dump
576 channel containing anti-B220 and anti-CD11b antibodies, CD3+CD4+ T helper cells were identified.
577 From this population, EM T cells were defined as CD44+CD62L-, and T_{FH} cells were defined as
578 CXCR5+PD-1+ (Supplementary Figure 2A). For B cell staining, B220+CD19+ B cells were identified
579 following live/dead discrimination and exclusion of myeloid cells and T cells using a dump channel
580 containing anti-CD3 and anti-CD11b antibodies. CD138+ plasma cells were excluded from further
581 analysis. From the remaining B cell population, GC B cells were defined as Fas+GL7+, and memory B
582 cells were defined as CD38+IgD- (Supplementary Figure 2B).

583 Following surface staining, all samples were inactivated for safe removal from the CL3 facility. Cells
584 were pelleted and resuspended in 200 μ L of 4% paraformaldehyde (PFA) in PBS and incubated for 10
585 minutes at room temperature. Cells were then pelleted, resuspended in 200 μ L of 10% neutral buffered
586 formalin (NBF), and incubated overnight at 4°C. The following day, cells were pelleted, resuspended in
587 fresh 200 μ L of 10% NBF, and transferred out of CL3. After transfer, cells were washed and resuspended
588 in 200 μ L PBS before acquisition. All samples were acquired on a CytoFLEX S flow cytometer
589 (Beckman Coulter). Data were analyzed using FlowJo software version 10 (BD Biosciences). Absolute
590 cell counts or the percentage frequency of cells relative to the total live cells were calculated from the
591 percentage of each gated population.

592

593

594 **Lung histopathology**

595 For histopathological analysis, lungs were perfused with 10% neutral buffered formalin (NBF)
596 immediately following collection at necropsy and kept fully submerged in formalin within the CL3
597 facility for a minimum of 7 days to ensure complete viral inactivation. Formalin-fixed lung tissues were
598 subsequently submitted to Prairie Diagnostic Services (Saskatoon, Saskatchewan, Canada) for paraffin
599 embedding, sectioning, slide mounting, and hematoxylin and eosin (H&E) staining. H&E-stained slides
600 were scanned and digitized at 10× magnification using an Aperio ScanScope XT digital slide scanner
601 (Leica Biosystems, Nußloch, Germany). All histopathological analysis and scoring were performed by a
602 board-certified veterinary pathologist blinded to group assignments using the following criteria:

603 *Percentage Affected:* proportion of pulmonary parenchyma involved by inflammatory or pathological
604 changes: 0, no lesion; 1, <25%; 2, 26–50%; 3, 51–75%; 4, 76–100%.

605 *Inflammation:* density of the parenchymal inflammatory infiltrate: 0, absent (no lesion); 1, slight or
606 questionable; 2, clearly present but not conspicuous; 3, marked; 4, severe.

607 *Blood Vessels:* presence of perivascular aggregates of inflammatory cells, with or without infiltration of
608 the vascular wall: 0, absent; 1, present.

609 *Bronchioles:* degree of bronchiolar epithelial involvement, including hydropic degeneration,
610 intraluminal cellular debris, necrosis, and/or hyperplasia or hypertrophy of the lining epithelium: 0,
611 absent (no lesion); 1, slight or questionable; 2, clearly present but not conspicuous; 3, marked; 4, severe.

612 *Overall Score:* composite assessment of total lung pathology incorporating all above criteria, assigned
613 by the pathologist on the same 0-4 scale. A minimum of three animals per group per time point were
614 assessed for histopathology. Ordinal scores were analyzed and recorded. Representative
615 photomicrographs were captured at 10× magnification; scale bars represent 300 µm throughout.

616 **Data Availability**

617 RNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive
618 (SRA) under BioProject accession number PRJNA1403614. All other data supporting the findings of
619 this study are available within the paper and its supplementary information.

620 **Statistical analysis**

621 All statistical analyses were performed in GraphPad Prism version 11.0.2 (GraphPad Software, San
622 Diego, USA). Comparisons across time points within a single group, and comparisons among more than
623 two groups at a single time point, were performed using the Kruskal-Wallis test with Dunn's correction
624 for multiple comparisons; the non-parametric test was chosen because group sizes were three to five
625 animals per time point and because histopathology data are ordinal. Comparisons between two time-
626 matched groups across the post-inoculation time course were performed using two-way ANOVA with
627 Sidak's correction. Multiple-comparison corrections were applied within each panel. No correction was
628 applied across panels or across the study as a whole, and p values should therefore be interpreted within
629 the comparison family of the panel in which they are reported. TCID₅₀ values were log₁₀-transformed
630 before analysis. Neutralizing antibody data are reported as geometric mean titers (GMT). Fold-change
631 values from PrimePCR and row-scaled expression values from RNA sequencing are presented
632 descriptively; no statistical comparisons were performed on these data. Differential gene expression and
633 Gene Ontology enrichment were analyzed as described above. A two-sided p-value of 0.05 or less was
634 considered significant.

635 Significance is indicated throughout as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$; ns,
636 not significant.

637

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741 **Figure 1: Time-dependent loss of protection from homologous SARS-CoV-2 Alpha reinfection in**
742 **C57BL/6J mice.**

743 **(A)** Experimental schematic. C57BL/6J mice were inoculated intranasally with SARS-CoV-2 Alpha
744 ($10^{4.15}$ TCID₅₀) on day 0 as a primary inoculation (Alpha 1°, red). Secondary exposure groups received
745 a homologous intranasal inoculation with SARS-CoV-2 Alpha on day 21 (D21 Alpha 2°, light pink), day
746 56 (D56 Alpha 2°, teal), or day 84 (D84 Alpha 2°, purple) post-primary inoculation (ppi). All groups
747 were sampled on days 0, 2, 6, 9, and 14 post-primary (ppi) and secondary inoculations (psi). Created
748 with BioRender.com. **(B)** Body weight expressed as a percentage of each animal's baseline weight
749 recorded immediately before inoculation and for 14 days post-inoculation. Data are presented as group
750 mean $\pm$ standard deviation (SD). $n \geq 10$ animals per group. **(C)** Live virus quantification (TCID₅₀/mL,
751 Reed-Muench method) in the right lung (upper row) and nasal turbinates (lower row) at days 0, 2, 6, 9,
752 and 14 ppi and psi. Each point represents one animal. Dashed lines indicate the limit of detection.

