

Supplementary Information

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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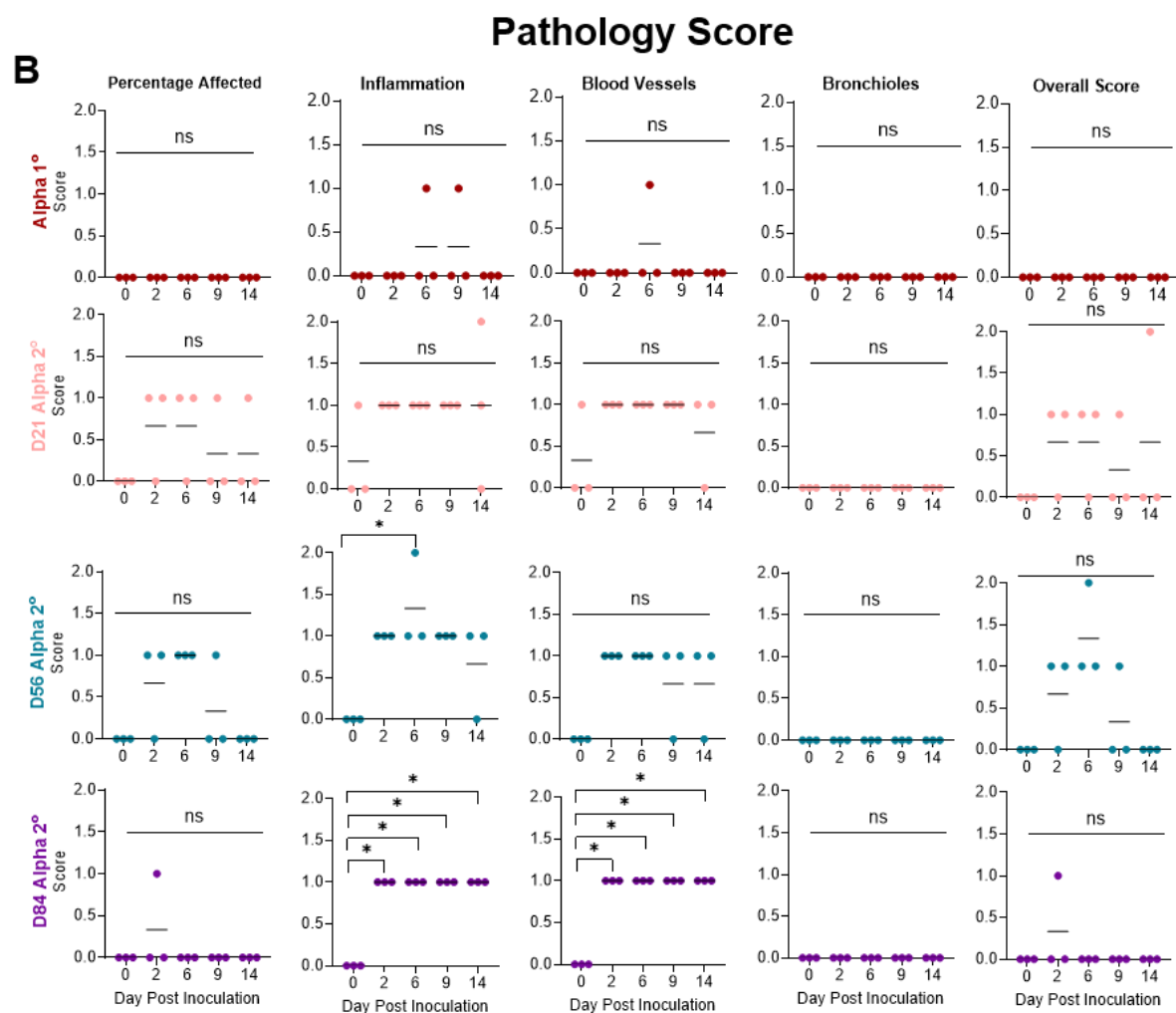
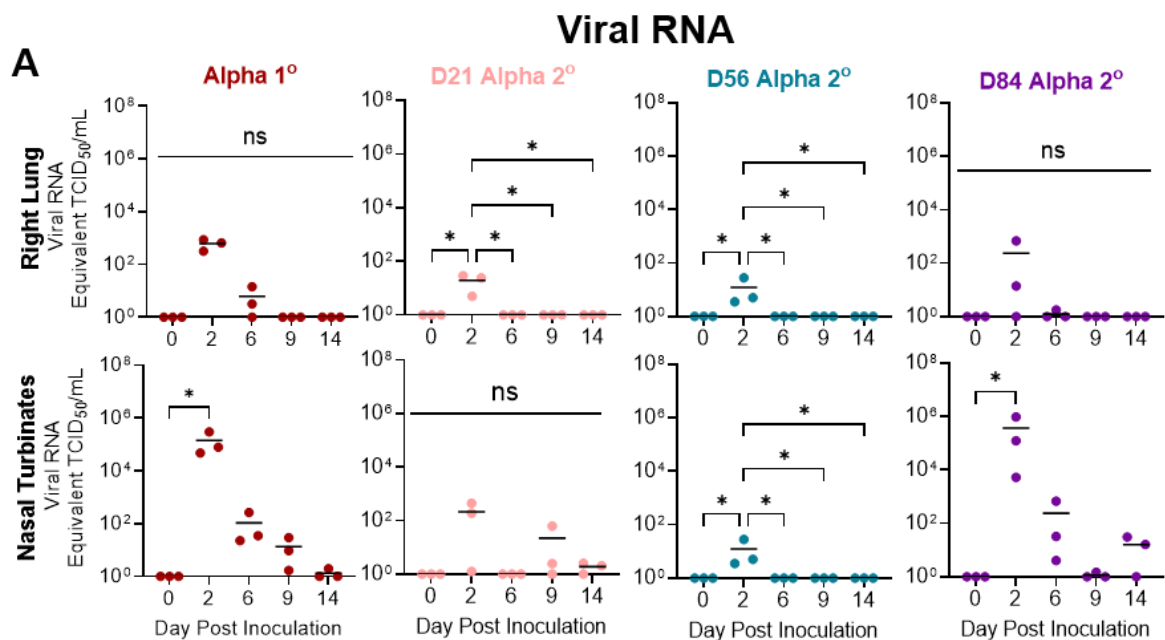
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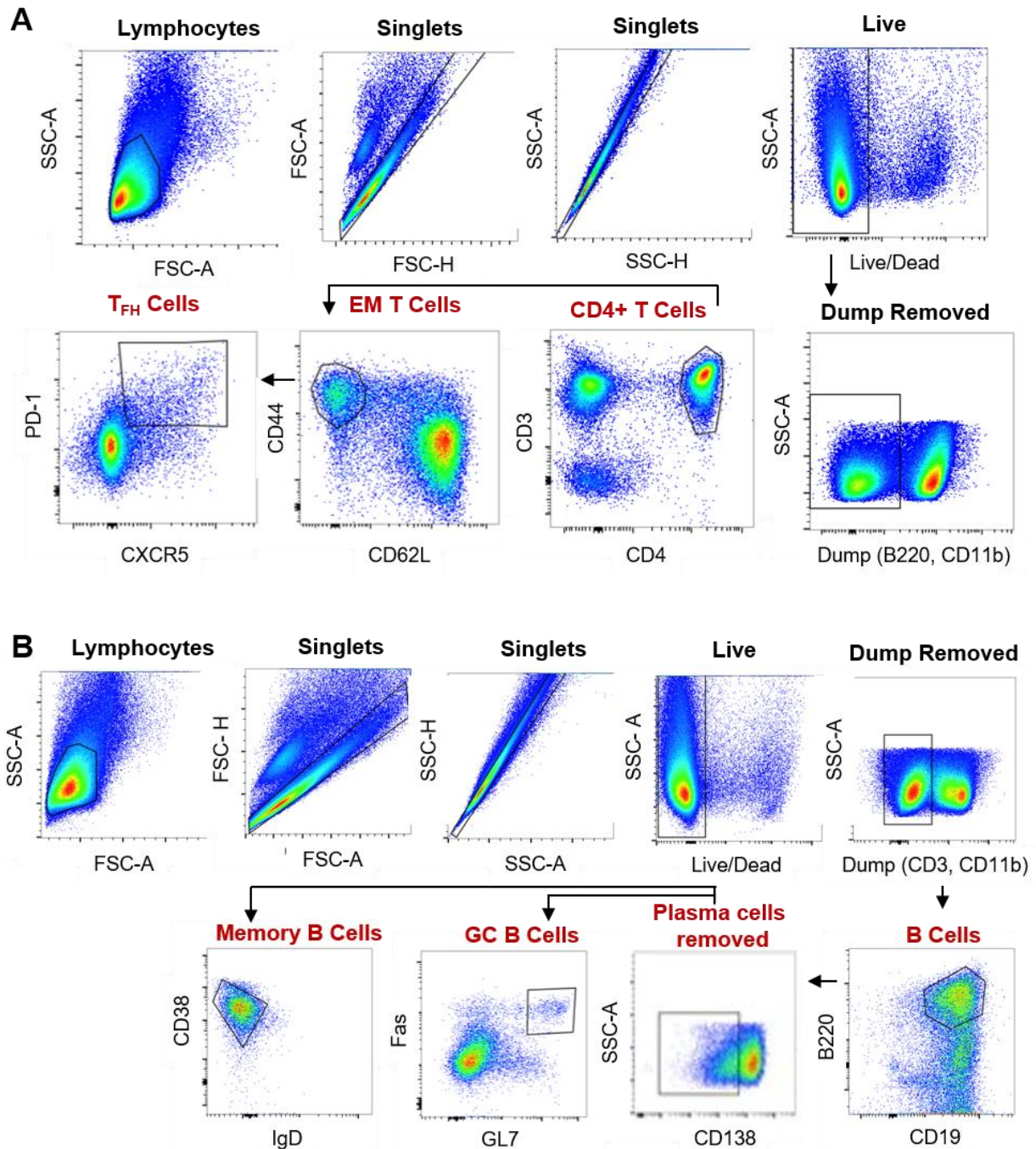
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Supplementary Figure 1: Viral RNA burden, lung histopathology, and immune cell mobilization during homologous SARS-CoV-2 Alpha secondary exposure. (A) Genomic viral RNA (vRNA) quantified by qRT-PCR (Equivalent TCID₅₀/mL) in the right lung (upper panels) and nasal turbinates (lower panels) at days 0, 2, 6, 9, and 14 ppi and psi. Each point represents one animal. **(B)** Semi-quantitative lung histopathology scores assessed from H&E-stained sections for: percentage affected (proportion of pulmonary parenchyma involved); inflammation (density of inflammatory infiltrate); blood vessels (perivascular inflammatory aggregates); bronchioles (epithelial damage); and overall score. Each point represents one animal. Statistical comparisons in Figures A and B were performed using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant.

