

1 **CD4+ and CD8+ T cells are required to prevent SARS-CoV-2**
2 **persistence in the nasal compartment**

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31

32 **ABSTRACT**

33 SARS-CoV-2 is the causative agent of COVID-19 and continues to pose a significant public
34 health threat throughout the world. Following SARS-CoV-2 infection, virus-specific CD4+
35 and CD8+ T cells are rapidly generated to form effector and memory cells and persist in the
36 blood for several months. However, the contribution of T cells in controlling SARS-CoV-2
37 infection within the respiratory tract are not well understood. Using C57BL/6 mice infected
38 with a naturally occurring SARS-CoV-2 variant (B.1.351), we evaluated the role of T cells in
39 the upper and lower respiratory tract. Following infection, SARS-CoV-2-specific CD4+ and
40 CD8+ T cells are recruited to the respiratory tract and a vast proportion secrete the cytotoxic
41 molecule Granzyme B. Using antibodies to deplete T cells prior to infection, we found that
42 CD4+ and CD8+ T cells play distinct roles in the upper and lower respiratory tract. In the
43 lungs, T cells play a minimal role in viral control with viral clearance occurring in the absence
44 of both CD4+ and CD8+ T cells through 28 days post-infection. In the nasal compartment,
45 depletion of both CD4+ and CD8+ T cells, but not individually, results in persistent and
46 culturable virus replicating in the nasal compartment through 28 days post-infection. Using *in*
47 *situ* hybridization, we found that SARS-CoV-2 infection persisted in the nasal epithelial layer
48 of tandem CD4+ and CD8+ T cell-depleted mice. Sequence analysis of virus isolates from
49 persistently infected mice revealed mutations spanning across the genome, including a
50 deletion in ORF6. Overall, our findings highlight the importance of T cells in controlling virus
51 replication within the respiratory tract during SARS-CoV-2 infection.

52 **MAIN**

53 The global impact of Severe Acute Respiratory Syndrome Coronavirus 2 remains
54 devastating, with over 773 million confirmed cases and around 7 million deaths reported
55 worldwide by the end of December 2023 (World Health Organization COVID-19 dashboard;
56 <https://covid19.who.int>). SARS-CoV-2 is primarily transmitted through respiratory droplets
57 and targets ciliated epithelial cells in the nasal cavity, trachea, and lungs^{1,2}. SARS-CoV-2
58 primarily infects epithelial cells within the respiratory tract by utilizing the cellular receptor
59 angiotensin-converting enzyme 2 (ACE2)³⁻⁵. Infection of the upper respiratory tract is
60 generally associated with a milder disease outcome whereas dissemination to the lungs, in
61 particular infection of the bronchi, bronchioles, and alveoli, can cause pneumonia, severe
62 disease, acute respiratory distress syndrome and death³.

63

64 The development of mouse models of SARS-CoV-2 has enabled the study of transmission,
65 immunity, and pathogenesis of this virus⁶⁻¹⁰. The ancestral SARS-CoV-2 strain does not
66 replicate in conventional laboratory mice due to inefficient spike protein binding to the murine
67 ACE2^{11,12}. To overcome this limitation, several mouse models have been developed,
68 including human ACE2 (hACE2) transgenic mice that express hACE2 transiently after
69 transduction of hACE2 with viral vectors (e.g., Adenovirus)^{13,14}, K18-hACE2 transgenic
70 mice¹⁵), or the use of mouse-adapted strains^{7,16,17}. A naturally occurring spike mutation at
71 position N501Y, which is found in many SARS-CoV-2 variants (Alpha, Beta, Gamma and Mu
72 variants), increases the affinity of SARS-CoV-2 spike protein for the murine ACE2 receptor
73 and allows for infection of inbred mice¹¹. Infection of conventional laboratory mice with
74 naturally occurring N501Y spike mutations show inflammatory infiltrates, alveolar edema,
75 and alveolitis^{13,16,18,19}. Using this model, we recently identified that the CCR2-monocyte
76 signaling axis is important for controlling virus replication and dissemination within the lungs
77 and protection against the SARS-CoV-2 B.1.351 ("Beta") variant¹⁸.

78

79 Following SARS-CoV-2 infection, both CD4+ and CD8+ T cells are detectable within the
80 peripheral blood of patients with COVID-19²⁰⁻²³. These circulating virus-specific CD4+ and
81 CD8+ T cells target several SARS-CoV-2 proteins, including Spike and Nucleocapsid, and
82 are polyfunctional and durable with estimated half-lives of over 200 days²⁰. SARS-CoV-2-
83 specific CD4+ T cells are important in promoting antibody responses and mitigating disease
84 severity as reduced responses associate with increased disease severity²⁴⁻²⁷. CD8+ T cells
85 appear to play a protective role with reduced responses correlating with adverse
86 prognoses²⁸. Rapid type I interferon (IFN) responses and virus-specific CD8+ T cell
87 responses coincide with milder SARS-CoV-2 infections, preceding the development of
88 antibodies by one to two weeks²⁹. However, the role of CD4+ and CD8+ T cells within the
89 respiratory tract is not well understood and is only beginning to be studied in the context of
90 SARS-CoV-2 infection in humans^{30,31}.

91

92 In this study, we investigated the role of CD4+ and CD8+ T cells on SARS-CoV-2 infection in
93 both the upper respiratory tract (i.e., nasal turbinates) and the lower respiratory tract.
94 Following infection, the respiratory tract recruits SARS-CoV-2-specific CD4+ and CD8+ T
95 cells, a significant proportion of which release the cytotoxic substance Granzyme B. Through
96 T cell depletion using antibodies prior to infection, we discovered that CD4+ and CD8+ T
97 cells have distinct roles in the upper and lower respiratory tract. In the lungs, T cells have a
98 limited impact on viral control, as viral clearance occurs even in the absence of both CD4+
99 and CD8+ T cells up to 28 days post-infection (pi). Conversely, in the nasal compartment,
100 depleting both CD4+ and CD8+ T cells (but not individually) leads to persistent and
101 culturable virus replication in the nasal compartment for 28 days pi. Utilizing *in situ*
102 hybridization, we observed persistent SARS-CoV-2 infection in the nasal epithelial layer of
103 mice depleted of both CD4+ and CD8+ T cells. Sequence analysis of virus isolates from
104 persistently infected mice revealed mutations spanning the entire genome. In summary, our
105 findings underscore the crucial role of T cells in controlling virus replication within the
106 respiratory tract during SARS-CoV-2 infection.

107 RESULTS

108 **SARS-CoV-2 infection kinetics and immune response in C57BL/6 mice.** The emergence
109 of SARS-CoV-2 variants encoding an N501Y mutation within the spike protein allows for
110 productive infection of conventional laboratory mice. Previously, we had shown that infection
111 of C57BL/6 mice with the B.1.351 strain results in virus replication in the lungs that
112 corresponds with induction of innate immune responses^{18,32}. To further characterize this
113 model, we infected C57BL/6 mice with doses of B.1.351 ranging from 10^5 to 10^6 PFU
114 through the intranasal route. We found that increasing virus inoculum led to increased weight
115 loss with mice infected with 5×10^6 PFU showing nearly 20% weight loss with 30% mortality
116 (**Fig. 1a**). For subsequent experiments, we selected 1×10^6 PFU as the viral dose as we
117 observed consistent weight loss in the absence of mortality. We next evaluated the kinetics
118 of virus replication in the upper and lower respiratory tracts. Infectious virus in the lungs, as
119 measured by plaque assay on Vero-ACE2-TMPRSS2 over-expressing cells, peaked
120 between days 2 and 4 post-infection (pi) and reached undetectable levels by day 10 pi (**Fig.**
121 **1b**). As a more sensitive measure of virus replication, we measured viral RNA-dependent
122 RNA polymerase (RdRp) levels by qRT-PCR and observed 116-fold reduction in virus
123 replication in the lungs and nasal turbinates (**Fig. 1c**). A hallmark of SARS-CoV-2 infection is
124 the generation of systemic spike- and nucleocapsid-specific antibody responses³³.
125 Following infection, B.1.351-infected mice generated robust IgG responses against the
126 receptor-binding domain (RBD) (GMT 107839), Spike (GMT 249211) and Nucleocapsid
127 (GMT 39803) that corresponded to live virus neutralization activity (FRNT₅₀ GMT 175) (**Fig.**
128 **1d-f**).

129

130 **SARS-CoV-2 B.1.351 infection triggers antigen-specific T cell responses in the upper**
131 **and lower respiratory tract.** Previous studies in humans have shown that SARS-CoV-2
132 infection can trigger antigen-specific T cell responses within the nasal compartment, lungs,
133 and periphery (e.g. blood)^{20,30,34}. We next evaluated T cell responses within the respiratory

134 tract and periphery of SARS-CoV-2 infected mice. Following B.1.351 infection, we isolated
135 immune cells from the nasal compartment, lungs, and spleen followed by *ex vivo* peptide
136 restimulation to identify antigen-specific CD4⁺ and CD8⁺ T cells. Prior to harvesting tissues,
137 mice were intravitaly labelled with CD45 antibody conjugated to phycoerythrin (PE) to allow
138 identification of circulating (CD45-PE positive) and tissue-resident/ parenchymal cells
139 (CD45-PE negative) within the respiratory tract (gating strategy shown in **Extended Data**
140 **Fig. 1**). On day 7 pi, within the spleen, we observed a modest, yet significant, increase in the
141 frequency of CD8⁺ T cells, but not CD4⁺ T cells (**Fig. 2a**). However, we observed a
142 significant increase in the frequency of tissue-resident CD8⁺ T cells, but not CD4⁺ T cells,
143 within the lungs and nasal compartment (**Fig. 2b-c**). This corresponded to an increase in the
144 total number of CD8⁺ T cells by 32.3-fold within the nasal compartment and by 3.4-fold in
145 the lungs.

146

147 We next evaluated the antigen-specific T cell responses by performing *ex vivo* peptide
148 restimulation with a spike peptide pool followed by intracellular staining (**Fig. 3a**). For
149 antigen-specific CD4⁺ T cells, in both the lungs and nasal compartment, we observed higher
150 cell frequencies and counts of Granzyme B secreting as compared to cytokine secreting
151 cells (**Fig. 3b**). Specifically, we observed 2.35% GrB⁺, 0.3% TNF α ⁺, 0.67%IFN γ ⁺, and
152 0.24% TNF α +IFN γ ⁺ CD4⁺ T cells in the lungs and 6.45% GrB⁺, 0.56% TNF α ⁺,
153 0.56%IFN γ ⁺, and 0% TNF α +IFN γ ⁺ CD4⁺ T cells in the nasal compartment. In the spleen,
154 we observed 0.27% GrB⁺, 0.50% TNF α ⁺, 0.57%IFN γ ⁺, and 0.08%TNF α +IFN γ ⁺ CD4⁺ T
155 cells.

156

157 For antigen-specific CD8⁺ T cells, in the lungs, nasal compartment and spleen, we observed
158 higher cell frequencies and counts of Granzyme B secreting as compared to cytokine
159 secreting cells (**Fig. 3c-d**). We observed 28.3% GrB⁺, 0.34% TNF α ⁺, 1.91%IFN γ ⁺, and
160 0.97%TNF α +IFN γ ⁺ CD8⁺ T cells in the lungs, 39.25% GrB⁺, 0.40% TNF α ⁺, 0.21%IFN γ ⁺,
161 and 0.04%TNF α +IFN γ ⁺ CD8⁺ T cells in the nasal compartment, and 4.42% GrB⁺, 0.41%

162 TNF α +, 2.50%IFN γ +, and 0.20%TNF α +IFN γ + CD8+ T cells in the spleen. We compared
163 activation markers (CD44, KLRG1, CD69) in CD4+ and CD8+ T cells across lungs, URT,
164 and spleen (**Extended Data Fig. 2**). We discovered intriguing variations in activation
165 between upper and lower respiratory areas. In the lungs, infected samples showed
166 increased CD44 and CD69, and to a lesser extent, KLRG1 in contrast to mock samples.
167 However, in the URT there was no difference in CD44 and CD69 expression in CD4+ and
168 CD8+ T cells. Surprisingly, there was a significant rise in KLRG1 expression in CD8+ T cells
169 in the URT. No changes were observed in expression of T cells from the spleen. These data
170 indicate that SARS-CoV-2 infection triggers antigen-specific CD4+ and CD8+ T responses in
171 the respiratory tract, with a higher proportion of T cells expressing Granzyme B within the
172 respiratory tract.