753 **(D)** Representative H&E-stained lung sections from Alpha 1°, D21 Alpha 2°, D56 Alpha 2°, and D84
754 Alpha 2° groups at 0, 2, 6, 9, and 14 days post-inoculation. Scale bars = 300 μ m. Images are
755 representative of 3 animals per group per time point. Statistical analyses in Figure C were performed
756 using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p < 0.05$, ** $p < 0.005$,
757 *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant.

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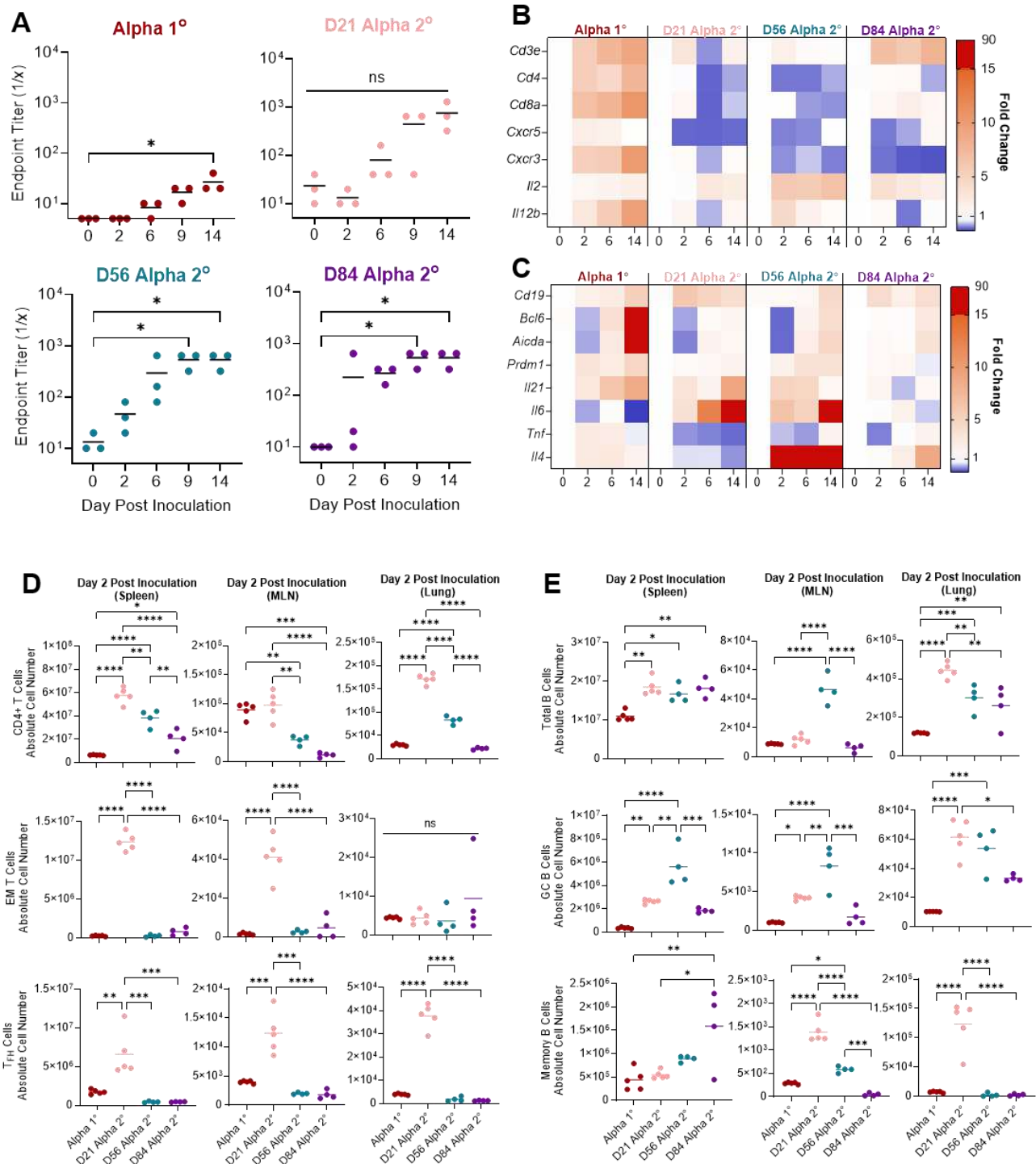


Figure 2: Germinal center cellular dynamics in the mediastinal lymph node and peripheral tissues distinguish protected from unprotected homologous SARS-CoV-2 secondary exposures independently of neutralizing antibody titers

(A) SARS-CoV-2 Alpha neutralizing antibody endpoint titers (1/x) in plasma from homologous secondary exposure groups assessed by microneutralization assay at days 0, 2, 6, 9, and 14 post primary

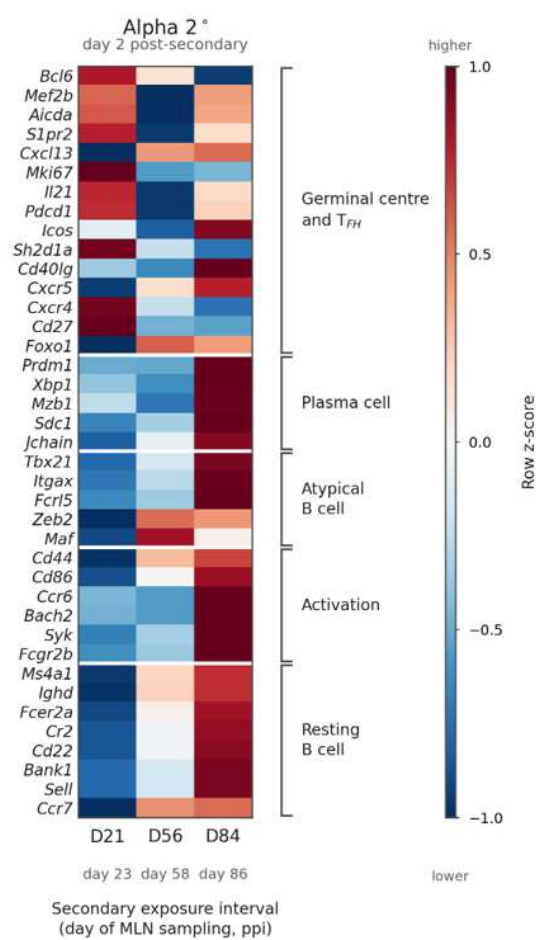
766 inoculation (ppi) and post secondary inoculation (psi). Alpha 1° primary animals are included for
 767 longitudinal comparison. Each data point represents one animal; the line represents the geometric mean.