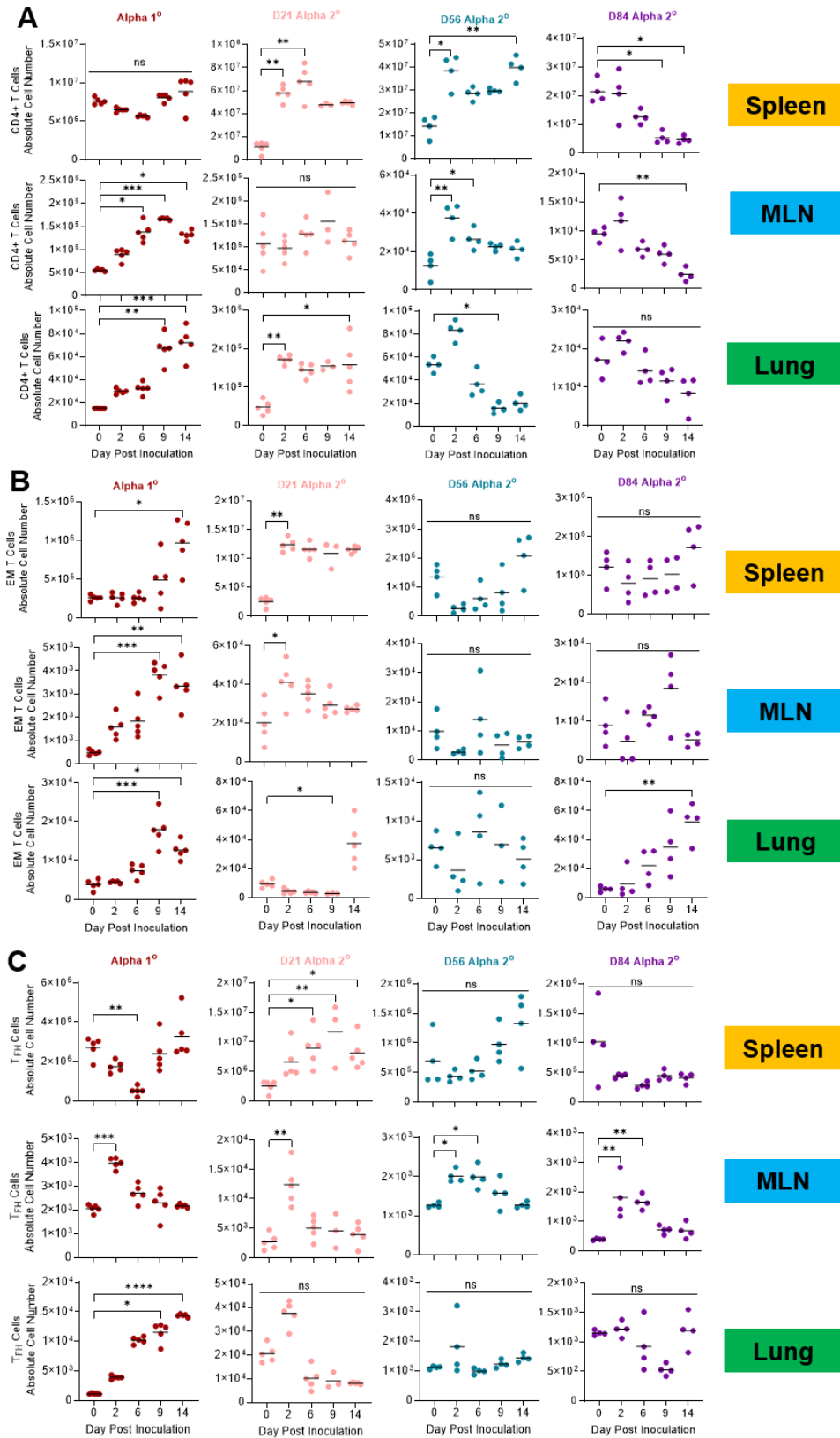


36 **Supplementary Figure 2: Flow cytometric gating strategy for T- and B-cell population**
 37 **identification.** (A) Representative flow cytometry gating strategy for T cell subsets applied to all
 38 tissues. Single cells were first identified by sequential forward scatter area vs. side scatter area
 39 (lymphocyte gate), forward scatter height vs. forward scatter area (singlets), and side scatter height
 40 vs. side scatter area (singlets). Live cells were selected using a live/dead viability stain. Myeloid