173

174 **CD4+ and CD8+ T cells are dispensable for protection against SARS-CoV-2 but**
175 **required for viral control within the respiratory tract.** To evaluate the contribution of T
176 cells during SARS-CoV-2 infection, we used antibody-based depletion to either individually
177 or tandem deplete CD4+ or CD8+ T cells (**Fig. 4a**). Anti-CD4 and/or anti-CD8 antibodies
178 were administered through the intraperitoneal route on days -5, -3, -1, +1, +7, +14, and +21
179 following SARS-CoV-2 infection. We assessed the efficiency of T cell depletions on days 0
180 and 28 pi and found near complete ablation of CD4+ and/or CD8+ T cells in whole blood,
181 lungs and nasal compartment (**Extended Data Fig. 3a-b**). Following SARS-CoV-2 infection
182 of CD4+ and/or CD8+ T cell-depleted mice, we observed similar peak weight loss on day 3
183 pi and recovery through day 10 pi. Further, we observed no mortality in any of the isotype or
184 T cell-depleted SARS-CoV-2 infected mice (**Fig. 4b**).

185

186 We next evaluated virus replication by qRT-PCR (nasal compartment and lungs) and plaque
187 assay (lungs). As a baseline for the depletions at later time points, we assessed viral
188 replication on day 3 pi and observed no difference in viral RNA in the nasal compartment or
189 lungs in the isotype or T-cell depleted mice (**Fig. 4c, d**). By day 10 pi, we observed a

190 modest, but not statistically significant, increase in viral RNA in the lungs of the individual or
191 tandem CD4+/CD8+ T cell-depleted mice as compared to the isotype control infected mice.
192 In contrast, viral RNA was higher in the nasal compartment of the CD4+/CD8+ T cell
193 tandem-depleted mice as compared to the isotype control. In the lungs by day 28 pi, the T
194 cell depleted groups showed similar viral RNA as compared to the isotype control infected
195 mice. However, in the nasal compartment, only the CD4+/CD8+ T cell tandem-depleted mice
196 showed significantly higher viral RNA (30.3-fold) as compared to the isotype control infected
197 mice (**Fig. 4c, d**).

198 We also compared deletion of CD8+ T cells using either CD8 β or CD8 α antibodies and no
199 difference in viral RdRp levels was detected between the CD8+ T cell-depleted mice in
200 either the CD8 β or CD8 α depletion group (**Extended Data Fig. 4**). These data demonstrate
201 that T cells are required for efficient viral control/clearance within the nasal compartment,
202 and to a lesser extent within the lungs during SARS-CoV-2 infection. Furthermore, both
203 CD4+ and CD8+ T cells are required for preventing viral persistence within the nasal
204 compartment and that CD4+ and CD8+ T cells can compensate for each other to control
205 virus replication within the nasal compartment.

206

207 **SARS-CoV-2 antibody responses are CD4+ T cell-dependent but not required for viral**
208 **control in the respiratory tract.** Infection and vaccine-mediated antibody responses are
209 essential protection against SARS-CoV-2 infection and have been identified as a correlate of
210 protection^{35,36}. To understand how T cells contribute to the antibody response and how these
211 antibodies may control acute SARS-CoV-2 infection, we evaluated binding and neutralizing
212 antibodies. On days 10 and 28 pi, we measured IgG binding antibodies to Spike, RBD and
213 Nucleocapsid (**Fig. 5a-c**). In mice in which CD4+ T cells were depleted (individual CD4+ or
214 CD4+/CD8+ tandem-depleted), we observed a significant reduction in anti-Spike, anti-RBD,
215 and anti- Nucleocapsid IgG binding antibodies as compared to isotype control. In contrast,
216 we observed no reduction in anti-Spike and anti-RBD IgG binding antibodies in the CD8+ T
217 cell depleted mice. Furthermore, we performed a live virus neutralization assay against

218 B.1.351 and found that CD4+ T cell depleted mice (individual CD4+ or CD4+/CD8+ tandem-
219 depleted) showed no neutralizing antibodies above the limit of detection (FRNT₅₀ = 20) (**Fig.**
220 **5d**). These findings demonstrate that the antibody response in mice during SARS-CoV-2
221 infection is CD4+ T cell dependent. Further, these data suggest that the antibody response
222 likely plays little to no role in preventing viral persistence in the upper respiratory tract.

223

224 **SARS-CoV-2 persists within the nasal epithelium in the absence of CD4+ and CD8+ T**
225 **cells.** To understand whether the persistent viral RNA in the upper respiratory tract
226 corresponds to infectious virus or not, we performed a TCID₅₀ infectious virus assay from
227 nasal compartment suspensions of isotype and CD4+/CD8+ tandem-depleted mice on day
228 28 pi (**Fig. 6a**). We found that 10/10 mice showed infectious virus from the tandem-depleted
229 infected mice, but not the isotype control infected mice, with an average viral titer of 1.26 x
230 10⁴ TCID₅₀/ml. To determine where the virus is replicating within the nasal compartment, we
231 performed *in situ* hybridization (ISH) using RNAscope on the entire head of a mouse, which
232 includes the nasal compartment, olfactory bulbs, and brain, with a spike-specific RNA probe
233 (**Fig. 6a, c**). We observed no viral RNA staining within brain or olfactory bulbs in either the
234 isotype control (n=5) or tandem depleted mice (n=10), suggesting that in the presence or
235 absence of T cells, SARS-CoV-2 does not infect the brain. CD4+/CD8+ T cell tandem-
236 depleted mice, but not isotype control mice, consistently showed viral RNA staining within
237 the nasal compartment. We observed viral RNA staining within the epithelial layer and
238 infection with little replication within the basement membrane. Additionally, infection was
239 more commonly seen in the nasal epithelium than the olfactory epithelium found in the
240 ethmoturbinates (**Fig. 6d**). These findings demonstrate that both CD4+ and CD8+ T cells are
241 required to prevent viral persistence within the nasal epithelium.

242

243 **Viral persistence leads to increased viral diversity.** In humans, persistent SARS-CoV-2
244 infection of immunocompromised individuals can lead to increased viral genetic diversity³⁷⁻⁴¹
245 . To understand the impact of viral persistence on SARS-CoV-2 evolution, we isolated virus

246 on day 28 pi from the nasal turbinates of CD4+/CD8+ tandem-depleted mice and propagated
247 it once using Vero-TMPRSS2 cells, which we have shown previously to minimize genetic
248 diversity during virus isolation and propagation⁴². We refer to these samples as 'isolate-28d'.
249 Concurrently, the B.1.351 stock virus was also cultured in Vero-TMPRSS2 cells (denoted:
250 'inoculum-Vero) to identify changes that may occur during a single viral passage. The
251 'inoculum-Vero' and stock virus used to infect the mice ('inoculum-stock') serve as the
252 baseline for virus diversity. These sequences were compared with the stock virus used to
253 infect the mice, referred to as 'inoculum-stock' in the text. To account for sequence
254 mutations resulting from mouse adaptations, mice were also infected with inoculum stock
255 virus and samples obtained from nasal turbinates on day 3 post-infection (referred to in this
256 text as 'inoculum-3d) where we observe peak viral titers (Fig. 1b). These mouse isolates and
257 inoculum controls were sequenced to characterize emergent intrahost SARS-CoV-2
258 variants.

259

260 To quantify the variation in the virus populations between the mouse isolates-28d and
261 inoculum controls, we calculated the pairwise genetic distance using the L1-norm. All the
262 three controls; the inoculum-Vero, inoculum-3d and inoculum-stock were similar in diversity
263 to each other (**Fig. 7a**) and differed only slightly likely due to variations in their low-frequency
264 minor variant populations (**Fig. 7a inset, Extended Data Fig. 5d-e**). Compared to the
265 inoculum-3d controls, all virus populations in the mouse isolates significantly diverged from
266 the inoculum-stock (p-value = 6.49e-16).

267

268 To identify mutations that emerged *de novo* in the tandem T-cell depleted mice by day 28 pi,
269 we filtered for intrahost single nucleotide variants (iSNVs) that were not present in the
270 standing diversity of the inoculum-stock and inoculum-Vero samples (0.5%-100%). The
271 location, number, and frequency of *de novo* iSNVs varied by mouse isolate (**Fig. 7b-c**), with
272 isolate 10 having zero iSNVs that reached $\geq 50\%$ while isolate 9 had 15 (6 of which were
273 amino acid substitutions) (**Extended Data Fig. S6**). The ORF10, nsp7, and envelope had

274 the highest mutational densities with averages of 14.5 (± 5.8), 3.2 (± 2.5), and 2.6 (± 2.3) *de*
275 *novo* iSNVs/kb, respectively. Out of the 57 unique *de novo* mutations, 19 emerged in more
276 than one mouse, 8 of which generated amino acid substitutions in the ORF1a (nsp3, nsp4,
277 nsp7, nsp8), ORF1b (nsp12, nsp15), E, and ORF10 gene regions. All 19 mutations were
278 present in a small subset ($<1\%$) of globally circulating SARS-CoV-2 sequences (CoV-
279 Spectrum 01/06/2020-12/05/2023)⁴³. Additionally, the nsp4 T295I mutation has previously
280 been reported in mouse-adapted strains of SARS-CoV-2¹⁷. The remaining nonsynonymous
281 mutations have not been characterized, though others have identified mouse-specific
282 adaptations that emerged in nsp6, nsp7, and nsp8^{44,45}. Interestingly, iSNVs shared in more
283 than one mouse isolate were more likely to be present as minor variants ($<50\%$) compared
284 to iSNVs found in only a single mouse isolate (p-value = 0.0003, Fisher's Exact), indicating
285 that the shared iSNVs may emerge at different points during infection.

286

287 In the mice where both CD4+ and CD8+ T cells were depleted, the synonymous divergence
288 rates (mean = $1.12e-05$ per site per day) were higher than the nonsynonymous (mean =
289 $5.84e-06$ per site per day) (**Fig. 7d**, p-value = 0.043 Mann Whitney U), indicating that
290 purifying selection is occurring in the CD4+/CD8+ tandem depleted mice. Further, the rates
291 observed in our mouse isolates fall into similar ranges observed in immunocompromised
292 individuals with prolonged infections (≥ 21 days)⁴⁶. Over 76% of the iSNVs were C-to-T
293 transitions (**Fig. 7e**), a signature of SARS-CoV-2 evolution observed in human intrahost
294 studies and globally circulating strains⁴⁷.

295

296 **Genetic diversity within SARS-CoV-2 leads to reduced virus replication in the lungs.**

297 We next selected five mice isolates based on their distance from the inoculum-stock and *de*
298 *novo* iSNV populations (**Fig. 7a**, **Extended Data Fig. 6a**, **8a**). Isolates 4, 7 and 9 diverged
299 from the inoculum-stock the most with 13, 20, and 21 *de novo* iSNVs, respectively
300 (**Extended Data Fig. 6**). Isolates 4 and 9 had a consensus level deletion at nucleotide
301 positions 27264-27290 (amino acids 22-30) in ORF6 (**Extended Data Fig. 7a**). This deletion

302 was also found at <1% in the inoculum-stock (**Extended Data Fig. 7b**). Isolate 7 had *de*
303 *novo* mutations emerge in only the nucleocapsid gene (**Fig. 8a**). We propagated these five
304 isolates on VeroE6-TMPRSS2 cells (“isolate-28d-P2”) to generate working *in vivo* stocks.
305 After sequencing the isolate-28d-P2 samples, we confirmed that no new mutations emerged
306 upon viral propagation except for one mutation in isolate 7 (nsp4: T461A) and two mutations
307 in isolate 10 (nsp13: S80G, E: L37F) increased in frequency after propagation.