768 **(B)** Host gene expression heatmap for T cell-associated genes (*Cd3e*, *Cd4*, *Cd8a*, *Cxcr5*, *Cxcr3*, *Il2*,
 769 *Il12b*) assessed by PrimePCR in RNA extracted from the mediastinal lymph node (MLN) of Alpha 1°,
 770 D21 Alpha 2°, D56 Alpha 2°, and D84 Alpha 2° animals at days 0, 2, 6, and 14 ppi and psi. Fold-change
 771 was calculated relative to each group's day 0 baseline, using *BACT* as the housekeeping gene.
 772 Upregulation (fold change >1) is shown from white (= 1) to orange; fold changes >15 and <90 are shown
 773 in red. Downregulation (fold change <1) is shown in blue. Minimum 3 animals analyzed per time point.

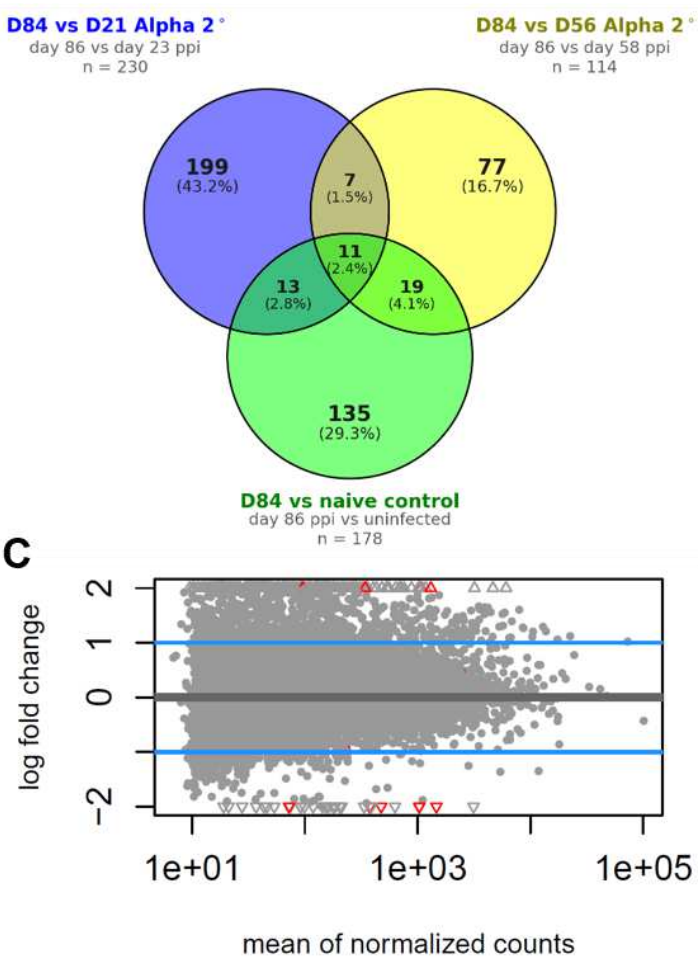
774 **(C)** Host gene expression heatmap for B cell and germinal center (GC)-associated genes (*Cd19*, *Bcl6*,
 775 *Aicda*, *Prdm1*, *Il21*, *Il6*, *Tnf*, *Il4*) in the MLN, presented as described in (B). **(D)** Absolute cell numbers
 776 of CD4⁺ T cells (upper row), EM T cells (middle row; CD44⁺CD62L⁻ gated from CD3⁺CD4⁺
 777 lymphocytes), and T follicular helper (T_{FH}) cells (lower row; CXCR5⁺PD-1⁺ gated from
 778 CD3⁺CD4⁺CD44⁺ lymphocytes), quantified by flow cytometry in the spleen, MLN, and lung at day 2
 779 ppi and psi Alpha inoculation. Bar graphs represent the group mean, and each data point represents one
 780 animal. **(E)** Absolute cell numbers of total B cells (upper row), GC B cells (middle row; Fas⁺GL7⁺ gated
 781 from B220⁺CD19⁺CD138⁻ cells), and MCB (lower row; CD38⁺IgD⁻ gated from B220⁺CD19⁺CD138⁻
 782 cells), quantified by flow cytometry in the spleen, MLN, and lung at day 2 ppi and psi Alpha inoculation.
 783 Bar graphs represent the group mean, and each data point represents one animal. Statistical analyses in
 784 Figures A, D, and E were performed using the Kruskal-Wallis test with Dunn's correction for multiple
 785 comparisons. $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and **** $p < 0.0001$, ns, not significant. Note that
 786 the y-axis scales in Figures D and E differ among immune cell phenotypes and tissue panels.

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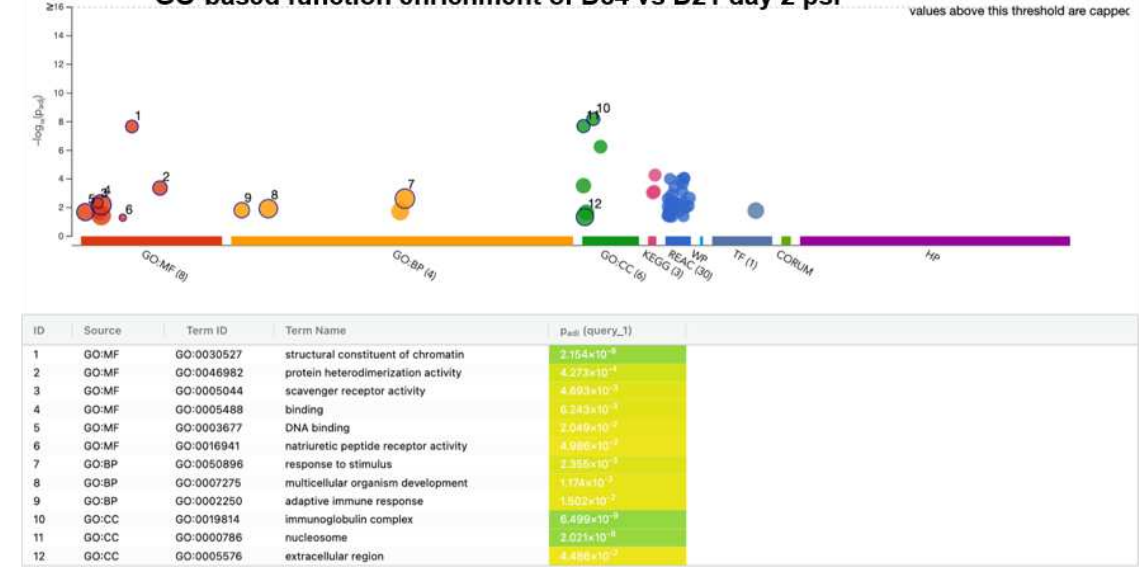