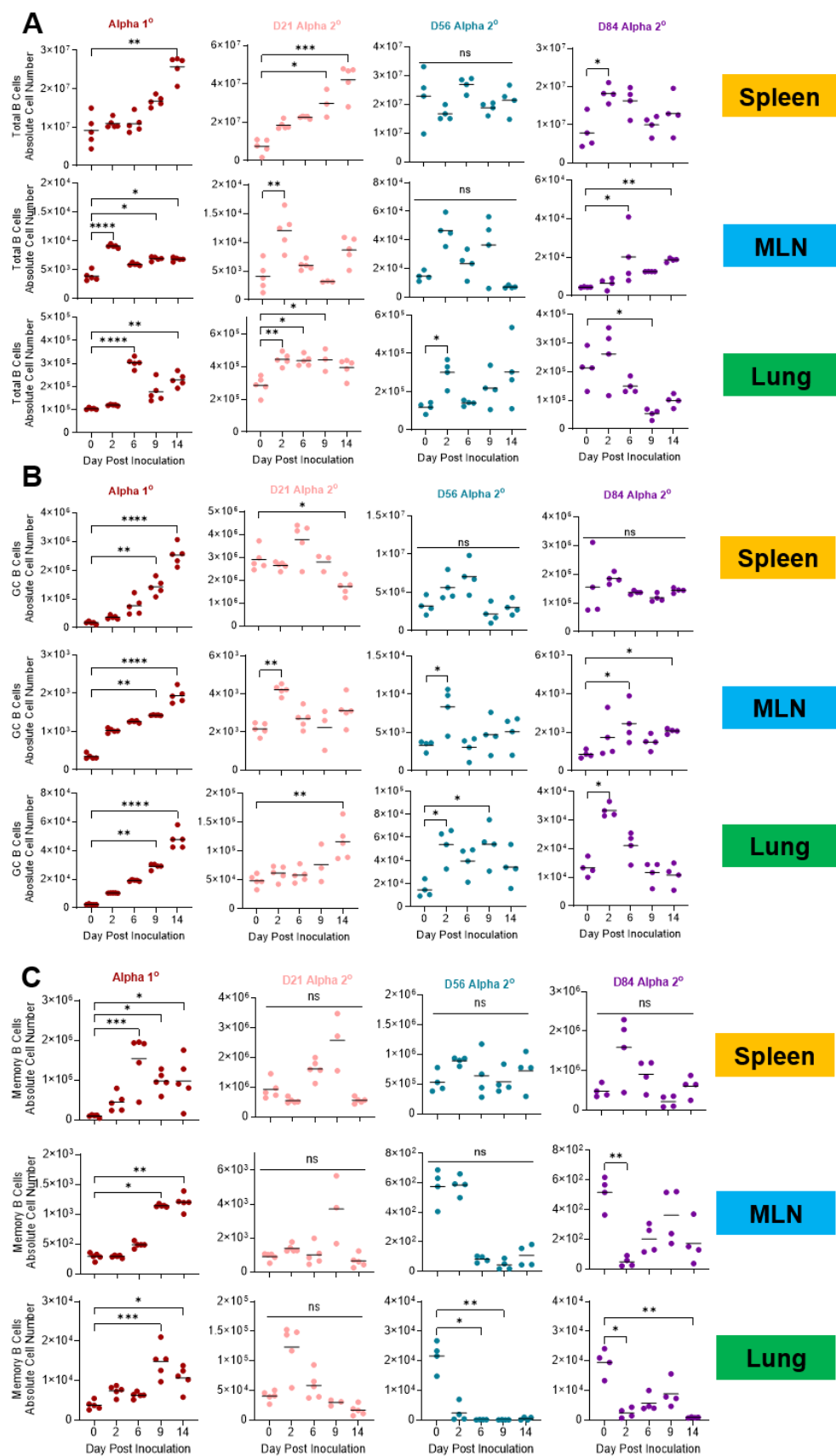
cells and B cells were excluded using a dump channel, gating out CD11b⁺ and B220⁺ events. CD3⁺CD4⁺ T helper cells were identified, from which effector memory (EM) T cells were defined as CD44⁺CD62L⁻, and T follicular helper (T_{FH}) cells were defined as CD44⁺CXCR5⁺PD-1⁺. **(B)** Representative flow cytometry gating strategy for B cell subsets applied to all tissues. Single live lymphocytes were identified as described in Supplementary Figure 2A. Myeloid cells and T cells were excluded using a dump channel, gating out CD11b⁺ and CD3⁺ events. B220⁺CD19⁺ B cells were identified, and CD138⁺ plasma cells were excluded from further analysis. From the remaining B cell population, germinal center (GC) B cells were defined as Fas⁺GL7⁺, and memory B cells (MBCs) were defined as CD38⁺IgD⁻.