308

309 We next infected C57BL/6 mice with the five mice isolates-28d-P2 at 10^6 PFU/mL and
310 measured weight loss throughout the infection and infectious viral titers in the lungs at day 3
311 pi. We observed similar weight loss dynamics across the five isolates compared to the mice
312 infected with the B.1.351 stock virus (**Fig. 8b**). Isolate 10, which was the most similar to the
313 stock B.1.351 virus, had similar titers to B.1.351-infected mice (**Fig. 7a, 8c**). The most
314 divergent isolates, 4, 7, and 9, showed a 6.44, 5.44, and 3.71-fold reduction, respectively, in
315 virus replication in the lung as compared to B.1.351-infected mice (**Fig. 7a, 8c**). Collectively,
316 these findings demonstrate that viral persistence leads to increased SARS-CoV-2 intrahost
317 genetic diversity and these changes can lead to differences in virus replication.

318

319

320 **DISCUSSION**

321 The magnitude and quality of the T cell response is essential for driving protection against
322 SARS-CoV-2 infection as well as promoting vaccine efficacy⁴⁸. Several groups have
323 investigated T cell responses against SARS-CoV-2 infection in the lungs⁴⁸⁻⁵², however, the
324 contribution of these cells in promoting viral control and clearance within the upper
325 respiratory tract is not well understood. In our study, we demonstrate that T cells are
326 necessary for controlling virus replication in the upper respiratory tract, but not the lower
327 respiratory tract, during SARS-CoV-2 infection. Using antibodies to deplete T cells prior to
328 infection, we found that CD4+ and CD8+ T cells play distinct roles in the upper and lower
329 respiratory tract. Tandem depletion of both CD4+ and CD8+ T cells, but not individual,
330 results in persistent virus replication in the nasal compartment through 28 days post-
331 infection. The persistent virus was culturable as we were able to recover infectious virus
332 from the nasal compartment of all tandem depleted mice nearly a month after infection.
333 Further, we used *in situ* hybridization to determine that the virus was replicating within the
334 nasal epithelium, and not the olfactory bulbs or brain, during persistent infection. Through
335 deep sequencing, we found that the virus isolates from persistently infected mice show
336 mutations across the viral genome, with several mice showing deletions within the ORF6
337 gene. Combined, these findings highlight the importance of T cells in controlling virus
338 replication within the respiratory tract during SARS-CoV-2 infection.

339

340 Our studies show that T cells are critical for viral clearance within the upper respiratory tract,
341 especially within the nasal compartment but not the lungs. In support, previous studies with
342 the related SARS-CoV-1 virus found that B and T cells were dispensable for controlling virus
343 replication within the lungs- highlighting the importance of innate immunity within the lungs
344 for viral control against coronaviruses⁵³. Our data also supports an important role for the
345 innate response in controlling virus replication within the respiratory tract. Indeed, studies
346 have found that type I and III IFNs can control SARS-CoV-2 replication in the upper and
347 lower respiratory tracts^{54,55}. Further, pDCs and alveolar macrophages have been implicated

348 in modulating inflammation and viral control⁵⁶⁻⁵⁹. We recently determined the importance of
349 the CCR2-monocyte signaling axis in promoting virus control and dissemination within the
350 lungs and mediating protection¹⁸. Altogether these studies show that a combination of the
351 innate and adaptive immune response is important for controlling virus replication, mitigating
352 viral dissemination, limiting inflammation and protecting against lethal disease outcome.

353

354 One interesting observation was that neutralizing antibodies do not appear to play an
355 important role in controlling SARS-CoV-2 infection. First, we show that depletion of CD4+ T
356 cells dramatically reduces SARS-CoV-2-specific spike and nucleocapsid binding antibodies.
357 This is consistent with studies in humans which have shown that antibody responses during
358 SARS-CoV-2 infection and vaccination are T cell dependent^{20,60,61}. Second, we show that the
359 lack of CD8+ T cells does not alter SARS-CoV-2 spike binding or neutralizing antibody
360 responses. Lastly, we show similar control of virus replication within the upper and lower
361 respiratory tracts on day 10 and 28 post-infection in the absence of either CD4+ or CD8+ T
362 cells, suggesting that antibody response is not a major driver of viral control within the
363 respiratory tract during infection. It is possible that non-neutralizing and neutralizing
364 antibodies play a subtle role in the kinetics of viral clearance within the respiratory tract and
365 future studies should specifically look at the timing of the antibody response and viral control
366 within the respiratory tract.

367

368 Under normal conditions, following respiratory virus infection, effector CD4+ and CD8+ T
369 cells are primed in the lung-draining lymph nodes and then traffic back to the infected lung to
370 control virus replication through their effector mechanisms⁴⁸. There are many factors that
371 can influence T cell programming, including but not limited to the local cytokine environment,
372 engagement with antigen-presenting cells (APCs), interaction with co-stimulatory molecules
373 and antigen load. Our findings suggest that T cell priming maybe different between the upper
374 and lower respiratory tract. While Granzyme B-secreting T cells were similar between the
375 URT and lungs, we did observe differences in cytokine-producing T cells. This suggests that

376 anatomic compartmentalization could influence T cell responses within the respiratory tract
377 during SARS-CoV-2 infection. Analogously, we have found during West Nile virus infection,
378 an encephalitic RNA virus that targets the brain, shows differences in T cell activation and
379 effector functions between the brain and spleen⁶². In the context of LCMV infection, we have
380 previously shown that anatomic location is a driver of memory precursor CD8+ T cells into
381 long-term memory cells within the white pulp region of the spleen⁶³. Future studies should
382 focus on identifying APC-T cell interactions within the lung-draining lymph node and within
383 the lungs as well as how the inflammatory milieu may influence T cell responses within the
384 upper and lower respiratory tract.

385

386 In individuals with weakened immune systems, the clearance of viruses may be delayed,
387 resulting in prolonged shedding of the virus and accumulation of mutations in the virus
388 genome^{37,64}. One leading hypothesis is that chronic infections in immunocompromised
389 individuals are an important source of SARS-CoV-2 genetic diversity, driving the emergence
390 of highly divergent SARS-CoV-2 variants of concern^{37,38}. However, data are limited for
391 studying how virus persistence, antiviral therapeutics, and host response during chronic
392 infections impact the intrahost evolution of SARS-CoV-2. In our study, we detected viral RNA
393 persisting in the upper respiratory tract and successfully isolated and sequenced infectious
394 virions from the nasal turbinates 28 days post-infection. Sequence analysis of the virus
395 populations collected from persistent infections revealed *de novo* mutations that
396 accumulated at high frequencies across the viral genome, including mutations in ORF10 and
397 ORF6. ORF10 had the highest density of mutations and has been shown to degrade antiviral
398 innate immunity by mitophagic degradation of MAVS⁶⁵. Interestingly, we observed a 27-
399 nucleotide long deletion in ORF6 that was present at <1% in the stock inoculum but reached
400 >50% in mouse isolates 4 and 9. The significance of the ORF-6 region in antagonizing
401 interferon signalling during SARS-CoV-1 infections is well-established. However, conflicting
402 findings exist regarding the role of ORF6 in SARS-CoV-2 infection, with studies showing
403 both direct interference with IFN signaling through binding to nucleoprotein Nup98 and

404 contradictory outcomes in animal models, including mice and hamsters⁶⁶⁻⁷⁰. Interestingly,
405 there was limited diversity found in the Spike protein. Among the three observed Spike
406 mutations, only one was present in the receptor binding domain (P384S, isolate 9) and out of
407 the 19 mutations shared, only one (nsp4 T295I) has been identified as a mouse adaptation.
408 Our findings, which include elevated rates of synonymous mutations and a higher
409 occurrence of C-to-T transitions, align with observations made in prolonged SARS-CoV-2
410 infections of immunocompromised humans^{39,47,71}. These studies collectively highlight how
411 mutations within the viral genome can result in strategies to evade the immune system,
412 underscoring the importance of thorough investigation into these mutations. To the best of
413 our knowledge, our study represents one of the initial animal models illustrating SARS-CoV-
414 2 viral persistence, leading to sequence alterations, offering a valuable tool for exploring
415 persistent viral infections and studying the intrahost evolutionary dynamics of SARS-CoV-2.

416

417 In summary, our studies highlight the importance of T cells in mediating viral control within
418 the respiratory tract. These findings directly impact the future design of mucosal vaccines
419 and demonstrate the importance of promoting T cell immunity within the upper and lower
420 respiratory tract. Further, these studies now provide a model to study innate immunity and
421 virus-host interactions in the context of viral persistence within the upper respiratory tract.

422 **METHODS**

423

424 **Viruses and cells.** VeroE6-TMPRSS2 cells were generated as previously described⁷² and
425 cultured in complete DMEM consisting of 1x DMEM (VWR, #45000-304), 10% FBS, 2mM L-
426 glutamine, and 1x antibiotic in the presence of Puromycin 10mg/mL (Gibco). The SARS-
427 CoV-2 B.1.351 variant, kindly obtained from Andy Pekosz at John Hopkins University in
428 Baltimore, MD, was plaque purified, propagated in VeroE6-TMPRSS2 cells to create a
429 working stock, and sequence confirmed. Viral titers were determined through plaque assays
430 conducted on VeroE6-TMPRSS2-hACE2 cells (kindly provided by Dr. Barney Graham,
431 Vaccine Research Center, NIH, Bethesda, MD) as described here¹⁸.

432

433 **Mouse experiments:** C57BL/6J mice were purchased from Jackson Laboratories or bred in-
434 house at the Emory National Primate Research Center rodent facility at Emory University. All
435 mice used in these experiments were females between 8-12 weeks of age. Mice were
436 anesthetized using isoflurane and infected with SARS-CoV-2 B.1.351 at 10⁶ PFU intranasally
437 in a final volume of 50 µL in saline in accordance with the institutional standard operating
438 procedure for working in animal biosafety level 3 facility. Post-infection, mice were monitored
439 daily for any clinical pathology and mortality. For experiments involving T cell depletion, mice
440 were administered 200µg/mouse of either anti-CD4 (clone GK1.5, BioXCell) or anti-CD8α
441 (clone 2.43, BioXCell), or a combination of both anti-CD4 and anti-CD8α antibodies, or an
442 isotype mAb IgG1 (clone HRPN, BioXCell). The administration was done via the
443 intraperitoneal (IP) route on days -5, -3, -1, +1, +7, +14, and +21 following SARS-CoV-2
444 infection. Unless otherwise specified, all CD8+ T cell depletions were carried out using the
445 anti-CD8α antibody. The impact of CD8+ T cell depletions on SARS-CoV-2 infection was
446 also evaluated using anti-CD8β (clone 53-5.8, BioXCell) and isotype mAb IgG2b (clone LTF-
447 2, BioXCell). All experiments and the mouse handling and care procedures followed the
448 guidelines of the Emory University Institutional Animal Care and Use Committee.

449

450 **Antibody binding assays.** Serum collected from infected mice was tested to assess the
451 binding of IgG antibodies against B.1.351 spike, RBD, and nucleocapsid using using the V-
452 PLEX SARS-CoV-2 Panel 7 (Mouse IgG) Kit (Meso Scale Discovery, #K15484U-2) per
453 manufacturer protocol⁷². In brief, plates coated with the specific SARS-CoV-2 antigens were
454 blocked using MSD blocker at room temperature, with shaking at 700 rpm for 30 minutes.
455 Samples were diluted 1:1000 and incubated with the plates for two hours at room
456 temperature. Following this, SULFO-TAGTM conjugated Goat Anti-Mouse IgG Antibody was
457 added. The plates were washed with 1X MSD Wash Buffer, and then MSD Gold Read Buffer
458 B was added to each well. Between each step, the plates were washed three times with PBS
459 containing 0.05% Tween. An MSD plate reader was used to read the plates, and the results
460 were analyzed using Discovery Workbench[®] software, version 4.0 and reported as arbitrary
461 units per ml (AU/mL) against SARS-CoV-2.

