

1 **SARS-CoV-2 spike-specific memory B cells express markers of durable immunity after**
2 **non-severe COVID-19 but not after severe disease**

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24 **ABSTRACT**

25 SARS-CoV-2 infection elicits a robust B cell response, resulting in the generation of long-lived
26 plasma cells and memory B cells. Here, we aimed to determine the effect of COVID-19 severity
27 on the memory B cell response and characterize changes in the memory B cell compartment
28 between recovery and five months post-symptom onset. Using high-parameter spectral flow
29 cytometry, we analyzed the phenotype of memory B cells with reactivity against the SARS-CoV-
30 2 spike protein or the spike receptor binding domain (RBD) in recovered individuals who had
31 been hospitalized with non-severe (n=8) or severe (n=5) COVID-19. One month after symptom
32 onset, a substantial proportion of spike-specific IgG⁺ B cells showed an activated phenotype. In
33 individuals who experienced non-severe disease, spike-specific IgG⁺ B cells showed increased
34 expression of markers associated with durable B cell memory, including T-bet, FcRL5, and
35 CD11c, which was not observed after severe disease. Five months post-symptom onset, the
36 majority of spike-specific memory B cells had a resting phenotype and the percentage of spike-
37 specific T-bet⁺ IgG⁺ memory B cells decreased to baseline levels. Collectively, our results
38 suggest that the memory B cell response elicited during non-severe COVID-19 may be of higher
39 quality than the response after severe disease.

40 INTRODUCTION

41 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the global
42 coronavirus disease 2019 (COVID-19) pandemic resulting in more than 4 million deaths
43 reported worldwide as of September 2021 (1). Highly efficacious vaccines have limited SARS-
44 CoV-2 transmission and significantly reduced morbidity and mortality in regions of the world with
45 access to these vaccines. However, the majority of the world's population lives in areas with low
46 vaccination rates and remain at higher risk of SARS-CoV-2 infection and COVID-19. Both
47 vaccination and natural infection elicit immunological protection against SARS-CoV-2 (re-
48)infection. Although mRNA vaccination elicits higher antibody titers and more diverse antibody
49 responses against the SARS-CoV-2 spike protein than natural infection (2, 3), results from the
50 first longitudinal studies in unvaccinated individuals with prior COVID-19 suggest that naturally
51 acquired immune responses are maintained for at least a year after infection and that these
52 responses protect from subsequent re-infection (4-11). Because many people remain
53 unvaccinated, it will be important to understand the immune response elicited by natural
54 infection and whether the durability of naturally acquired immunity is influenced by the severity
55 of disease.

56

57 Although SARS-CoV-2 infection elicits robust responses in both the T cell and B cell arms of the
58 adaptive immune system (12, 13), the majority of research efforts have focused on B cell and
59 antibody responses, which are thought to be critical for the control of infection and protection
60 against re-infection. Humoral immune responses against pathogens consist of multiple
61 components. The early B cell response is dominated by short-lived antibody-secreting cells,
62 called plasmablasts. Simultaneously, other B cell populations undergo selection for high affinity
63 antigen-binding in germinal centers of the secondary lymphoid organs, and can differentiate into
64 long-lived, antibody-secreting plasma cells that migrate to the bone marrow or into memory B
65 cells that remain in the circulation (reviewed in (14)). COVID-19 patients rapidly generate potent

66 neutralizing IgG antibodies against the spike protein (15, 16). Anti-spike antibody titers peak
67 within the first two months post-infection, decline in the subsequent 3 – 4 months, and then
68 plateau at titers higher than those detected in pre-pandemic samples, but are lower than anti-
69 spike antibody titers elicited by vaccination (3, 5, 7, 17). These phases of the anti-spike IgG
70 profile in the circulation are the result of early short-lived plasmablast responses, followed by the
71 secretion of antibodies by longer-lived bone marrow plasma cells (5). In addition, spike-specific
72 IgG⁺ memory B cells are maintained or even increase in numbers for at least six months to one
73 year following SARS-CoV-2 infection (6-8, 18). Analysis of monoclonal antibodies obtained from
74 these memory B cells revealed continued evolution of the B cell response over time, as
75 evidenced by higher levels of somatic hypermutation, resulting in increased binding affinity and
76 neutralizing capacity (7, 19, 20).