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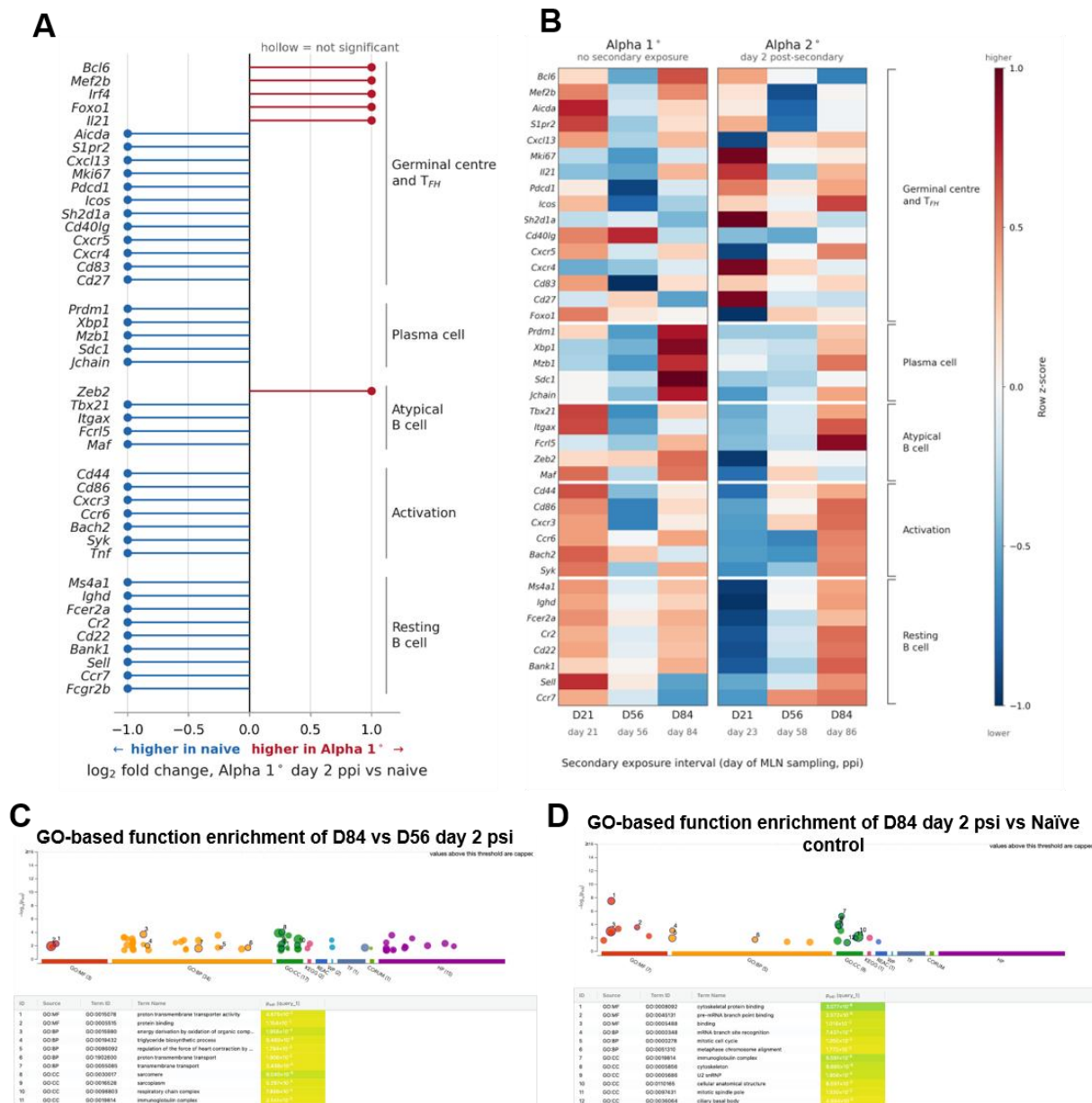
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Supplementary Figure 3: Quantification of total CD4⁺ T cells, effector memory T cells, and T follicular helper cells following homologous SARS-CoV-2 Alpha secondary exposure. (A) Absolute cell numbers of total CD4⁺ T cells in the spleen (yellow header), mediastinal lymph node (MLN, blue header), and lung (green header) from homologous secondary exposure groups quantified by flow cytometry at days 0, 2, 6, 9, and 14 post-primary and post-secondary Alpha inoculation. Each data point represents one animal. **(B)** Absolute cell numbers of effector memory (EM) T cells (CD44⁺CD62L⁻ gated from CD3⁺CD4⁺ lymphocytes) quantified by flow cytometry in the spleen (yellow header), mediastinal lymph node (MLN, blue header), and lung (green header) at days 0, 2, 6, 9, and 14 post-primary and post-secondary Alpha inoculation. Bar graphs represent the group mean, and each data point represents one animal. **(C)** Absolute cell numbers of T follicular helper (T_{FH}) cells (CXCR5⁺PD-1⁺ gated from CD3⁺CD4⁺CD44⁺ lymphocytes) quantified by flow cytometry in the spleen (yellow header), mediastinal lymph node (MLN, blue header), and lung (green header) at days 0, 2, 6, 9, and 14 post-primary (ppi) and post-secondary (psi) Alpha inoculation. Bar graphs represent the group mean, and each data point represents one animal. Statistical comparisons in A-C were performed using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant. Note that the Y-axis scales in A-C differ between immune cell phenotypes and tissue panels.