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790 **Figure 3. Transcriptional profiling of the mediastinal lymph node at day 2 post-secondary exposure**
791 **distinguishes a germinal centre recall program in protected animals from a plasma cell and**
792 **atypical B cell program during breakthrough infection.**

793 Mice primed with SARS-CoV-2 Alpha received a homologous Alpha secondary inoculation on day 21,
794 56, or 84 post-primary infection (D21, D56, or D84 Alpha 2°), and the mediastinal lymph node was
795 profiled by bulk RNA sequencing on day 2 post-secondary inoculation (days 23, 58, and 86 ppi,
796 respectively; n = 3 per group). **(A)** Row-scaled expression of curated immune-program genes across the
797 three secondary intervals, grouped by program (germinal centre/T follicular helper, plasma cell, atypical
798 B, activation, and mature/resting B; blocks separated by horizontal lines). Colour indicates the row z-
799 score computed across the three Alpha 2° groups and therefore encodes relative rank across these groups,
800 not expression magnitude or fold change. **(B)** Venn diagram of uniquely upregulated and shared
801 upregulated differentially expressed genes (DEGs), adjusted $p < 0.05$, $|\log_2 \text{fold change}| \geq 2$, for the D84
802 Alpha 2° group relative to D21 Alpha 2° (84 vs 21), D56 Alpha 2° (84 vs 56), and naïve control (86 vs
803 Naïve control). **(C)** MA plot of the D21 and D56 versus D84 Alpha 2° contrast; each point is a gene,
804 plotted as $\log_2$ fold change against mean normalized counts, with horizontal lines at the fold-change
805 threshold and DEGs marked. **(D)** Gene Ontology term enrichment among genes upregulated in D84
806 relative to D21 Alpha 2° (g: Profiler; terms coloured by source, with the tabulated adjusted p-values
807 below; values exceeding the y-axis cap are plotted at the threshold). Group labels denote the secondary
808 inoculation interval; colour scaling is confined to the groups shown in each panel.

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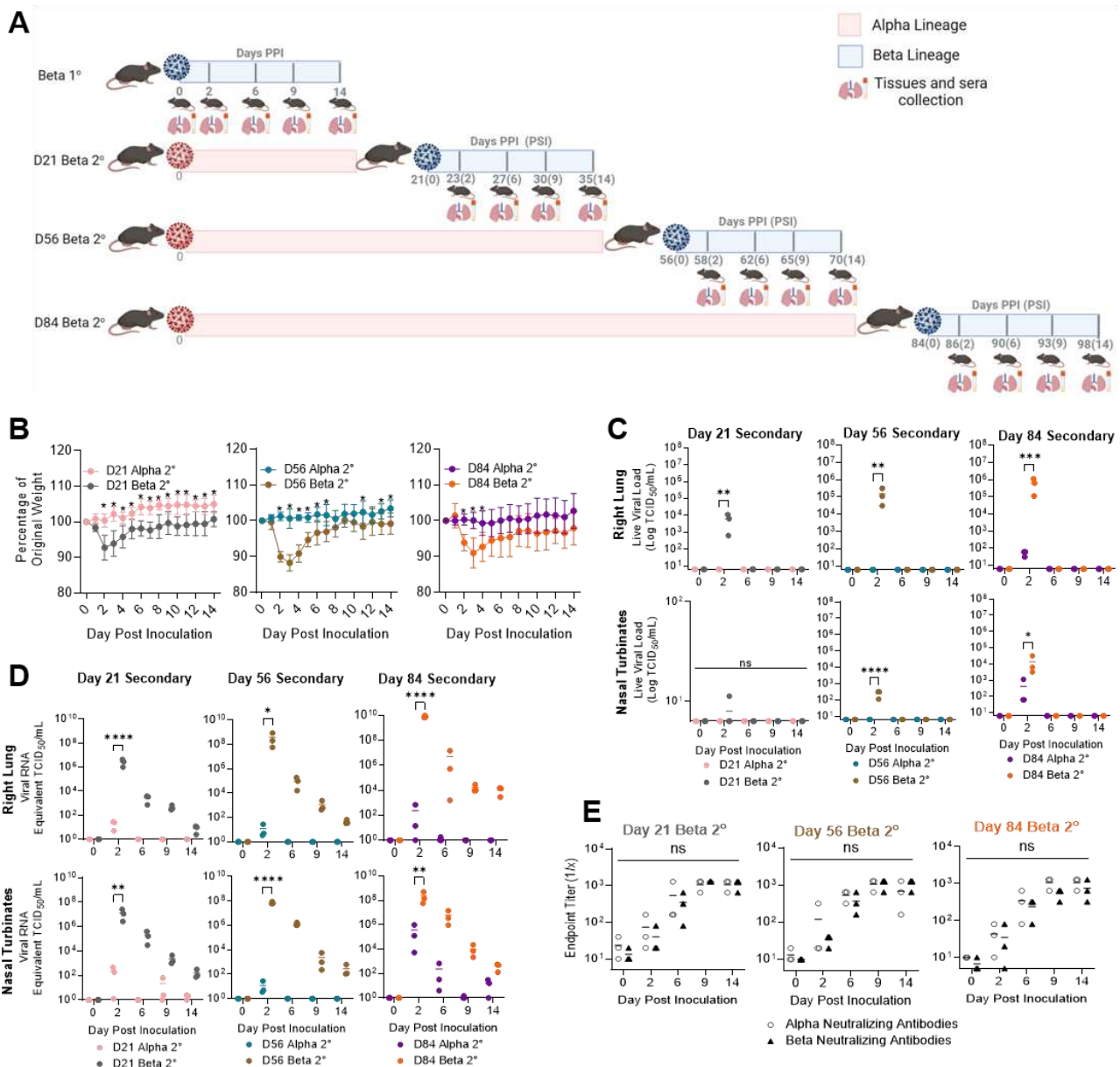


Figure 4: Heterologous SARS-CoV-2 Beta secondary exposure results in productive infection and weight loss at all timepoints, with evidence of immune imprinting in the neutralizing antibody response.