77
78 In recent years, novel subsets of activated B cells and memory B cells have been identified.
79 These novel B cell subsets have mainly been defined by their phenotype, that is, the collection
80 of markers expressed on their cell surface or intracellularly, and are thought to possess either
81 protective or pathogenic functions in the immune response. For example, several subsets of
82 double negative (DN; CD27⁻ IgD⁻) B cells have been defined in recent years (21, 22). Type 1
83 double negative cells (DN1 cells), defined as DN cells that express CXCR5 or CD21 and lack
84 CD11c and FcRL5, are transcriptionally similar to class-switched memory B cells and seem to
85 be part of a functional B cell response (21, 23). On the other hand, type 2 DN cells (DN2 cells),
86 defined as CD11c⁺/FcRL5⁺ and CXCR5⁻/CD21⁻ DN cells, are thought to arise from the activation
87 of naïve B cells outside of the B cell follicle in the lymph node. Type 3 DN cells (DN3 cells),
88 defined as CD11c⁻/FcRL5⁻ and CXCR5⁻/CD21⁻ DN cells, have also been associated with this
89 extrafollicular response and may be DN2 cell precursors (22, 23). DN2 cells are more abundant
90 in patients with autoimmune disorders, such as systemic lupus erythematosus, and are thought
91 to differentiate into auto-antibody-secreting cells. DN2 cells are also observed in the circulation

92 of patients with severe COVID-19 (22), an observation that has been linked to lack of germinal
93 center formation, presumably leading to an impaired B cell response (24).

94
95 Interestingly, memory B cells (CD27⁺ IgD⁻) expressing some of the same markers as DN2 cells,
96 including CD11c and FcRL5, as well as the transcription factor T-bet, have been described as
97 part of normal, functional immune responses (25, 26). Specifically, these cells have been
98 implicated in long-lived B cell memory after tetanus toxoid and influenza virus vaccination (27,
99 28). In recovered COVID-19 patients, memory B cells present 1 – 3 months after symptom
100 onset are also found to express FcRL5 and T-bet, suggesting that these markers may delineate
101 a subset of long-lived memory B cells against SARS-CoV-2 (13, 29). However, it is unclear
102 whether disease severity influences the development of memory B cells that express T-bet or
103 FcRL5, and whether the phenotype of memory B cells changes with the continued evolution of
104 this B cell compartment in the months following SARS-CoV-2 infection. A more detailed analysis
105 of B cell subsets in recovered COVID-19 patients will therefore be needed to acquire insight into
106 the early B cell response and the evolution of memory B cells over time.

107
108 Here, we performed spectral flow cytometry to characterize the phenotype of SARS-CoV-2-
109 specific B cells in unvaccinated patients who recovered from non-severe or severe COVID-19.
110 We focused on two comparative analyses. First, we compared the phenotype of spike-specific B
111 cells between convalescent patients who were hospitalized with non-severe and severe disease
112 to determine whether the B cell response developed differently in these two groups. Second, we
113 compared the phenotype of memory B cells shortly after recovery to that at five months post-
114 symptom onset to analyze the ongoing evolution of the B cell response. The results from this
115 study provide insight into naturally acquired B cell memory against SARS-CoV-2 and a better
116 understanding of the characteristics of durable B cell immunity.

117 **RESULTS**

118 **Study criteria**

119 Study participants (n=16) were enrolled in the Adaptive COVID-19 Treatment Trial (ACTT)-1 or
120 ACTT-2 clinical trials which were designed to evaluate the effect of remdesivir or baricitinib plus
121 remdesivir for the treatment of COVID-19 in hospitalized patients (30, 31). Participants identified
122 predominantly as white (94%) and Hispanic (75%), and all except one individual had one or
123 more comorbidities (**Table S1**). Participants were classified as non-severe (n = 11) or severe (n
124 = 5) COVID-19 cases based on their worst ordinal score for disease severity during hospital
125 stay. In summary, non-severe cases did not require supplemental oxygen (score = 4) or
126 required supplemental oxygen or non-invasive ventilation (score = 5 or 6), while severe cases
127 needed invasive mechanical ventilation (MV) or extracorporeal membrane oxygenation (ECMO)
128 (score = 7). The definition of severe disease was made based on the need for MV or ECMO,
129 because this distinguishes the most critical patients, who are the most likely to develop impaired
130 immune responses (24, 32). Blood was collected after discharge from the hospital (a median of
131 30 days after symptom onset for non-severe cases (n = 8) and 32 days after symptom onset for
132 severe cases (n = 5)) (**Figure 1**). For participants with non-severe disease, additional samples
133 were collected at an earlier time point (a median of 18 days post-symptom onset; n = 9) and at a
134 follow-up visit approximately 5 months post-symptom onset (median, 147 days; n = 7) based on
135 availability and consent. Peripheral blood mononuclear cells (PBMCs) from healthy donors (n =
136 3) collected before the start of the pandemic (2018 or early 2019) were used as negative
137 controls.