462

463 **Focus reduction neutralization assay.** FRNT assays were conducted following the
464 methods outlined in the protocol described previously⁷³. In summary, the samples were
465 diluted in 3-fold increments, creating 8 serial dilutions using DMEM in duplicates. The initial
466 dilution was set at 1:10, and the total volume reached 60 μ L. The serially diluted samples
467 were then incubated with virus at 37°C for 1 hour in a round-bottomed 96-well culture plate.
468 After the incubation, the antibody-virus mixture was added to Vero-TMPRSS2 cells and
469 incubated again at 37°C for 1 hour. Following this step, the antibody-virus mixture was
470 removed, and a 0.85% methylcellulose overlay (Sigma-Aldrich) was added to each well. The
471 plates were further incubated at 37°C for 18 hours. Once the incubation was complete, the
472 methylcellulose overlay was removed, cells were washed with PBS and fixed using 2%
473 paraformaldehyde in PBS. Plates were washed with PBS and permeabilized using a buffer
474 containing 0.1% BSA and Saponin in PBS for 20 minutes. Following this, cells were
475 incubated overnight at 4°C with the anti-SARS-CoV spike primary antibody directly
476 conjugated to Alexa Fluor-647 (CR3022-AF647). Subsequently, the cells underwent two
477 washes with 1x PBS before imaging on an ELISPOT reader (CTL Analyzer).

478

479 **TCID50 assay.** VeroE6-TMPRSS2 cells were seeded at a density of 25,000 cells per well in
480 complete DMEM media in quadruplicates for each sample. Once confluent, the medium was
481 removed, and 180 μ L of DMEM containing 2% FBS and was added. Serial dilutions of
482 samples, along with positive controls (virus stock with a known infectious titer) and negative
483 controls (medium only), were included. The plates were further incubated at 37°C with 5.0%
484 CO₂ for 2 to 5 days. Cells were fixed and stained with a crystal violet solution containing 2%
485 paraformaldehyde. Visual inspections were carried out on cell monolayers to detect any
486 cytopathic effect and TCID50 was determined using the Read–Muench formula⁷⁴.

487

488 ***In situ* hybridization of brain tissues.** Mice heads collected on day 28 pi were subjected to
489 decalcification in ethylenediaminetetraacetic acid (EDTA) for approximately two weeks,
490 followed by rehydration in PBS for two days. Subsequently, the samples were immersed in a
491 30% sucrose solution prepared in PBS for three to four days to achieve sucrose
492 equilibration. Once equilibrated, the heads were rapidly frozen in 100% Optimal Cutting
493 Temperature (O.C.T.) compound and stored at -80°C. Tissues were sliced using a cryostat
494 and stored at -80°C. RNA in situ hybridization (RNA-ISH) was performed following the
495 RNAscope Brown Kit protocol, with a modification for OCT-frozen tissues where tissue is
496 post-fixed onto the slide. The tissue was stained for Spike RNA using a custom RNAscope
497 Probe V-nCoV2019-S (ACD, #848561). Images were obtained using a Zeiss Axiolmager Z2
498 system with Zeiss software at 20X on the Slide Scanner, an automated imaging system.

499

500 **Processing of mouse tissues for flow cytometry analysis.** On the specified day following
501 infection, mice were anesthetized using isoflurane and given a retro-orbital injection of
502 CD45:PE (BD Biosciences, clone 30-F11) diluted 1:20 in saline in a final volume of 100 μ L
503 per mouse. After a 5-minute recovery period, the mice were euthanized using isoflurane
504 overdose. Lung, URT tissues and spleens were harvested from each mouse and placed in
505 1% FBS-HBSS. The spleens underwent mechanical homogenization on a 70 μ M cell

506 strainer, and the resulting cell suspension was collected in 10% FBS-RPMI. Splenocytes
507 were processed by centrifugation (1250 rpm, 5 min, 4C), followed by lysis in ACK Lysis
508 buffer for 5 minutes on ice. After washing with 10% FBS-RPMI, the splenocytes were kept
509 chilled until they were ready for further applications. The lungs were mechanically disrupted
510 in 6-well plates and then subjected to a 30-minute digestion at 37C using a solution of
511 DNaseI and collagenase in HBSS. The digestion was halted with 10% FBS-RPMI, and the
512 lung cells were filtered through a 70 μ M filter to obtain a single-cell suspension. The
513 obtained cells were layered onto a Percoll-PBS gradient, centrifuged, and the top layer of
514 cell debris was removed. The resulting cell pellet was lysed with ACK lysis buffer for 5
515 minutes on ice, followed by washing and resuspension in 10% FBS-RPMI, keeping the cells
516 chilled until they were ready for the staining process.

517

518 **Flow cytometry analysis.** Single-cell suspensions were centrifuged and were then
519 resuspended in a blocking solution containing anti-CD16/32 (Tonbo, Clone 2.4G2) for 20
520 minutes at 4°C. After centrifugation, the cell suspensions were stained using surface stain
521 antibodies including Live/Dead Ghost Dye stain (Tonbo Biosciences) for 20 minutes at 4°C.
522 Following this, the stained cells were washed and fixed in 2% PFA-PBS for 30 minutes at
523 room temperature. Finally, cells were washed, resuspended in 300 μ L of FACS buffer (1%
524 FBS in 1x PBS). Precision count beads (Biolegend) were added to the samples to facilitate
525 cell counting. These samples were then processed using a BD FACS Symphony A5
526 instrument. The anti-mouse surface staining antibodies utilized in this study were: CD45:PE
527 (Biolegend, Clone: 30-F11), CD45.2:BV650 (Biolegend, Clone: 104), CD44: FITC
528 (Biolegend, Clone: NIM-R8), CD3e: PerCP Cy5.5 (Tonbo, Clone: 145-2C11), IL-7R: PE/
529 Cyanine 5 (Biolegend, Clone: A7R34), KLRG1:APC-Cy7 (Biolegend, Clone: 2F1), CD8b:
530 BV421 (Biolegend, Clone: YTS156.7.7), Live/Dead Ghost Dye™ UV 450 (Tonbo), CD69:
531 BV785 (Biolegend, Clone: H1.2F3), CD103: AF700 (Biolegend, Clone: 2E7), CD4: PE-Cy7
532 (Biolegend, Clone: GK1.5).

533

534 **Ex vivo T cell assays.** For T cell peptide restimulation, approximately 1×10^6 cells harvested
535 from tissues were placed per well in a 96-well round bottom plate and stimulated for six
536 hours at 5% CO₂, 37°C, with the addition of 10 µg/mL brefeldin A (BFA) in complete RPMI
537 media. For positive control, splenocytes were stimulated with BFA and PMA/Ionomycin. For
538 negative control, cells were stimulated with BFA and vehicle DMSO in the same media. To
539 measure antigen-specific T cell responses, splenocytes, URT and lung cells were stimulated
540 with 1 µg/ml of SARS-CoV-2 spike peptide pool (BEI resources) in the presence of BFA.
541 After T cell stimulation, cells were washed with FACS buffer and stained for the surface
542 antigens as described in the previous section. Cells were then washed with FACS buffer and
543 incubated with 1x Fix/Perm solution (Tonbo) at room temperature for one hour. Following
544 this, cells were washed with 1x Perm buffer (Tonbo) and stained for intracellular antigens for
545 30 mins at 4°C with the following antibodies: GranzymeB: AF647 (Biolegend, Clone: GB11),
546 IL-2: BV605 (Biolegend, Clone: JES6-5H4), TNFα (Biolegend, Clone: MP6-XT22) and IFNγ:
547 PE Dazzle 594 (Biolegend, Clone: XMG1.2). Following intracellular staining, cells were
548 washed twice with the 1x Perm buffer and once with FACS buffer. Cells were resuspended
549 in 300 µL of FACS buffer. Precision count beads (Biolegend) were added to the samples to
550 facilitate cell counting. These samples were then processed using a BD FACS Symphony A5
551 instrument. For data analysis, splenocytes were gated on lymphocytes, single cells, live,
552 parenchymal lymphocytes, CD3+ and then further categorized as either CD4-, CD8+ for
553 CD8 T cell response analysis, or CD4+, CD8- for CD4 T cell analysis. Antigen-specific cells
554 were identified based on their production of IFN-γ, TNF-α, or both cytokines in response to
555 SARS-CoV-2 peptide restimulation.

556

557 **Quantitative reverse transcription-PCR (qRT-PCR).** To prepare tissue samples for
558 evaluating viral RNA levels and mRNA expression, lung and nasal turbinate tissues were
559 collected in an Omni Bead Ruptor tube containing Tri reagent (Zymo). Subsequently, tissues
560 were homogenized using the Omni Bead Ruptor 24 instrument with a program set at 5.15
561 m/s for 15 seconds. The samples were then stored at -80°C until further analysis. Samples

562 in Tri reagent were briefly spun and then RNA extracted using Direct-zol RNA miniprep kit
563 (Zymo) and cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit
564 (Thermo Fisher Scientific) as per the manufacturer's protocol. Viral RNA levels and
565 replication were measured as previously described⁵⁴. Briefly, qRT-PCR was set up using IDT
566 Prime Time gene expression master mix on a QuantStudio5 qPCR system using the cycling
567 conditions recommended by the manufacturer. To measure viral RNA levels, SARS-CoV-2
568 RDRP-specific forward primer: GTGARATGGTCATGTGTGGCGG; reverse primer:
569 CARATGTTAAASACACTATTAGCATA, and probe 56-6-carboxyfluorescein
570 [FAM]/CAGGTGGAA/ZEN/CCTCATCAGGAGATGC/3IABkFQ were used. To measure virus
571 replication, levels of SARS-CoV-2 E gene subgenomic RNA (sgRNA) was measured using
572 forward primer sgLeadSARSCoV2-F: 5'-CGATCTCTTGTAGATCTGTTCTC-3' (IDT) and the
573 E_Sarbeco R2 reverse primer (IDT; #10006890) and P1 FAM probe (IDT; #10006892).
574 GAPDH was used as a reference gene to normalize viral RNA levels which were
575 represented as fold change over mock samples.

576

577 **Illumina library preparation, sequencing, and alignment.** SARS-CoV-2 RNA was isolated
578 using RNAzol® RT Column Kit (Molecular Research Center, Inc.) as per manufacturer's
579 instructions from the B.1.351 stock samples (n=4, "inoculum-stock"), B.1.351 stock samples
580 passaged once in VeroE6-TMPRSS2 cells (n=3, "inoculum-Vero"), nasal turbinates of
581 C57BL/6 control mice at day 3 pi (n= 5, "inoculum-3d"), nasal turbinates of CD4+/CD8+
582 tandem depleted mice on day 28 pi and passaged once in VeroE6-TMPRSS2 cells (n=10,
583 "isolate-28d"), and five mouse isolates passaged once more in VeroE6-TMPRSS2 cells
584 ("isolate-28d-P2"). Approximately 400bp long amplicons were generated from the isolated
585 SARS-CoV-2 viral RNA using the ARTIC V4 primers and protocol ([https://artic.network/2-
586 protocols.html](https://artic.network/2-protocols.html)). Amplicons were cleaned using AMPure beads and input into the Illumina
587 DNA Prep Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. The
588 concentration and fragment size of the libraries were determined using the Qubit dsDNA
589 high-sensitivity assay (ThermoFisher Scientific, Waltham, MA) and a high-sensitivity D1000

590 screentape (Agilent, Santa Clara, CA), respectively. The final libraries were pooled at equal
591 molarity and sequenced on the MiSeq (v3 600 cycles, Illumina, San Diego, CA).
592 Amplification, library preparation, and sequencing were done twice on the same RNA
593 sample.

594

595 Reads were trimmed with trimmomatic v0.39⁷⁵ and aligned to the Wuhan/Hu-1 SARS-CoV2
596 genome (NC_045512.2) using bwa mem v0.7.17⁷⁶. ARTIC v4 primer sequences were
597 removed using iVar v1.3.1⁷⁷ with a minimum quality threshold of 0, including all reads with
598 no primer sequences found. Consensus sequences and variants were called using an in-
599 house variant calling pipeline, timo (v4) (<https://github.com/GhedinLab/timo>)⁷⁸. The
600 amplification and library preparation protocol and alignment pipeline are available
601 at https://github.com/GhedinSGS/SARS-CoV-2_analysis. The sequencing data are available
602 at (PRJNA1064978).