(A) Experimental schematic. Mice that received a primary intranasal inoculation with SARS-CoV-2 Alpha ($10^{4.15}$ TCID₅₀) on day 0 received a heterologous secondary inoculation with SARS-CoV-2 Beta (10^5 TCID₅₀) on day 21 (D21 Beta 2°, grey), day 56 (D56 Beta 2°, golden-brown), or day 84 (D84 Beta 2°, orange) post-primary inoculation (ppi). Beta 1° animals received a primary inoculation with SARS-CoV-2 Beta only. All groups were sampled at days 0, 2, 6, 9, and 14 psi. Created with BioRender.com.

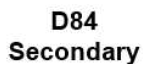
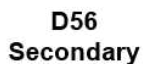
819 **(B)** Body weight expressed as a percentage of pre-inoculation baseline weight for heterologous Beta 2°
820 groups compared to time-matched homologous Alpha 2° groups at each secondary exposure interval
821 (Day 21, Day 56, Day 84). Data are presented as group mean $\pm$ SD. $n \geq 10$ animals per group. **(C)** Live
822 infectious virus quantified by TCID₅₀ assay (Reed-Muench method, Log TCID₅₀/mL) in the right lung
823 (upper row) and nasal turbinates (lower row) at days 0, 2, 6, 9, and 14 psi, organized by secondary
824 exposure time point (Day 21, Day 56, Day 84 Secondary). Heterologous Beta 2° groups are compared
825 to their time-matched homologous Alpha 2° counterparts. Each data point represents one animal. **(D)**
826 Genomic viral RNA (vRNA) quantified by qRT-PCR (Equivalent TCID₅₀/mL) in the right lung (upper
827 row) and nasal turbinates (lower row) at days 0, 2, 6, 9, and 14 post-secondary inoculation (psi),
828 organized by secondary exposure time point. Each data point represents one animal. **(E)** Neutralizing
829 antibody endpoint titers (1/x) against SARS-CoV-2 Alpha (open circle) and SARS-CoV-2 Beta (filled
830 triangles) measured by microneutralization assay in plasma from D21 Beta 2°, D56 Beta 2°, and D84
831 Beta 2° animals at days 0, 2, 6, 9, and 14 psi. Each data point represents one animal; lines represent the
832 geometric mean. Statistical comparisons between Alpha and Beta within each group in figures B-E were
833 performed using two-way ANOVA followed by Sidak's multiple-comparisons post hoc test. ns, not
834 significant. Statistical notation for all panels: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$,
835 ns, not significant.

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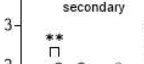
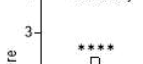
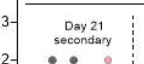
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D21
Secondary