138

139 **The proportions of B cell subsets in convalescent patients are similar between non- 140 severe and severe COVID-19 cases**

141 Severe COVID-19 is associated with an extrafollicular B cell response characterized by a high
142 percentage of DN2 B cells in the circulation, similar to what is observed in patients with

143 autoimmune disorders (21, 22). Previous data suggest that the percentage of DN2 B cells in
144 patients with severe COVID-19 had returned to normal levels approximately 1.5 months after
145 hospital discharge (33). To determine whether COVID-19 severity affects the distribution of B
146 cell subsets shortly after recovery (four to five weeks after symptom onset), we analyzed the
147 relative proportions of B cell subsets individuals who recovered from non-severe (n = 8) or
148 severe (n = 5) disease by spectral flow cytometry using an antibody panel against 19 cell
149 surface and intracellular markers (**Table S2**). B cells were first gated on live, CD19⁺ CD20⁺
150 CD38^{-/lo} cells to exclude transitional B cells and antibody-secreting cells, and were subsequently
151 divided into naïve (CD27⁻ IgD⁺), unswitched memory (CD27⁺ IgD⁺), resting switched memory
152 (CD27⁺ IgD⁻ CD21⁺), activated switched memory (CD27⁺ IgD⁻ CD21⁻) and double negative (DN;
153 CD27⁻ IgD⁻) B cells (**Figure 2A**). Apart from a significant increase in the percentage of
154 unswitched memory B cells in individuals who had experienced severe disease compared to
155 those with non-severe COVID-19, no differences in the distribution of these B cell subsets were
156 observed between the two groups of recovered COVID-19 patients (**Figure 2B-C, Figure S1A**).
157 Among DN cells, DN1 (FcRL5⁻ CXCR5⁺) B cells were the dominant subset in both groups of
158 recovered COVID-19 patients, and we did not detect significant differences in the frequencies of
159 DN1, DN2 (FcRL5⁺ CXCR5⁻), and DN3 (FcRL5⁻ CXCR5⁻) B cells between the two groups
160 (**Figure 2D, Figure S1B**). In combination with previous reports (22, 33), these results suggest
161 that expansion of the DN2 B cell population during severe COVID-19 might be transient and that
162 these cells disappear soon after recovery.

163

164 **The percentage of spike-specific and RBD-specific B cells and their isotype distributions** 165 **are similar between non-severe and severe cases**

166 Our flow cytometry panel also included antigen probes to detect B cells reactive with the SARS-
167 CoV-2 spike protein and the spike receptor binding domain (RBD). RBD is the part of the spike
168 protein that interacts with angiotensin-converting enzyme 2, the viral receptor on host cells, and

169 is the dominant target of neutralizing antibodies (34). However, the majority of antibodies
170 generated against the spike protein bind to epitopes outside RBD (35). Whereas the RBD
171 sequence is highly specific for SARS-CoV-2 (36), the non-RBD parts of spike, in particular the
172 S2 subunit, share epitopes with other coronaviruses that circulate in the human population (37-
173 39). Evidence for pre-existing memory B cells that are cross-reactive with other coronaviruses
174 has been reported (40). Because pre-existing immunity could affect the development of the
175 immune responses against these two parts of the spike protein, we analyzed B cell responses
176 against RBD and non-RBD spike epitopes separately.

177

178 We constructed spike tetramers in two fluorochrome formats and defined the population of cells
179 staining positive for both tetramers as spike-specific (**Figure 3A**). The spike-specific B cells
180 were then divided into non-RBD-specific and RBD-specific B cells based on reactivity with the
181 RBD tetramer (**Figure 3A**). Non-RBD-specific and RBD-specific B cells were strongly enriched
182 for antigen-experienced B cells (**Figure 3B**, compare to **Figure 2B**), with no statistically
183 significant differences in the proportions of various B cell populations between individuals who
184 recovered from non-severe or severe disease (**Figure 3B**, **Figure S2**). In addition, reactivity
185 against spike and RBD tetramers among control donor B cells was minimal (**Figure 3A,C**),
186 suggesting that these probes selectively bind to antigen-specific B cells.