603

604 **Single nucleotide variant analysis.** Coverage and variant data were pulled from the timo
605 outputs (**Extended Data Fig. 5a-c**). Minor variants in the inoculum and inoculum-Vero
606 samples were required to be present in both sequencing replicates with an average
607 frequency of 0.5%-49% and read depth of 200X. Nucleotides $\geq 50\%$ at positions with $\geq 10X$
608 read depths (dp) were considered the consensus nucleotide for the sample. All consensus
609 sequences for the inoculum-stock and inoculum-Vero samples were confirmed to be
610 identical. Passaging the inoculum stock once in the VeroE6-TMPRSS2 filtered out a large
611 proportion of low-frequency (mean = 1%, median = 0.7%) minor variants in the inoculum-
612 stock used for infections (**Extended Data Fig. 5d-e**). Therefore, intrahost single nucleotide
613 variants (iSNVs) that were generated *de novo* were required to be present at 2%-49% and at
614 positions with 200X dp for minor variants or 50%-100% 10X dp for consensus variants, and
615 not present in any inoculum stock or inoculum-Vero samples, including as a minor variant at
616 0.5%-49%. Tables outlining the *de novo* iSNVs are located at
617 [https://github.com/GhedinSGS/TCD Mice](https://github.com/GhedinSGS/TCD_Mice).

618

619 **Genetic distance and divergence calculations.** Divergence rates were calculated as
620 previously outlined⁷⁹. The number of nonsynonymous and synonymous sites were estimated
621 for each sample using the number of nucleotide positions in the coding sequence with at
622 least 200X read depth. All mouse isolates had >90% coverage of the coding sequence at
623 200X. Sites that lacked any minor variant and were identical in their consensus nucleotide
624 across all samples were not used for distance calculations, as the distance for these sites
625 equals 0. All other sites were used to calculate the pairwise genetic distance using the L1-
626 norm. Data and code are located at https://github.com/GhedinSGS/TCD_Mice.

627

628 **Quantification and Statistical Analysis.** All experiments in mice were repeated at least
629 twice, with representative results from one experiment shown. Statistical analysis was
630 performed in GraphPad Prism 10.1.1 (Prism, La Jolla, CA, USA) using Student's t test to
631 compare two groups and unpaired one-way analysis of variance (ANOVA) to compare more
632 than two groups. Statistical significance was defined as *P* values less than 0.05.

633

634 Antibody neutralization was quantified by determining the foci count for each sample done in
635 duplicates with the aid of the Viridot program⁸⁰. To calculate the neutralization titers, the
636 following formula was applied: $1 - (\text{mean number of foci in the presence of sera} / \text{foci at the highest dilution of the corresponding sera sample})$. The FRNT-50 titers were
637 estimated through 4-parameter nonlinear regression in GraphPad Prism 8.4.3. Samples that
638 did not exhibit neutralization at 50% were plotted at 20 and used for calculating the
639 geometric mean.

641

642 RT-qPCR results are expressed relative to mouse Gapdh expression for the same sample
643 and were calculated using the $\Delta\Delta\text{CT}$ relative quantitation method as compared to mock age-
644 matched controls.

645

646

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653

654 **Author Contributions**

655 M.K. and M.S.S. contributed to the acquisition, analysis, and interpretation of the data, as
656 well as the conception and design of the work, and writing of the manuscript. K.E.E.J.
657 contributed to the acquisition, analysis, and interpretation of the data, and writing of the
658 manuscript. K.F. and A.P. contributed to the acquisition, analysis, and interpretation of the
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660 the acquisition and analysis of the data. A.P., R.K., A.G. and E.G., contributed to the
661 interpretation of the data and conception and design of the work.

662

663 **FIGURE LEGEND**

664

665 **Figure 1- SARS-CoV-2 infection kinetics and immune response in C57BL/6 mice.**

666 C57BL/6 mice were infected with the SARS-CoV-2 B.1.351 (Beta) variant or an equal
667 volume of saline for mock mice (A) Percent of initial weight for Beta infected mice at
668 indicated PFUs over ten days. (B) Quantification of infectious virus at indicated days post-
669 infection as measured by plaque assay and expressed as PFU/gm of lung tissue. (C)
670 Quantification of viral RNA by qRT-PCR for SARS-CoV-2 RNA-dependent RNA polymerase
671 (RdRp) in lungs (left) and nasal turbinates (right). CT values represented as relative fold
672 change over mock (log10). IgG antibody titers against (D) RBD and spike and (E)
673 nucleocapsid as measured by an electrochemiluminescent multiplex immunoassay and
674 reported as arbitrary units per ml (AU/mL) and normalized by a standard curve for the
675 B.1.351 SARS-CoV-2 variant. (F) Neutralizing antibody response measured as 50%
676 inhibitory titer (FRNT50) by focus reduction neutralization assay. Graphs show mean \pm SD.
677 Results are representative of data from two independent experiments. Day 2 pi (n=4), day 4
678 pi (n=8), day 10 pi (n=10).

679

680 **Figure 2- SARS-CoV-2 B.1.351 infection leads to increased infiltration of CD8+ T cells**

681 **in the respiratory tract but not in the periphery.** C57BL/6 mice were infected with the
682 SARS-CoV-2 B.1.351 (Beta) variant at 10^6 PFU intranasally and at day 7 pi spleen, lungs
683 and URT tissues were harvested, processed for flow cytometry and analyzed via FlowJo.
684 Frequency and cell count for CD4+ and CD8+ T cells in (A) Spleen, (B) Lungs, and (C) URT;
685 representative flow plots on the left, frequency of cells in the middle and cell counts on the
686 right. Graphs show mean \pm SD. A two-way ANOVA statistical test was performed. *P < 0.05;
687 **P < 0.01; ***P < 0.001; ****P < 0.0001; no symbol, not significant. Results are
688 representative of data from three independent experiments with 5 mice per group.

689

690 **Figure 3- SARS-CoV-2 B.1.351 infection triggers antigen-specific T cell responses in**
691 **the upper and lower respiratory tract.** C57BL/6 mice were infected with the SARS-CoV-2
692 B.1.351 (Beta) variant at 10^6 PFU intranasally and at day 7 pi, virus-specific CD4⁺ and
693 CD8⁺ T cell responses were evaluated by ex-vivo peptide stimulation using spike peptide
694 pools in spleen, lungs and URT. (A) Representative flow plots for GzB (top), TNF α and IFN γ
695 (bottom) expression in CD4⁺ T cells in spleen, lungs and URT. (B) Frequency (top) and cell
696 counts (bottom) of CD4⁺ T cells positive for GzB, TNF α and IFN γ expression in spleen,
697 lungs and URT. (C) Representative flow plots for GzB (top), TNF α and IFN γ (bottom)
698 expression in CD8⁺ T cells in spleen, lungs and URT. (D) Frequency (top) and cell counts
699 (bottom) of CD4⁺ T cells positive for GzB, TNF α and IFN γ expression in spleen, lungs and
700 URT. Graphs show mean \pm SD. A two-way ANOVA statistical test was performed. *P <
701 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; no symbol, not significant. Results are
702 representative of data from three independent experiments with 5 mice per group.

703

704 **Figure 4- CD4⁺ and CD8⁺ T cells are dispensable for protection against SARS-CoV-2**
705 **but required for viral control within the respiratory tract.** (A) Study design: C57BL/6
706 mice were depleted of either CD4⁺ or CD8⁺ or both T cells using 200 μ g anti-mouse CD4 or
707 anti-mouse CD8 α or both respectively via i.p. route at day -5, -3, -1, 1, 7, 14 and 21 post-
708 infection. (B) Percent weight loss in SARS-CoV-2 (B.1.351) infected mice through 10 days pi
709 Viral RNA levels as measured by relative RdRp levels (top) and sgRNA levels (bottom) at
710 indicated time points in (C) lungs (D) URT. Graphs show mean \pm SEM. Multiple Mann-
711 Whitney statistical test was performed. *P < 0.05; **P < 0.01; ***P < 0.001; no symbol, not
712 significant. Data are an aggregate of two independent experiments with group sizes between
713 6-30 mice.

714

715 **Figure 5- SARS-CoV-2 antibody responses are CD4+ T cell-dependent but not**
716 **required for viral control in the respiratory tract.** C57BL/6 mice were depleted of either
717 CD4+ or CD8+ or both T cells and at indicated days post-infection, binding and neutralizing
718 antibody response against SARS-CoV-2 B.1.351 spike, RBD, and nucleocapsid were
719 measured by electrochemiluminescent multiplex immunoassay and reported as arbitrary
720 units per ml (AU/mL) against SARS-CoV-2. IgG antibody responses were measured against
721 (A) Spike (B) receptor-binding domain (RBD) and (C) Nucleocapsid. (D) The 50% inhibitory
722 titer (FRNT50) on the focus reduction neutralization test (FRNT) was measured at day 10 pi
723 and day 28 pi The dotted line in the FRNT assay represents the maximum concentrations of
724 the serum tested (1/20). A two-way ANOVA statistical test was performedwas performed.
725 *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; no symbol, not significant.

726

727 **Figure 6- SARS-CoV-2 persists predominantly within the nasal epithelium in the**
728 **absence of CD4+ and CD8+ T cells.** C57BL/6 mice were depleted of both CD4+ or CD8+
729 and assessed for localization of viral antigen in mice heads at 28 days pi (A) Tissue culture
730 infectious dose (TCID₅₀) per mL of nasal turbinate suspension assessed in mice where both
731 CD4+ and CD8+ T cells were depleted. (B) Representation of the sagittal section of a mouse
732 skull showing various parts of the nasal cavity and brain (created with BioRender.com). (C)
733 Representative images of ISH for RNA Spike Antigen performed on heads of mice where
734 both CD4+ and CD8+ T cells were depleted and compared to Isotype control mice. (D)
735 Representative images of nasal epithelium (top panel) and olfactory epithelium (bottom
736 panel) of ISH for RNA Spike Antigen. Arrows represent anti-spike RNA (dark brown).
737 Representative images from three of ten T cell-depleted mice.

738

739 **Figure 7- Intrahost SARS-CoV-2 variants emerge during infection in tandem**
740 **CD4+/CD8+ T-cell depleted mice.** (A) MDS plot of the pairwise genetic distance (L1-norm)

741 calculations across all samples in the dataset. Point color and shape represent the sample
742 and collection type. For the inoculum-stock, the color gradient represents different aliquots of
743 the stock. For inoculum-Vero and inoculum-3d, the color gradients represent different
744 infections. (B) The frequency distribution of *de novo* variants identified in the inoculum-3d
745 (black) and isolate-28d (1-10, gray) samples. (C) The location, frequency, and
746 characteristics of *de novo* variants in the inoculum-3d and isolate-28d (1-10) samples. The
747 color of each point represents the type of mutation including mutations that are
748 synonymous/in non-coding regions (black) or nonsynonymous/stop-codon mutations (red).
749 Point shape indicates the type of sample collection. Dashed lines highlight the genomic
750 positions where a *de novo* mutation was found in more than one mouse isolate-28d sample.
751 Labels are added for the nonsynonymous substitutions only. (D) The nonsynonymous and
752 synonymous divergence rates normalized by the expected number of sites in the coding
753 sequence for the inoculum-3d samples and T-cell depleted mouse isolate-28d samples.
754 Point color and shape represent the sample and collection type as outlined in Fig 7a. (E) The
755 density of transitions and transversions for all *de novo* mutations (2%-100%).

756

757 **Figure 8- *de novo* mutations lead to differences in virus replication.** (A) Mutation maps
758 of *de novo* SARS-CoV-2 consensus mutations ($\geq 50\%$) in the mouse isolates 4, 6, 7, 9, and
759 10, and compared to the isolate-Vero samples. Blue labels represent mutations that reached
760 $\geq 50\%$ in the isolate-Vero sample but were present at $< 50\%$ in the original mouse isolate.
761 Labels represent amino acid information for mutations in coding regions and nucleotide
762 information for deletions or mutations in the non-coding regions of the genome. (B) C57BL/6
763 mice were infected with mice isolates-P2 as indicated and weight loss measured over three
764 days. Graph represents percent weight loss compared to initial weight on the day of
765 infection. (C) Infectious virus from lungs of mice infected with indicated isolates at day 3 pi
766 was quantified by plaque assay in VeroE6-ACE2-TMPRSS2 over-expressing cells and
767 expressed as PFU/gm of lung tissue.