Percentage Affected

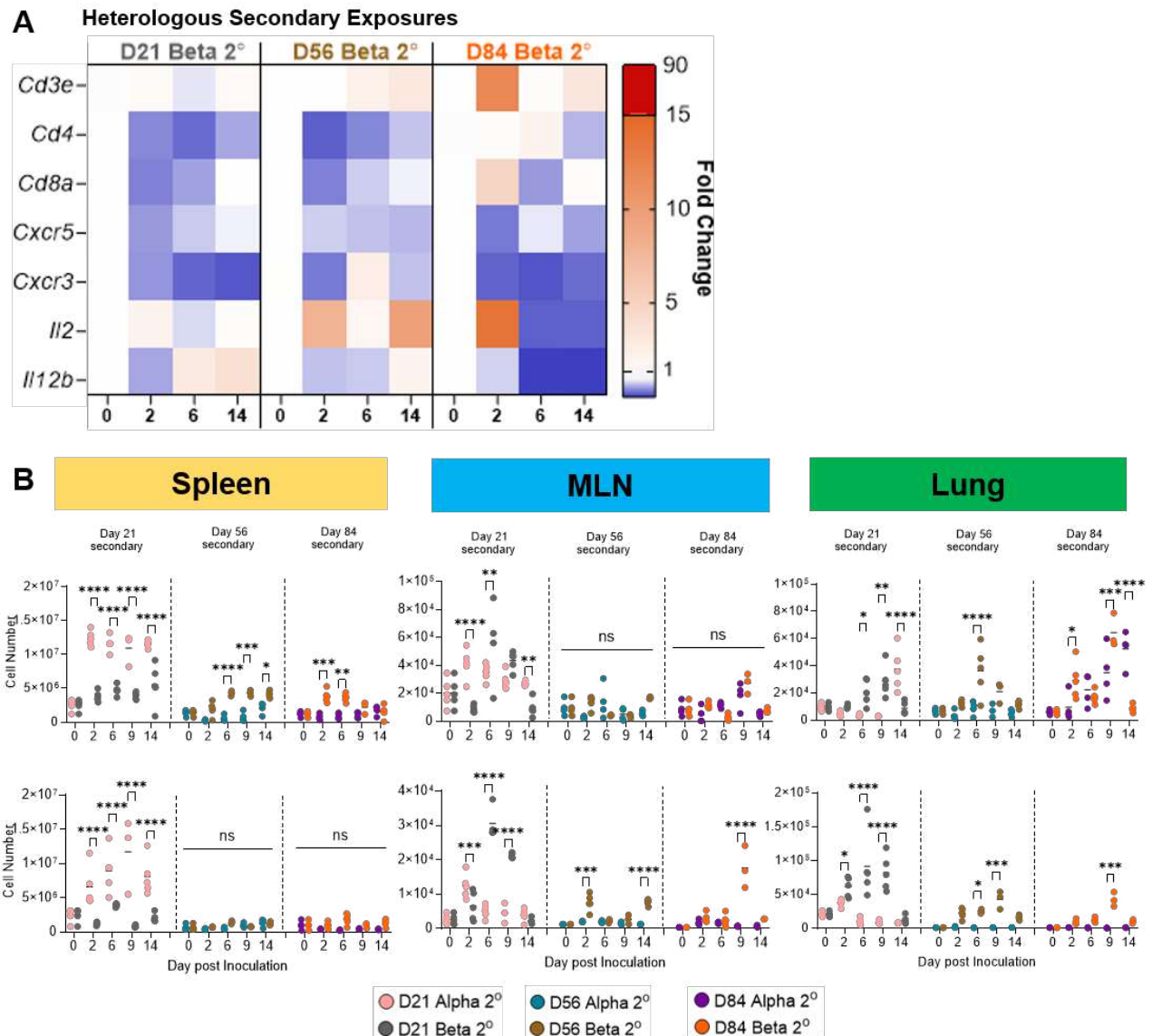


840 **Figure 5: Heterologous SARS-CoV-2 Beta secondary exposure produces bronchiolar lung**
841 **pathology that is absent during homologous rechallenge, regardless of secondary exposure timing.**

842 **(A)** Representative H&E-stained lung sections from homologous Alpha 2° (upper row of each panel) and
843 heterologous Beta 2° (lower row of each panel) animals at days 0, 2, 6, 9, and 14 psi, organized by
844 secondary exposure time point (Day 21, Day 56, Day 84 secondary). Images are representative of 3
845 animals per group per time point. Scale bars = 300 µm. **(B)** Semi-quantitative lung histopathology scores
846 for percentage affected, inflammation, blood vessels, bronchioles, and overall Score assessed at days 0,
847 2, 6, 9, and 14 psi across three secondary exposure windows (Day 21 secondary, Day 56 secondary, Day
848 84 secondary). Scoring criteria were for: percentage affected (proportion of pulmonary parenchyma
849 involved); inflammation (density of inflammatory infiltrate); blood vessels (perivascular inflammatory
850 aggregates); bronchioles (epithelial damage); and overall score. Each data point represents one animal.
851 Statistical comparisons between homologous and heterologous groups in Figure B at each time point
852 were performed using two-way ANOVA followed by Sidak's multiple-comparisons post hoc test. ** $p <$
853 0.005, **** $p < 0.0001$. ns, not significant.

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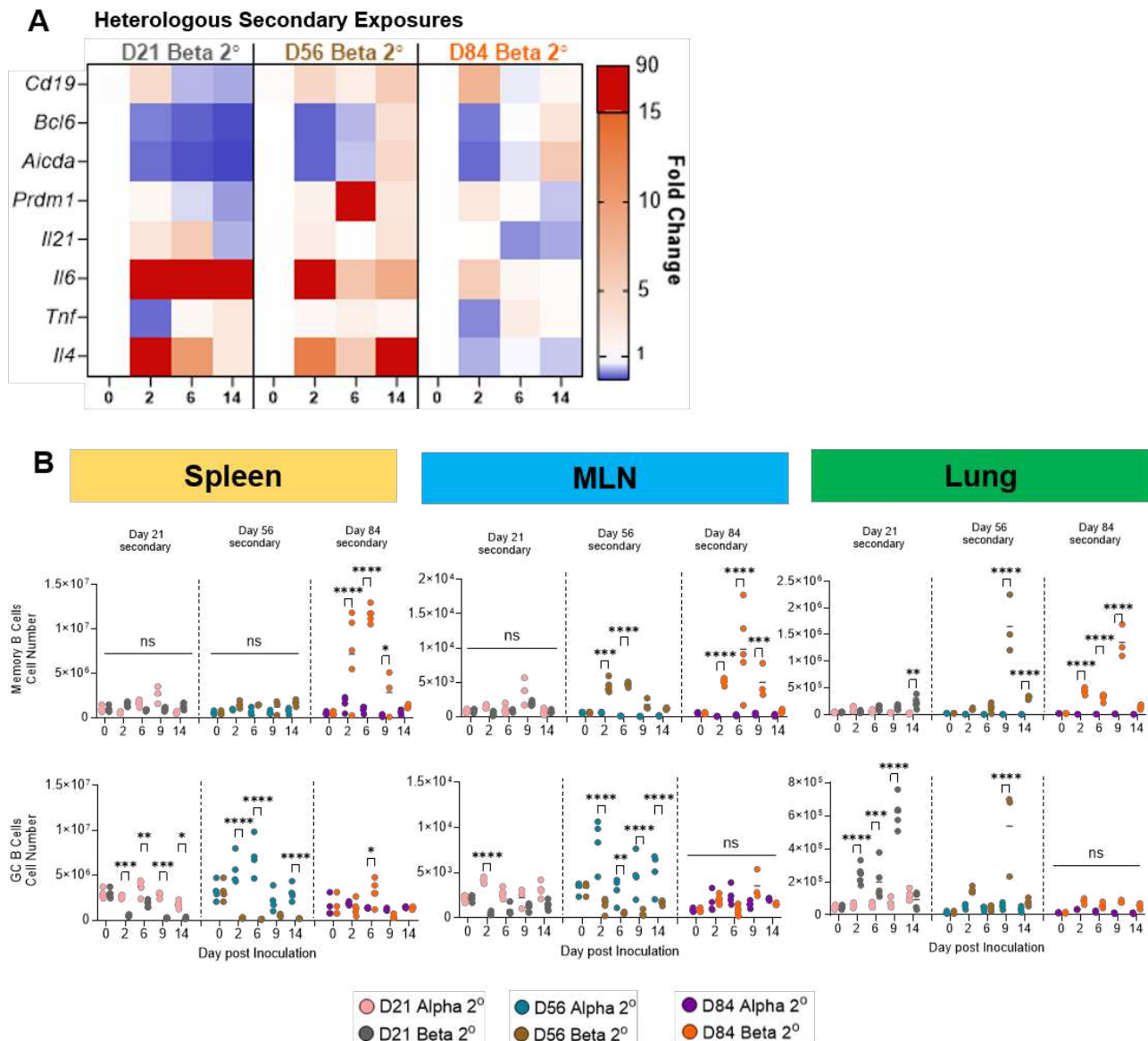
856 **Figure 6: Heterologous SARS-CoV-2 Beta secondary exposure elicits blunted T follicular helper**
 857 **cell responses in the mediastinal lymph node compared to time-matched homologous rechallenge**
 858 **(A)** Host gene expression heatmap for T cell-associated genes in the MLN of heterologous secondary
 859 exposure groups (D21 Beta 2°, D56 Beta 2°, D84 Beta 2°) at days 0, 2, 6, and 14 psi. Fold change
 860 calculated as relative to each group's respective day 0 psi baseline with *BACT* as housekeeping gene.
 861 Upregulation (>1) shown from white (= 1) to orange; fold changes >15 and <90 shown in red.
 862 Downregulation (<1) shown in blue. Minimum 3 animals per time point. **(B)** Absolute cell numbers of
 863 effector memory (EM) T cells (upper row; CD44+CD62L- gated from CD3+CD4+ lymphocytes; gating
 864 strategy in Supplementary Figure 2A) and T_{FH} cells (lower row; CXCR5+PD-1+ gated from