187

188 The percentage of spike-specific B cells among antigen-experienced B cell populations
189 (unswitched memory, switched memory, and DN B cells) among all patients ranged from 0.1%
190 to 1.8% (median, 0.6%) (**Figure 3C**). One donor (marked with # in Figure 3C) had only six
191 spike-specific B cells and was therefore not included in subsequent analyses that required
192 further subsetting of these cells. All other donors had between 42 and 450 spike-specific B cells.
193 In line with a previous study (4), a median of 22% of spike-specific B cells were reactive against
194 RBD (**Figure 3D**). Ogega *et al.* recently reported that patients who recovered from severe

195 COVID-19 harbored more RBD-specific B cells than COVID-19 patients who never required
196 hospitalization (29). We did not observe a difference in the percentage of RBD-specific cells
197 between individuals who recovered from non-severe and severe disease, potentially because
198 our non-severe group had been hospitalized and suffered from more severe disease than the
199 group with non-severe disease in the Ogega *et al.* study.

200

201 Next, we compared the isotype of class-switched spike-specific B cells between patients who
202 recovered from non-severe and severe COVID-19 by categorizing spike-specific antigen-
203 experienced B cells based on IgM, IgG, or IgA expression. The percentage of IgM⁺, IgG⁺, and
204 IgA⁺ class-switched B cells among spike-specific B cells did not differ significantly between the
205 two patient groups and in both groups, the majority of class-switched spike-specific B cells were
206 IgG⁺ (**Figure 3E**). We also determined plasma IgM and IgG reactivity to the spike protein and
207 RBD and observed no significant differences in IgM or IgG titers to the spike protein or RBD
208 between convalescent patients who recovered from non-severe or severe disease (**Figure 3F**).
209 Collectively, these results suggest that individuals who recovered from either non-severe or
210 severe COVID-19 have similar immune responses to the SARS-CoV-2 spike protein, in terms of
211 the prevalence of spike-specific B cells and their major phenotype.

212

213 **Non-severe COVID-19 is associated with an increased population of T-bet⁺ spike-specific** 214 **IgG⁺ B cells**

215 To gain deeper understanding of the differences in memory B cell responses between
216 individuals who had either non-severe or severe COVID-19, we combined the acquired flow
217 cytometry data for all 19 markers (**Table S2**) of all individuals and plotted a composite image
218 using Uniform Manifold Approximation Projection (UMAP). UMAP clusters cells in a 2D plot
219 based on similarity in phenotype and therefore provides meaningful organization of cell subsets.
220 For each recovered COVID-19 patient, we took a random sample of cells (n = 10,000) and

221 projected the spike-specific antigen-experienced B cells onto the composite UMAP (**Figure 4A**).
222 We also plotted contours for various B cell subsets to visualize their location within the UMAP
223 (**Figure 4B**, see **Figure S3** for heatmaps of all individual markers overlaid on the composite
224 UMAP). The composite image shows differences in the location of spike-specific B cells,
225 predominantly among IgG⁺ B cells (**Figure 4A, B**). The large majority of IgG⁺ B cells are
226 memory B cells, but a small fraction of these cells are DN1 B cells that are thought to contribute
227 to a functional immune response and were therefore included in a phenotypic analysis of spike-
228 specific IgG⁺ B cells.

229
230 Irrespective of disease severity, spike-specific IgG⁺ B cells expressed increased levels of
231 activation markers CD80, Ki-67, and CD95 (**Figure 4C**) compared to all IgG⁺ B cells, with no
232 differences between the two groups. In contrast, spike-specific IgG⁺ B cells after non-severe
233 disease showed an increase in the expression of the transcription factor T-bet that was not seen
234 after severe disease (**Figure 4D**). A median of 28% of spike-specific IgG⁺ B cells in individuals
235 who experienced non-severe disease expressed T-bet, which has been associated with strong
236 anti-viral immunity (41). Conversely, T-bet was expressed in only about 10% of spike-specific
237 IgG⁺ memory B cells in individuals who recovered from severe disease (**Figure 4D**). T-bet⁺ cells
238 often have a distinct cell surface marker profile, including expression of CD11c and FcRL5 and
239 the absence of CD21 (25, 26, 28). Indeed, in addition to T-bet, the percentage of both FcRL5⁺
240 and CD11c⁺ spike-specific IgG⁺ memory B cells was increased among spike-specific IgG⁺ B
241 cells in patients who recovered from non-severe COVID-19 as compared to all IgG⁺ B cells in
242 these patients (**Figure 4D**), while these differences were not seen in individuals who recovered
243 from severe COVID-19. Conversely, a lower percentage of CD21⁺ spike-specific IgG⁺ B cells
244 was observed after non-severe disease but not after severe disease (**Figure 4D**). To confirm
245 that the measurement of T-bet, FcRL5, CD11c, and CD21 expression was robust between
246 experiments, we processed and analyzed technical replicates for samples from one non-severe