769 **EXTENDED DATA FIGURES**

770 **Extended Data Figure 1- Gating strategy for flow cytometry analysis of CD4+ and**

771 **CD8+ T cells at day 8 pi** C57Bl/6 mice were infected with SARS-CoV-2 B.1.351 and spleen,
772 lungs and URT tissues were harvested at day 7 pi. Five minutes prior to euthanasia, mice
773 were intravitaly labelled with CD45:PE (injected via the retro-orbital route). Tissues were
774 processed to a single-cell suspension and analyzed via flow cytometry. Total cell populations
775 were gated on (FSC-SSC), then singlets, then on Live (Ghost Dye 780). Lung infiltrating
776 cells were identified as CD45 IV- and CD45 ex-vivo+. Tissue parenchymal lymphocytes in
777 case of lungs and URT and total CD45+ cells in case of spleen were then gated for CD3+
778 cells which were further gated for CD4 and CD8+ T cells.

779

780 **Extended Data Figure 2- KLRG1 and CD69 MFI in Cd4+ and CD8+ T cells.** C57Bl/6 mice

781 were infected with SARS-CoV-2 B.1.351 and spleen, lungs and URT tissues were harvested
782 at day 7 pi and analyzed by flow cytometry. Representative histogram overlay plots for mock
783 (in grey) vs infected (in pink) for CD44 (top panel), KLRG1 (middle panel), and CD69
784 (bottom panel) in Lungs (A), URT (B) and spleen (C) at day 7 pi.

785

786 **Extended Data Figure 3- CD4+ and CD8+ T cells depletion assessment at day 0 and**

787 **day 28 pi in lungs and URT.** (A) Whole blood from mice depleted for either CD4+ T cells or
788 CD8+ T cells or both were subjected to CD4+ and CD8+ staining to assess depletion
789 efficiency as compared to the isotype control at day 0 pi. (B) Cells isolated from lungs, URT,
790 and whole blood at day 28 pi, were assessed for depletion efficiency as compared to the
791 isotype control. The analysis was done on CD3+ T cells which were gated on live and single
792 CD45+ lymphocytes.

793

794 **Extended Data Figure 4- comparison of effect of CD8+T cell depletion using CD8 β or**
795 **CD8 α antibody on viral RNA levels in lungs and URT at day 28 pi** Mice were either
796 depleted using CD8 β or CD8 α antibody and assessed for viral RdRp levels in (A) lungs and
797 (B) URT at day 28 pi by qRT-PCR. RNA levels were compared with individual isotype
798 controls. N=5 mice for each group.

799

800 **Extended Data Figure 5- Quality checks for SARS-CoV-2 sequencing data.** (A-
801 C) Fraction of the genome covered (y-axis) across different read depths (x-axis) for (A) the
802 inoculum stock, inoculum-Vero, and inoculum-C57BL/6 controls, (B) day 28 pi CD4+/CD8+
803 tandem T-cell depleted mouse samples, and (C) the 5 mouse isolates passaged once in
804 VeroE6-TMPRSS2 cells. Data for each sequencing replicate are plotted and differentiated by
805 the point shape and line type. (D) The number of minor variants found at 0.5-49% in the
806 inoculum stock and inoculum-Vero controls. (E) The frequency of minor variants (y-axis)
807 found in a single stock sample versus the frequency of minor variants present in at least one
808 inoculum stock and one inoculum-Vero sample (x-axis).

809

810 **Extended Data Figure 6- iSNV richness for each mouse isolate.** The number of high-
811 frequency (dark gray) and low-frequency (light gray) *de novo* iSNVs (y-axis) in each mouse
812 isolate separated by mutation type (non-coding, synonymous “syn”, nonsynonymous
813 “nonsyn”, and nonsense).

814

815 **Extended Data Figure 7– Consensus level deletions in mouse isolates.** (A) ORF6
816 consensus sequences from nucleotide positions 27264-27290 across all inoculum and
817 mouse isolate samples. Box colors represent the nucleotide found at each position. Black
818 squares represent positions identified as a deletion. (B) The relative frequency of ORF6
819 27264-27290 deletions in other samples where it did not reach consensus levels.

821 References

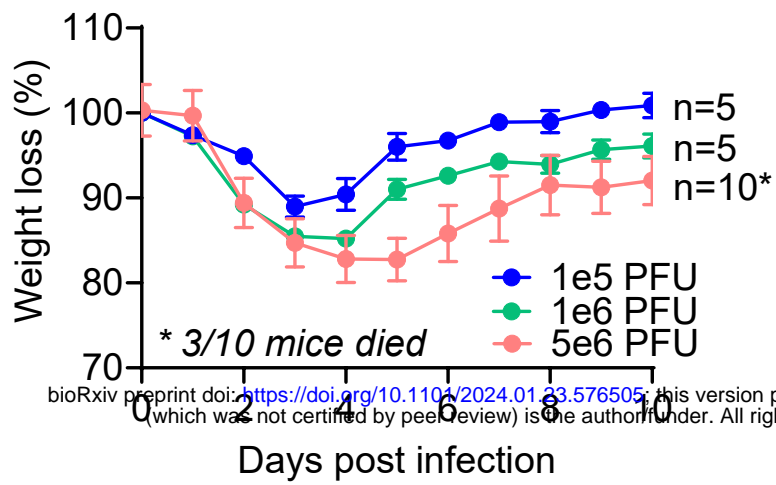
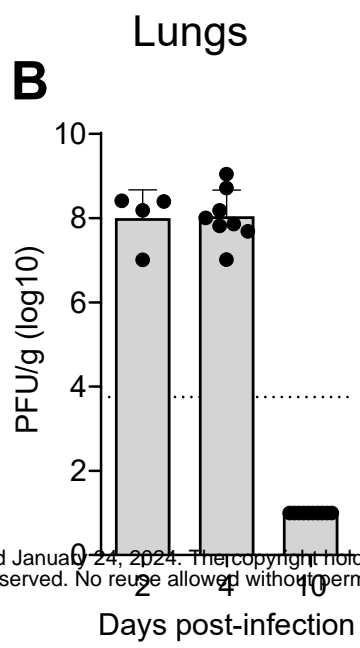
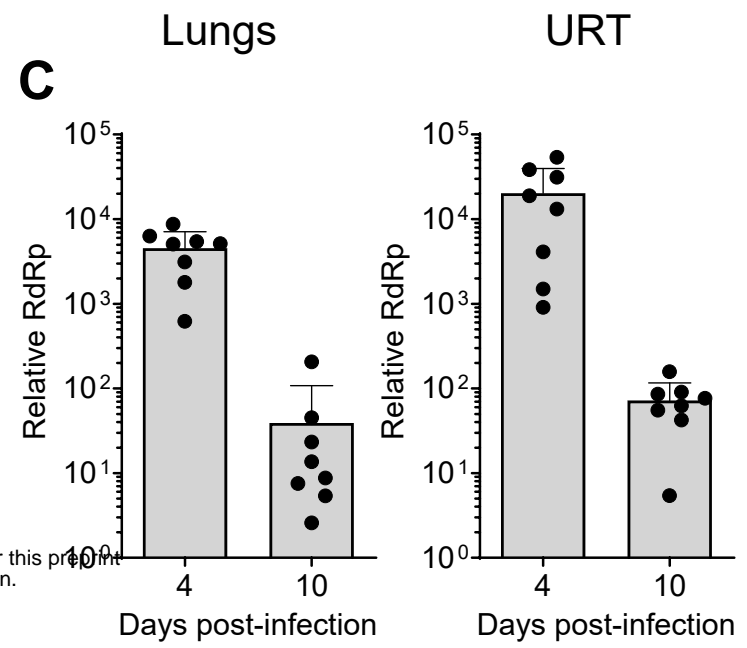
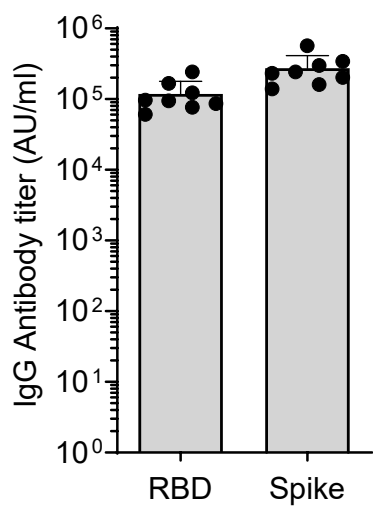
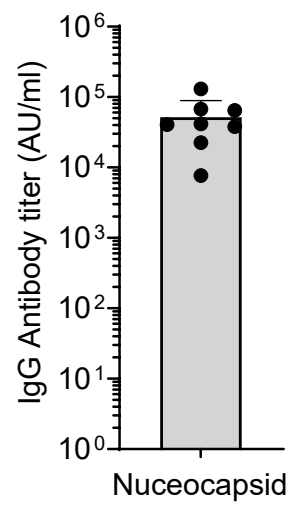
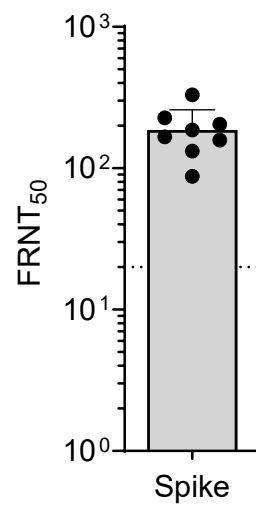
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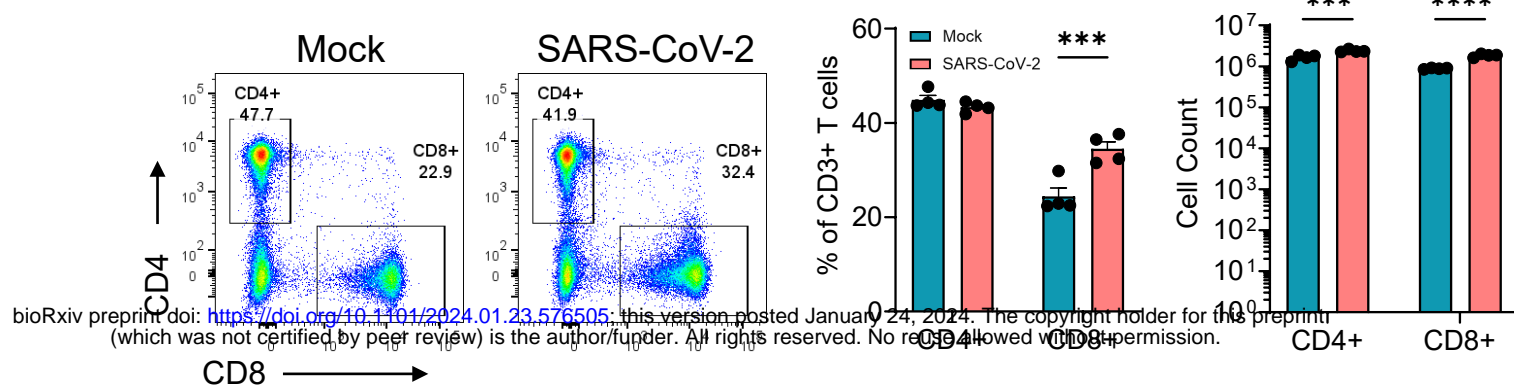
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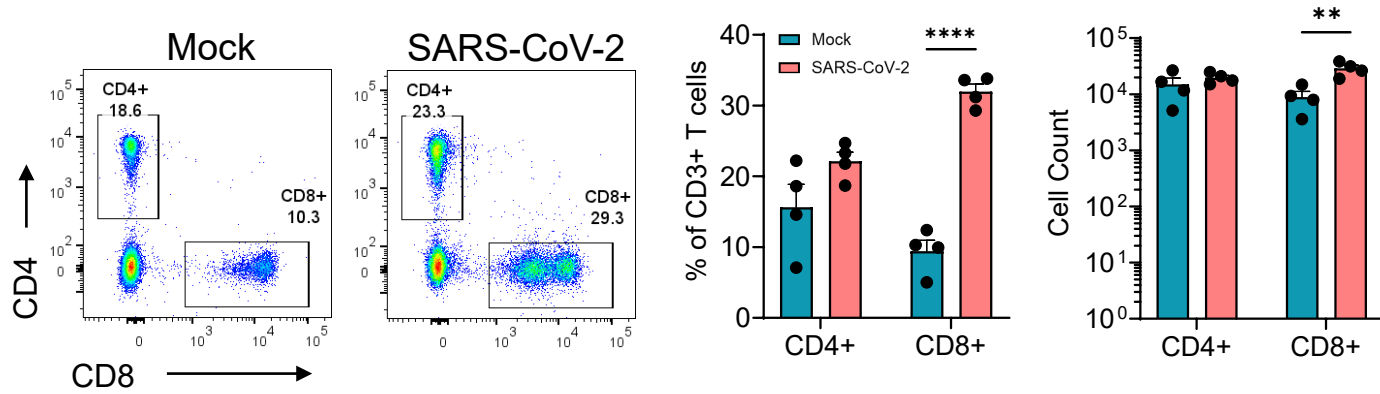
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A**B****C****D****E****F**