865 CD3+CD4+CD44+ lymphocytes) in the spleen (yellow header), mediastinal lymph node (MLN, blue
866 header), and lung (green header) for days 0, 2, 6, 9, and 14 psi across the Day 21, Day 56, and Day 84
867 secondary exposure intervals. Each data point represents one animal. Statistical comparisons between
868 time-matched homologous and heterologous groups in Figure B were performed using two-way ANOVA
869 followed by Sidak's multiple-comparisons post hoc test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, ****
870 $p < 0.0001$. Note that the Y-axis scales in B differ between immune cell phenotypes and tissue panels.

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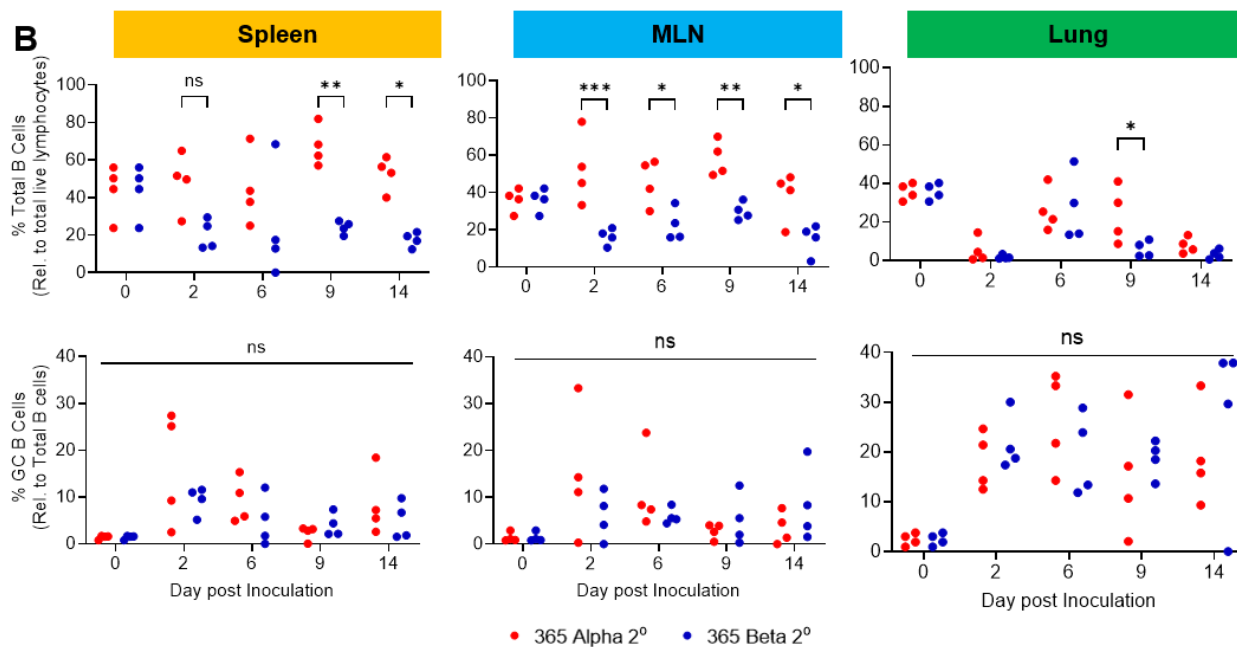
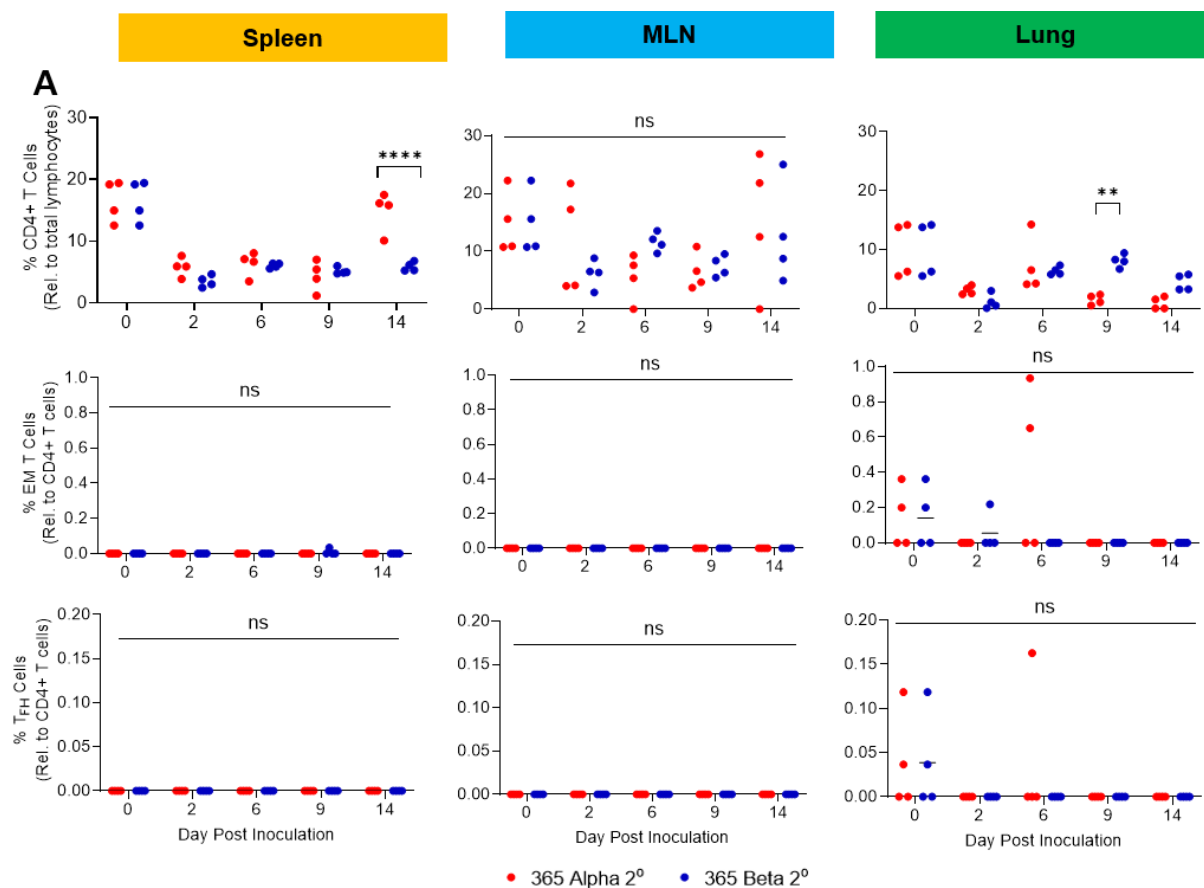
874 **Figure 7: Heterologous SARS-CoV-2 Beta secondary exposure drives inflammatory B cell gene**
 875 **expression with impaired germinal center B cell accumulation in the mediastinal lymph node**
 876 **compared to homologous rechallenge.**