247 and one severe case on different days, and observed almost perfect correlation between the
248 replicates (Spearman $r = 0.99$, $P = 0.0002$, **Figure S4**). We then analyzed the combinatorial
249 expression of these markers by combining spike-specific IgG⁺ T-bet⁺ B cells from all donors and
250 plotting contour plots depicting the expression of FcRL5, CD11c, CD21, CD27, CXCR3, and
251 CXCR5 (**Figure 5**). This revealed four subpopulations of T-bet⁺ IgG⁺ B cells, of which cells
252 expressing FcRL5, CD11c, CD27 and CXCR3, and lacking CD21 and CXCR5 (type I; activated
253 switched memory B cells) were most abundant in both non-severe and severe cases (**Figure 5**).
254 This phenotype has previously been observed in activated B cells after influenza virus
255 vaccination and is thought to delineate a B cell subset associated with long-lived humoral
256 immunity (26, 28, 41). Collectively, the higher prevalence of spike-specific B cells that express
257 markers associated with durable immunity in individuals who recovered from non-severe
258 COVID-19 than in those who experienced severe disease suggests that disease severity
259 influences the quality of the B cell response.

260

261 **SARS-CoV-2 spike-specific memory B cells return to a resting phenotype with baseline** 262 **levels of T-bet expression five months post-symptom onset**

263 Activated T-bet⁺ B cell subsets have been shown to expand and contract rapidly following
264 influenza virus vaccination, peaking between 14 and 28 days post-vaccination and returning to
265 baseline levels after three months (25). In addition, it has been shown that the memory B cell
266 response continues to mature up to one year following SARS-CoV-2 infection, giving rise to B
267 cell clones with higher levels of somatic hypermutation and resulting in increases in antigen-
268 binding affinity and ability to neutralize the virus (7, 19, 20). To evaluate the early dynamics of T-
269 bet⁺ spike-specific B cells and continued development of the phenotype of memory B cells in
270 parallel with ongoing selection and maturation, we analyzed additional blood samples from
271 recovered COVID-19 patients with non-severe disease collected at an earlier time point
272 (median, 19 days post-symptom onset) (T1, **Figure 1**) and at a follow-up visit five months post-

273 symptom onset (T3, **Figure 1**). Unfortunately, we were unable to recruit any individuals who had
274 experienced severe disease for a follow-up visit at five months post-symptom onset. In addition,
275 at the earlier time point, most individuals with severe disease were still hospitalized (**Figure 1**).
276 We therefore limited this analysis to individuals who recovered from non-severe COVID-19.
277
278 The percentages of total naïve, unswitched memory, resting switched memory, activated
279 switched memory, and double negative B cell subsets among total B cells did not differ between
280 the two time points (**Figure S5**). However, the phenotype of spike-specific B cells changed
281 considerably between the two early time points and five months post-symptom onset (**Figure**
282 **6A, Figure S6**), as described in further detail below. Similar to what has been reported by Dan
283 *et al.* and Sokal *et al.* (4, 8), the percentage of spike-specific B cells among antigen-experienced
284 B cells remained relatively stable at five months post-symptom onset (**Figure 6B**). In addition,
285 the reactivity against RBD among these spike-specific B cells increased between 2 – 3 weeks
286 and 4 – 5 weeks post-symptom onset, but did not change at the 5-month time point (**Figure 6C**).
287 The larger proportion of non-RBD-specific B cells early in infection may be the result of recall
288 responses of pre-existing memory B cells that are cross-reactive with other coronaviruses.
289
290 The percentage of antigen-experienced spike-specific B cells with an unswitched memory B cell
291 phenotype was reduced at the 5-month time point (**Figure 6D**). The percentage of spike-specific
292 activated switched memory B cells drastically decreased over time, accompanied by an
293 increase in resting memory B cells (**Figure 6E**). Among switched memory B cells, the
294 percentage of IgM⁺ and IgA⁺ memory B cells declined and the proportion of IgG⁺ memory B cells
295 increased at five months post-symptom onset (**Figure 6F**). In line with the decrease of IgM⁺
296 memory B cells, the anti-spike and anti-RBD plasma IgM responses were lost almost completely
297 at five months post-symptom onset. Plasma IgG titers against the spike protein remained stable,
298 whereas plasma IgG titers against RBD showed a modest decrease (**Figure 6G**). These results