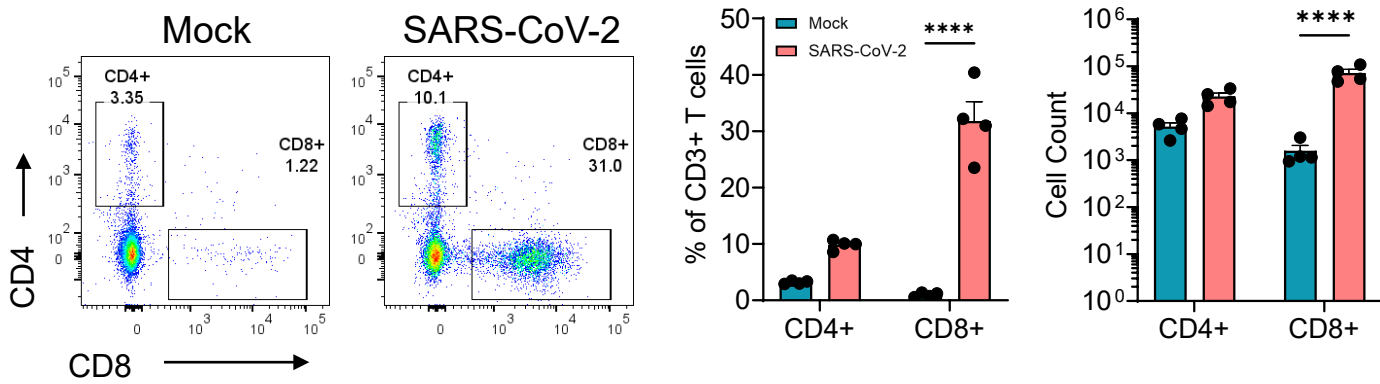
A. Spleen

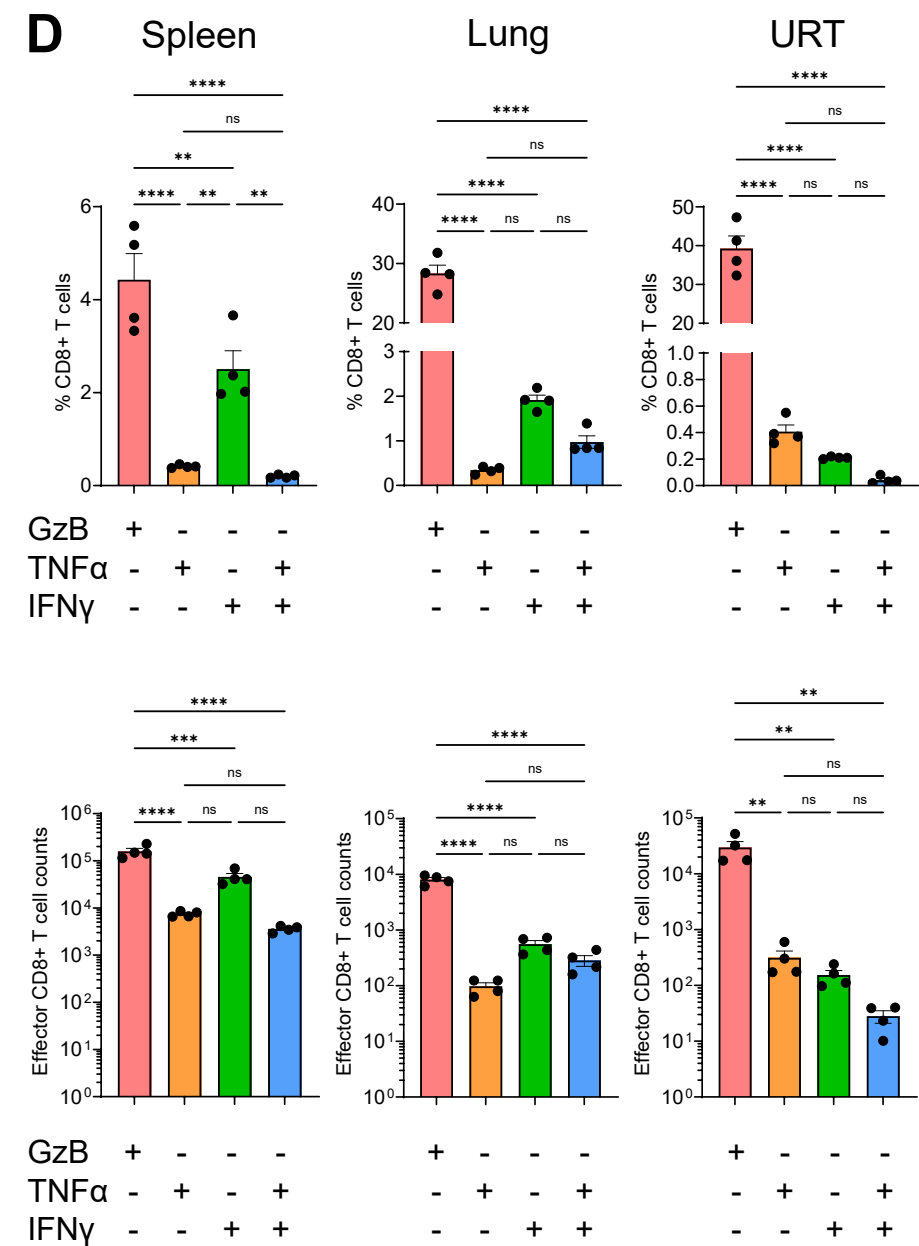
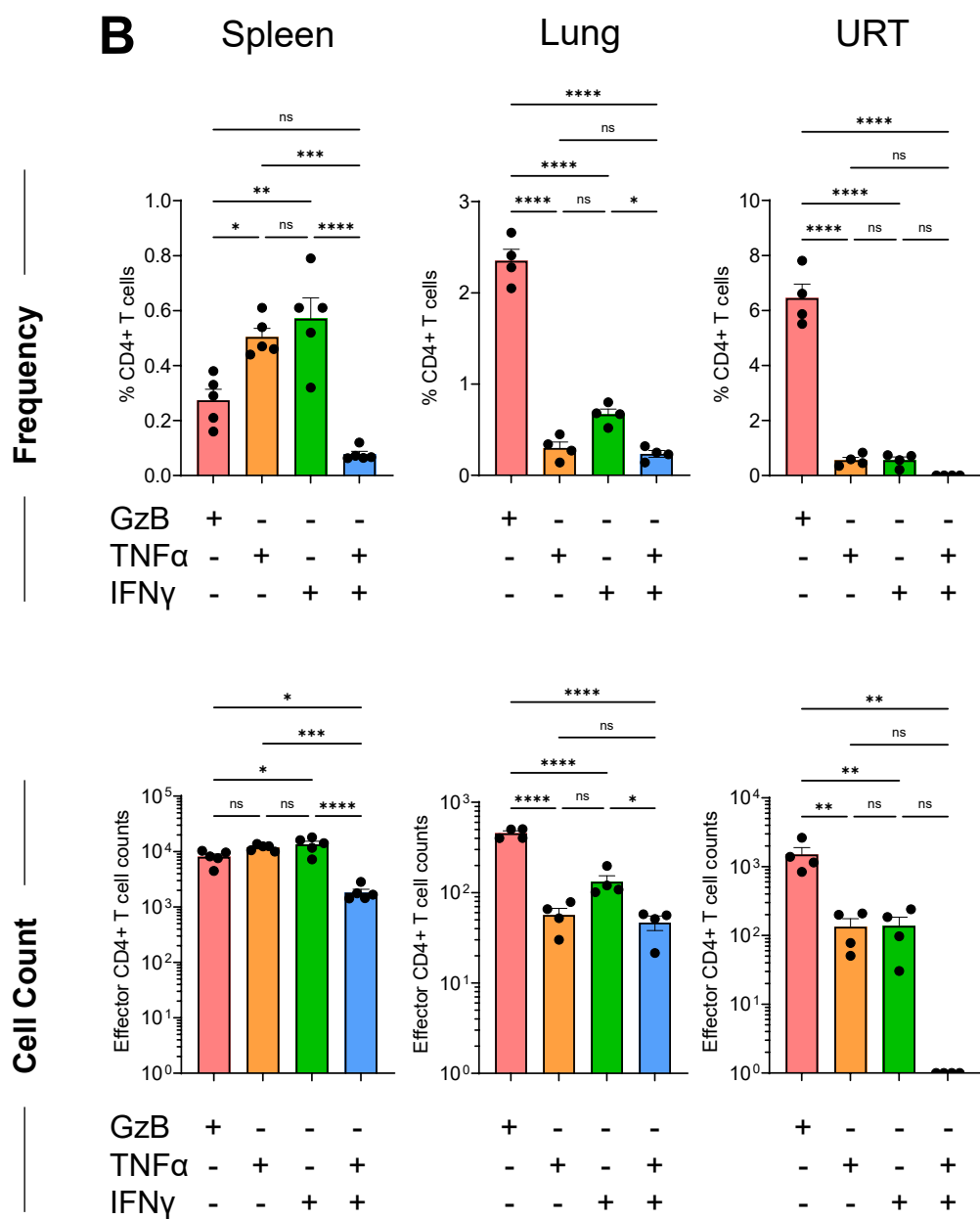
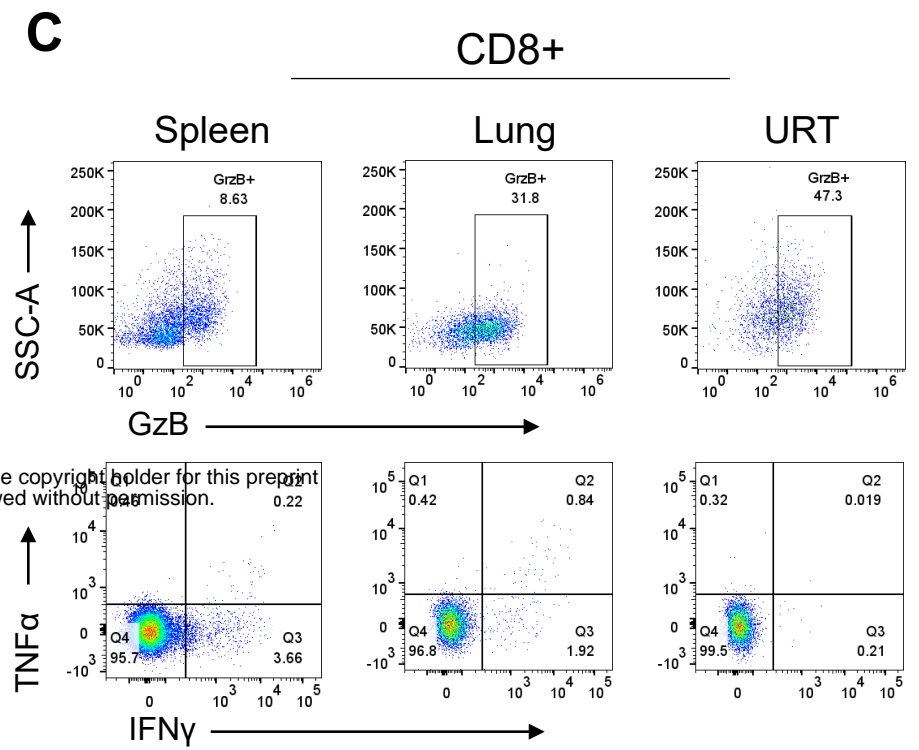
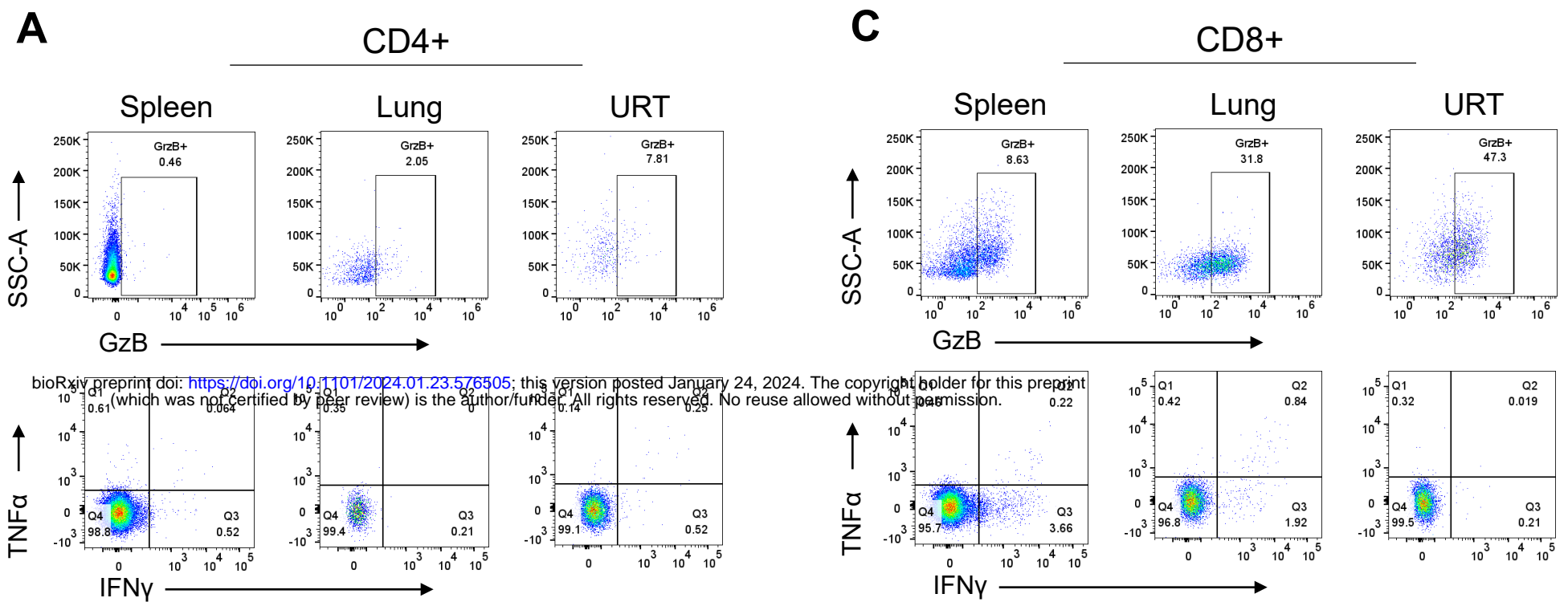