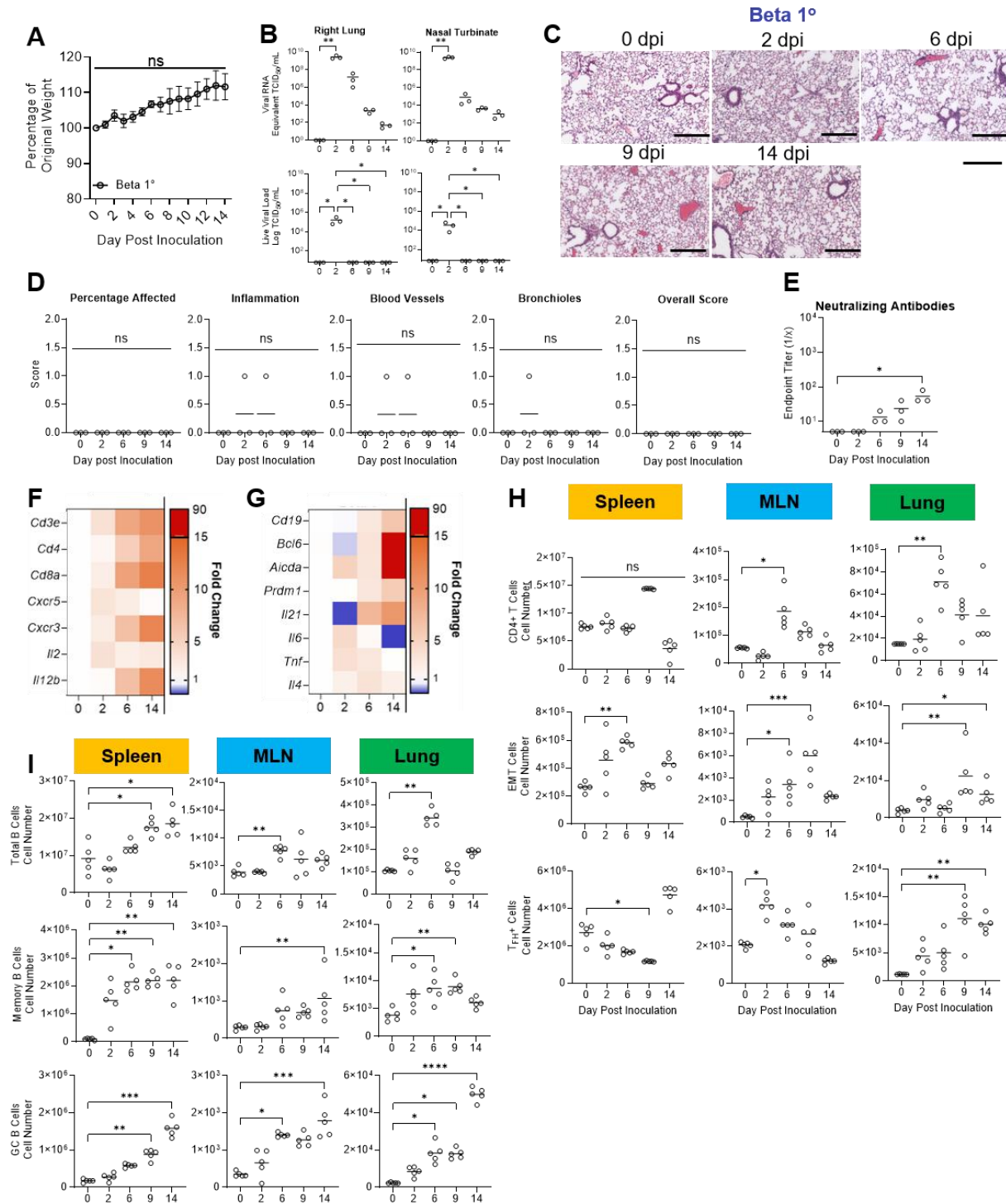


Supplementary Figure 4: Quantification of total B cells, germinal center B cells, and memory B cells following homologous SARS-CoV-2 Alpha secondary exposure. (A) Absolute cell numbers of total B cells in the spleen (yellow header), mediastinal lymph node (MLN, blue header), and lung (green header) from homologous secondary exposure groups quantified by flow cytometry at days 0, 2, 6, 9, and 14 post-primary (ppi) and post-secondary (psi) Alpha inoculation. (B) Absolute cell numbers of germinal center (GC) B cells (lower row; Fas+GL7+ gated from B220+CD19+CD138- cells) quantified by flow cytometry in the spleen (yellow header), MLN (blue header), and lung (green header) at days 0, 2, 6, 9, and 14 Alpha ppi and psi. Bar graphs represent the group mean, and each data point represents one animal. (C) Absolute cell numbers of memory B cells (upper row; CD38+IgD- gated from B220+CD19+CD138- cells) quantified by flow cytometry in the spleen, MLN, and lung at days 0, 2, 6, 9, and 14 Alpha ppi and psi. Bar graphs represent the group mean, and each data point represents one animal. Statistical comparisons in Figures A-C were performed using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant. Note that the Y-axis scales in A-C differ between immune cell phenotypes and tissue panels.