299 indicate that both the unswitched plasma cell and memory B cell response against the spike
300 protein are short-lived, while the class-switched IgG⁺ response is more durable, as has been
301 reported by others (4, 8).

302

303 Both the decrease of unswitched memory B cells and the change from an activated to a resting
304 switched memory B cell phenotype can be seen in composite UMAPs of the three time points
305 (**Figure 7A-B**). In line with the return to a resting memory B cell phenotype, expression of
306 CD80, Ki-67, and CD95 was decreased at 20 – 23 weeks post-symptom onset as compared the
307 two earlier time points (**Figure 7C**). The percentage of CD80⁺ spike-specific IgG⁺ B cells at 20 –
308 23 weeks post-symptom onset still showed an increase over baseline levels (P = 0.016,
309 Wilcoxon signed rank test), while expression of Ki-67 and CD95 had returned to levels seen in
310 non-spike-specific IgG⁺ B cells (**Figure 7C, Figure S7**). The expression of T-bet was higher at 2
311 – 3 weeks post-symptom onset than at 4 – 5 weeks post-symptom onset (median, 58% and
312 28%, respectively), although this difference was not statistically significant, and decreased
313 further to baseline levels (~ 10%) at 20 – 23 weeks post-symptom onset (**Figure 7D,E**). Similar
314 dynamics were observed for FcRL5 and CD11c, while CD21 expression increased significantly
315 over time (**Figure 7E**). The percentage of type I T-bet⁺ spike-specific IgG⁺ B cells (activated
316 switched memory B cells) that dominated the early response decreased dramatically over time
317 (**Figure 7D**). Instead, the T-bet⁺ spike-specific IgG⁺ B cells present at five months post-symptom
318 onset were mainly type II (FcRL5⁺ CD11c⁺ CD21⁻ CD27⁻, DN2 B cells) and type IV (FcRL5⁻
319 CD11c⁻ CD21⁺ CD27⁺, resting switched memory B cells) subsets, making up ~35% and ~40% of
320 T-bet⁺ spike-specific IgG⁺ B cells, respectively. These results suggest that the memory B cell
321 response continues to evolve until at least five months after infection. In addition, the decrease
322 of T-bet⁺ spike-specific B cells in the circulation over the course of five months post-symptom
323 onset suggests that their abundant presence in the blood may signify recent exposure.

324

325 **DISCUSSION**

326 In the current COVID-19 pandemic, an important question that has only partially been answered
327 is whether SARS-CoV-2 infection elicits durable immunity. Assessments of B cell responses up
328 to one year post-infection suggest that both neutralizing antibodies and memory B cells against
329 the SARS-CoV-2 spike protein remain detectable in the circulation of most recovered COVID-19
330 patients and are stable or decay slowly at this time point. In this study, we aimed to better
331 understand the memory B cell response and to determine whether the severity of COVID-19
332 disease course influences the development of B cell responses. Using high-parameter spectral
333 flow cytometry, we analyzed the phenotype of B cells reactive with both non-RBD and RBD
334 epitopes on the spike protein shortly after recovery and approximately five months post-
335 symptom onset.

336
337 Our study consisted of a relatively small number of individuals but recapitulated many of the
338 observations reported from much larger cohorts, including the frequency of spike-specific
339 memory B cells after infection and the fraction of spike-specific B cells that recognizes RBD
340 epitopes. We also confirmed the loss of IgM⁺ and IgA⁺ spike-specific memory B cells and anti-
341 spike and anti-RBD plasma IgM, as well as maintenance of IgG⁺ spike-specific memory B cells
342 and anti-spike plasma IgG five months post-symptom onset (4, 17). This gives us confidence in
343 the validity of several new observations that we discuss in more detail below.