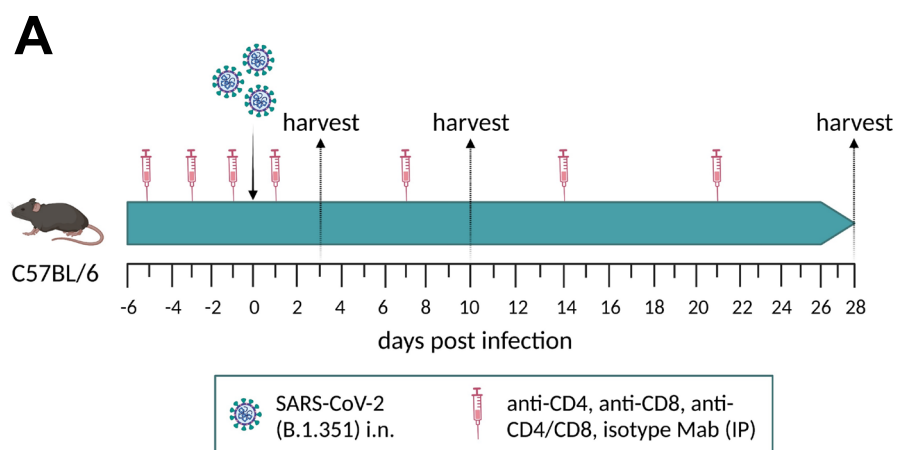
B. Lung



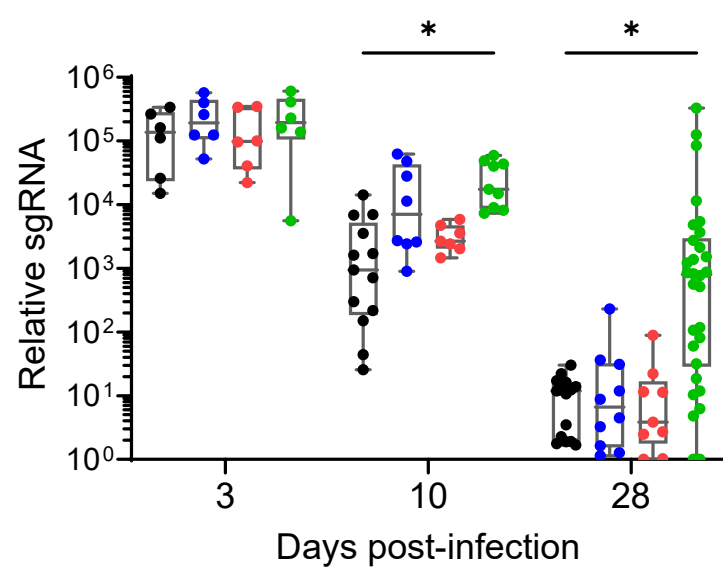
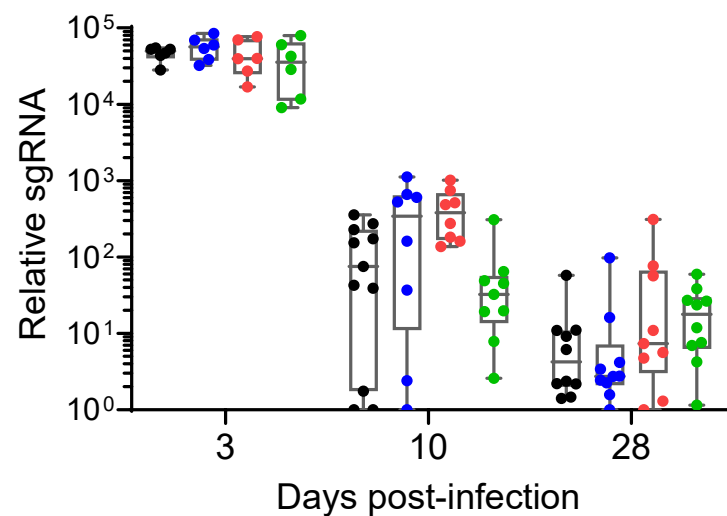
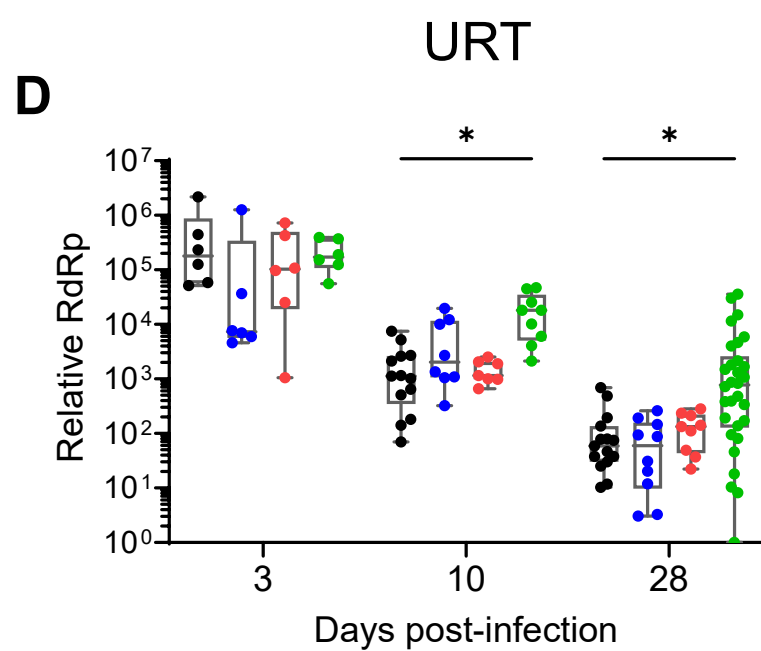
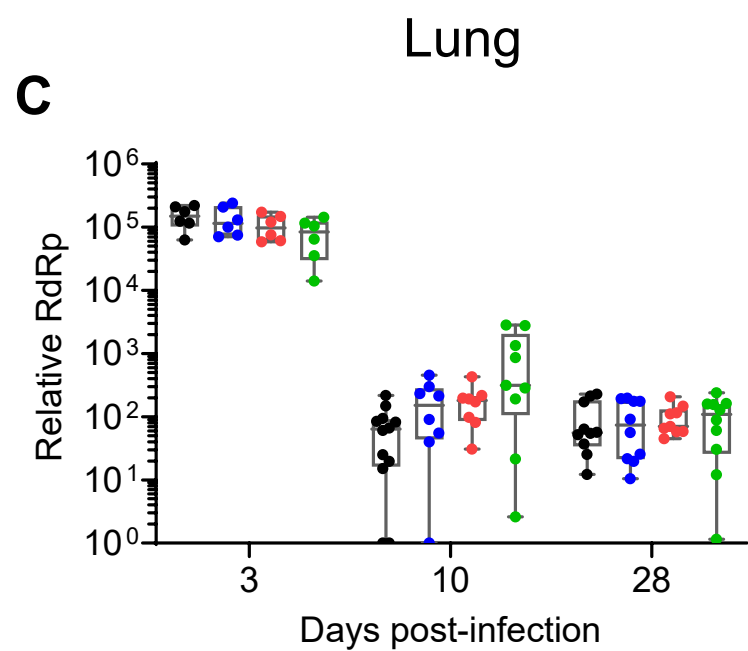
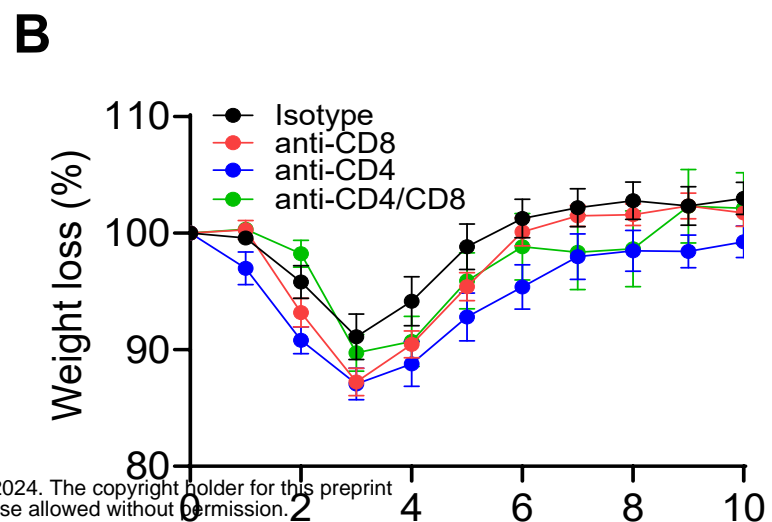
C. URT







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● Isotype control ● anti-CD4 ● anti-CD8 ● anti-CD4/CD8