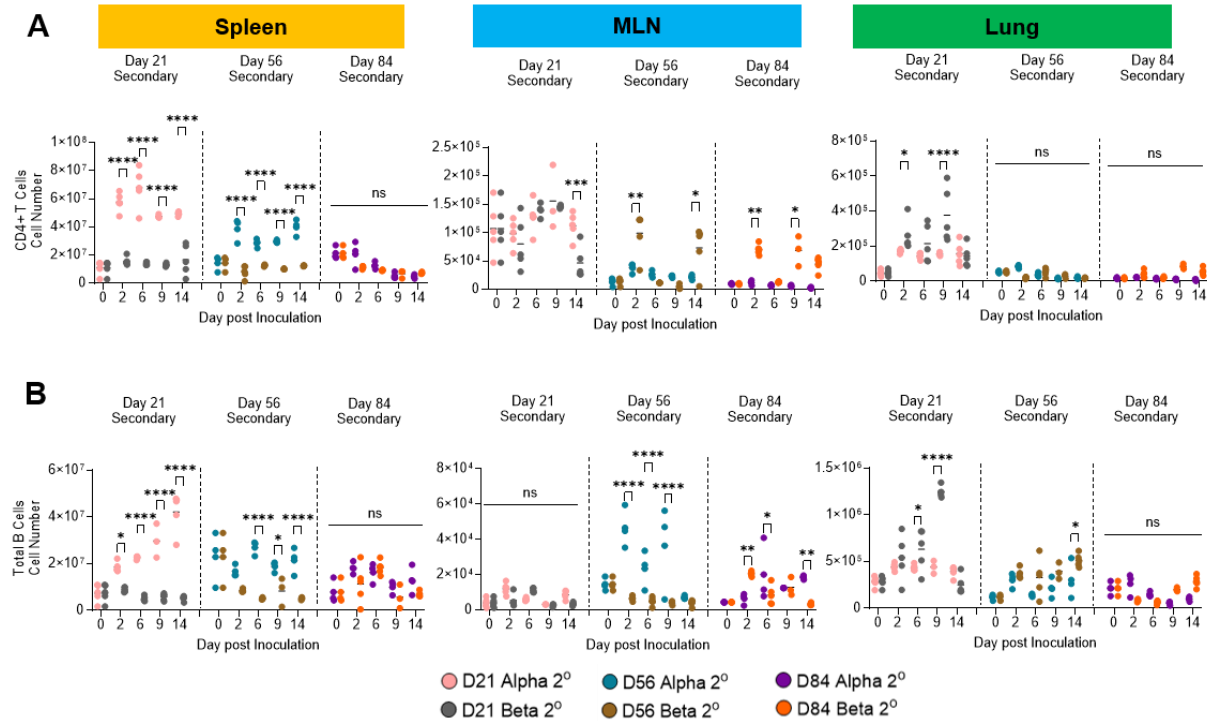
Supplementary Figure 5. Primary Alpha infection commits the lymph node to a germinal centre program, and the germinal centre recall signature is specific to secondary exposure rather than to time since priming. (A) Row-scaled expression of the immune-program genes in the mediastinal lymph node of naive control mice (day 0) versus mice at day 2 after primary Alpha infection (Alpha 1°, day 2 ppi; n = 3 per group), ordered with genes induced at day 2 (lower block: *Foxo1*, *Mef2b*, *Bcl6*, *Zeb2*, *Irf4*, *Il21*) separated from those below control (top block). Because

only two groups are compared, the row z-score is binary by construction (each gene is higher in one group or the other) and colour indicates direction of change. **(B)** Row-scaled expression of the same immune-program genes across the D21, D56, and D84 intervals for primary-only Alpha 1° animals (left) and secondary Alpha 2° animals (right), in the program-based gene order of Figure 3A. Here the row z-score is computed across all six groups; because the scaling includes the primary-only groups, the Alpha 2° columns appear less saturated than in the three-group scaling of Figure 3A. **(C and D)** Gene Ontology term enrichment among genes upregulated in D84 Alpha 2° relative to D56 Alpha 2° (C) and relative to naive control (D), displayed as in Figure 3D. Across all panels, colour encodes relative rank within the scaling scope stated for each panel, not expression magnitude.



111 **Supplementary Figure 6: Primary SARS-CoV-2 Beta infection in C57BL/6J mice produces**
 112 **a mild, self-limiting respiratory infection with a measurable primary adaptive immune**
 113 **response. (A)** Body weight of Beta 1° animals expressed as a percentage of pre-inoculation
 114 **baseline. Data presented as mean ± SD. (B)** Genomic vRNA (upper panels) and live virus by

115 TCID₅₀ assay (lower panels) in the right lung (left) and nasal turbinates (right) from Beta 1°
116 animals at days 0, 2, 6, 9, and 14 dpi. Each data point represents one animal. **(C)** Representative
117 H&E-stained lung sections from Beta 1° animals at 0, 2, 6, 9, and 14 dpi. Scale bars = 300 µm.
118 **(D)** Semi-quantitative lung histopathology scores (percentage affected, inflammation, blood
119 vessels, bronchioles, overall score) from Beta 1° animals assessed at days 0, 2, 6, 9, and 14 ppi.
120 Each data point represents one animal. **(E)** SARS-CoV-2 Beta neutralizing antibody endpoint titers
121 (1/x) in plasma from Beta 1° animals at days 0, 2, 6, 9, and 14 ppi. Each data point represents one
122 animal; the line represents the geometric mean. **(F)** Host gene expression heatmap for T cell-
123 associated genes (*Cd3e*, *Cd4*, *Cd8a*, *Cxcr5*, *Cxcr3*, *Il2*, *Il12b*) in the MLN of Beta 1° animals. Fold
124 change calculated relative to day 0 baseline with *BACT* as the housekeeping gene. Upregulation
125 (fold change >1) is shown from white (= 1) to orange; fold changes >15 and <90 are shown in red.
126 Downregulation (fold change <1) is shown in blue. Minimum 3 animals analyzed per time point.
127 **(G)** Host gene expression heatmap for B cell and GC-associated genes (*Cd19*, *Bcl6*, *Aicda*, *Prdm1*,
128 *Il21*, *Il6*, *Tnf*, *Il4*) in the MLN of Beta 1° animals, presented as described in (F). **(H)** Absolute cell
129 numbers of CD4+ T cells (upper row), EM T cells (middle row), and T_{FH} cells (lower row) in the
130 spleen (yellow header), MLN (blue header), and lung (green header) of Beta 1° animals quantified
131 by flow cytometry at days 0, 2, 6, 9, and 14 ppi. Each data point represents one animal. **(I)** Absolute
132 cell numbers of total B cells (upper row), memory B cells (middle row), and GC B cells (lower
133 row) in the spleen, MLN, and lung of Beta 1° animals at days 0, 2, 6, 9, and 14 ppi. Each data
134 point represents one animal. Note that the Y-axis scales in H and I differ between immune cell
135 phenotypes and tissue panels. Statistical comparisons in Figures A, B, D, E, H, and I were
136 performed using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p <$
137 0.05, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant.