877 **(A)** Host gene expression heatmap for B cell and GC-associated genes in the MLN of heterologous
 878 secondary exposure groups (D21 Beta 2°, D56 Beta 2°, D84 Beta 2°) at days 0, 2, 6, and 14 psi. Fold
 879 change calculated relative to each group's respective day 0 psi baseline with *BACT* as housekeeping gene.
 880 Colour scale as described in Figure 6A. Minimum 3 animals per time point. **(B)** Absolute cell numbers
 881 of memory B cells (upper row; CD38+IgD- gated from B220+CD19+CD138- lymphocytes; gating

882 strategy in Supplementary Figure 2B) and GC B cells (lower row; Fas+GL7+ gated from
883 B220+CD19+CD138- lymphocytes) in the spleen (yellow header), mediastinal lymph node (MLN, blue
884 header), and lung (green header) for all groups at days 0, 2, 6, 9, and 14 psi across the Day 21, Day 56,
885 and Day 84 secondary exposure intervals. Each data point represents one animal. Statistical comparisons
886 between time-matched homologous and heterologous groups in Figure B were performed using two-way
887 ANOVA followed by Sidak's multiple-comparisons post hoc test. * $p < 0.05$, ** $p < 0.005$, *** $p <$
888 0.0005 , **** $p < 0.0001$. ns, not significant. Note that the Y-axis scales in B differ between immune cell
889 phenotypes and tissue panels.

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893 **Figure 8. Long-term T and B cell immune dynamics at day 365 post-primary inoculation during**
 894 **homologous and heterologous SARS-CoV-2 secondary exposure.**
 895 C57BL/6J mice were primarily inoculated with SARS-CoV-2 Alpha and rechallenged at day 365 ppi
 896 with either homologous SARS-CoV-2 Alpha (D365 Alpha 2°, black circles) or heterologous SARS-CoV-
 897 2 Beta (D365 Beta 2°, blue circles). Tissues were collected on days 0, 2, 6, 9, and 14 post-secondary
 898 inoculation (psi) and analyzed by multiparameter flow cytometry. **(A)** T cell dynamics across the spleen
 899 (left, yellow header), mediastinal lymph node (MLN; middle, blue header), and lung (right, green
 900 header). Graphs show the percentage of CD4⁺ T cells (top row), effector memory (EM) T cells (middle
 901 row), and T follicular helper (T_{FH}) cells (bottom row), each expressed relative to the parent population.
 902 **(B)** B cell dynamics across the same three tissue compartments. Graphs show the percentage of total B
 903 cells relative to total live lymphocytes (top row) and germinal center (GC) B cells relative to total B cells
 904 (bottom row). Each data point represents one animal. Statistical comparisons between time-matched
 905 homologous and heterologous groups were performed using two-way ANOVA followed by Sidak's
 906 multiple-comparisons post hoc test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. ns, not
 907 significant.
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911 **Table(s)**

Table 1: C57BL/6J mouse group description based on SARS-CoV-2 exposures.					
Group	Name	Description	SARS-CoV-2		Exposure Combination
			Primary	Secondary	
1	Alpha 1°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha	Alpha	None	N/A
2	Beta 1°	Mice primary inoculated intranasally with 10 ⁵ TCID ₅₀ SARS-CoV-2 Beta	Beta	None	N/A
3	D21 Alpha 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha on day 21 ppi.	Alpha	Alpha on Day 21 ppi	Homologous
4	D56 Alpha 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha on day 56 ppi.	Alpha	Alpha on Day 56 ppi	Homologous
5	D84 Alpha 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha on day 84 ppi.	Alpha	Alpha on Day 84 ppi	Homologous
6	D365 Alpha 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha on day 365 ppi.	Alpha	Alpha on Day 365 ppi	Homologous
7	D21 Beta 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ⁵ TCID ₅₀ SARS-CoV-2 Beta on day 21 ppi.	Alpha	Beta on Day 21 ppi	Heterologous
8	D56 Beta 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ⁵ TCID ₅₀ SARS-CoV-2 Beta on day 56 ppi.	Alpha	Beta on Day 56 ppi	Heterologous
9	D84 Beta 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ⁵ TCID ₅₀ SARS-CoV-2 Beta on day 84 ppi.	Alpha	Beta on Day 84 ppi	Heterologous
10	D365 Beta 2°	Mice primary inoculated intranasally with 10 ^{4.15} TCID ₅₀ SARS-CoV-2 Alpha. Mice secondary inoculated intranasally with 10 ⁵ TCID ₅₀ SARS-CoV-2 Beta on day 365 ppi.	Alpha	Beta on Day 365 ppi	Heterologous

912 D: Day

913 1°: Primary

914 2°: Secondary

915 N/A: Non-applicable; ppi: post-primary inoculation

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [OludadaFrancisetalSupplementaryFiguresandTables.pdf](#)