Supplementary Figure 7: Total CD4+ T cell and B cell mobilization across tissues during homologous and heterologous SARS-CoV-2 Alpha secondary exposure. (A) Absolute cell numbers of total CD4+ T cells in the spleen (yellow header), MLN (blue header), and lung (green header) quantified by flow cytometry at days 0, 2, 6, 9, and 14 psi. Each data point represents one animal. **(B)** Absolute cell numbers of total B cells in the spleen, MLN, and lung quantified by flow cytometry at days 0, 2, 6, 9, and 14 psi. Each data point represents one animal. Statistical comparisons between Alpha and Beta within each group in figures A and B were performed using two-way ANOVA followed by Sidak's multiple-comparisons post hoc test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns, not significant.

149 **Supplementary Table 1. Prime PCR Assay IDs.**

Genes	Gene Symbol	Ensembl Gene ID	BioRad Assay ID
BACT	<i>Actb</i>	ENSMUSG00000029580	qMmuCED0027505
CD3	<i>Cd3e</i>	ENSMUSG00000032093	qMmuCED0047615
CD4	<i>Cd4</i>	ENSMUSG00000023274	qMmuCED0022320
CD8	<i>Cd8a</i>	ENSMUSG00000053977	qMmuCID0016523
CXCR5	<i>Cxcr5</i>	ENSMUSG00000047880	qMmuCED0039913
CXCR3	<i>Cxcr3</i>	ENSMUSG00000050232	qMmuCID0008568
IL-2	<i>Il2</i>	ENSMUSG00000027720	qMmuCED0060978
IL-12	<i>Il12b</i>	ENSMUSG00000004296	qMmuCID0022424
CD19	<i>Cd19</i>	ENSMUSG00000030724	qMmuCID0017517
BCL6	<i>Bcl6</i>	ENSMUSG00000022508	qMmuCID0005293
AID	<i>Aicda</i>	ENSMUSG00000040627	qMmuCID0008369
BLIMP-1	<i>Prdm1</i>	ENSMUSG00000038151	qMmuCID0013030
IL-21	<i>Il21</i>	ENSMUSG00000027718	qMmuCED0045758
IL-6	<i>Il6</i>	ENSMUSG00000025746	qMmuCED0045760
TNF	<i>Tnf</i>	ENSMUSG00000024401	qMmuCED0004141
IL-4	<i>Il4</i>	ENSMUSG00000000869	qMmuCED0044969

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Panel	Target	Fluorophore	Clone	Source	Catalogue No.
T Cell Panel	CD3	APC/Fire 750	17A2	BioLegend	100248
	CD4	Alexa Fluor 700	RM4-4	BioLegend	116022
	CD62L	PE	MEL-14	BioLegend	104408
	CD185 (CXCR5)	APC	L138D7	BioLegend	145506
	CD279 (PD-1)	eFluor 450	RMP1-30	ThermoFisher	48-9981-82
	CD44	PerCP	IM7	BioLegend	103036
	CD11b	Brilliant Violet 605	M1/70	BioLegend	101257
	CD45R(B220)	Brilliant Violet 605	RA3-6B2	BioLegend	103244
	Dead	Brilliant Violet 510	-	ThermoFisher	L34957
B Cell Panel	CD45R(B220)	Alexa Fluor 594	104	BioLegend	103254
	CD19	Alexa Fluor 700	1D3	BioLegend	152414
	CD138	APC	281-2	BioLegend	142506
	CD95 (Fas)	FITC	Jo2	BD Biosciences	554257
	T and B Cell Activation Antigen	Brilliant Violet 421	GL7	BD Biosciences	568851
	CD11a	PerCP/Cy5.5	M17/4	BioLegend	101124
	CD81	PE	Eat-2	BioLegend	104906
	CD38	APC/Fire 750	90	BioLegend	102738
	IgD	Brilliant Violet 650	11-26c.2a	BioLegend	405721
	CD11b	Brilliant Violet 605	M1/70	BioLegend	101257
	CD3	Brilliant Violet 605	17A2	BioLegend	100237
	Dead	Brilliant Violet 510	-	ThermoFisher	L34957

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