344
345 The most striking result from this study is the higher percentage of spike-specific IgG⁺ B cells
346 that express the transcription factor T-bet in individuals who recovered from non-severe disease
347 as compared to those who recovered from severe disease. Previously, it was observed that a
348 higher percentage of total CD19⁺ T-bet⁺ IgG₁⁺ B cells was associated with shorter symptom
349 duration (42). Here, we determined that this association predominantly involves SARS-CoV-2
350 antigen-specific B cells, suggesting implications for the development of B cell memory. The

351 expression of T-bet, in parallel with a surface marker profile that includes the presence of FcRL5
352 and CD11c and the absence of CD21, has previously been reported on activated B cells shortly
353 after vaccination and infection, and is associated with long-lived humoral immunity (26, 28, 41).
354 Here, we observed that an increased percentage of spike-specific B cells showed this activated
355 phenotype shortly after recovery from non-severe COVID-19, but not after severe COVID-19.
356 Ogega *et al.* reported higher FcRL5 expression on RBD-specific class-switched memory B cells
357 after non-severe COVID-19 (non-hospitalized patients) than after severe disease (hospitalized
358 patients) (29), findings that are in agreement with our results. In addition, higher levels of
359 somatic hypermutation were observed in IgG⁺ memory B cells following non-severe COVID-19
360 than severe disease (43), while patients who died from COVID-19 showed an absence of
361 germinal centers and a delay in the development of neutralizing antibodies (24, 44). Collectively,
362 these data are indicative of functional development of B cell immunity with germinal center
363 responses in patients with non-severe disease, as compared to a more dysfunctional B cell
364 response in severe disease cases.

365
366 T-bet expression in B cells can be induced by interferon gamma (IFN γ) signaling through the
367 IFN γ receptor (45, 46). IFN γ plays an important role in the immune response against viral
368 infections. Although many contradictory findings have been reported about the role of IFN γ in
369 SARS-CoV-2 infection, the induction of IFN γ early in infection seems important in the control of
370 viral replication [summarized in (47)]. Indeed, low IFN γ and low induction of interferon-
371 stimulated genes contribute to the development of COVID-19 (48). Failure to control viral load
372 leads to the induction of a hyper-inflammatory response later in disease, in parallel with
373 persistent high levels of IFN γ . However, these conditions may not promote the development of
374 T-bet⁺ memory B cells. Instead, patients with severe disease show a strong extrafollicular B cell
375 response, resulting in an accumulation of DN2 B cells [(22)]. In our study, no difference was
376 observed in the percentage of DN2 B cells shortly after recovery between patients with either

377 non-severe or severe disease, suggesting that these DN2 B cells may be relatively short-lived.
378 Of note, DN2 cells typically also express T-bet (21), highlighting that the expression of this
379 transcription factor alone is not the determining factor in the development of either a functional
380 or pathogenic B cell response. The difference that we observed in the percentage of T-bet⁺
381 spike-specific IgG⁺ B cells between patients recovered from non-severe or severe COVID-19
382 could reflect these two developmental pathways of anti-viral immune responses.

383
384 In individuals who experienced non-severe COVID-19, T-bet⁺ spike-specific IgG⁺ B cells almost
385 disappeared from the circulation five months post-symptom onset. This is in line with the
386 transient peak of activated memory B cells observed after influenza vaccination (25, 26). The
387 cell surface marker expression profile and dynamics of the T-bet⁺ B cells detected in this study
388 matches best with the AM2 subset of activated memory B cells defined by Andrews *et al.*, which
389 peaked 14 days after influenza vaccination and had almost disappeared 90 days post-
390 vaccination (25). However, this does not necessarily mean that these cells are short-lived. It
391 was recently shown that the spleen harbors a population of resident T-bet^{thi} memory B cells with
392 specificity to viral antigens (49). In addition, it has been reported that T-bet^{thi} memory B cells
393 elicited in response to influenza virus vaccination rapidly differentiated into antibody-secreting
394 cells upon reactivation one year later (28). T-bet⁺ memory B cells thus seem to contribute to
395 effective recall responses upon re-exposure, but may only be detectable in the circulation
396 shortly after infection. In this regard, it is important to point out that the timing of sampling can
397 strongly affect the frequency of transient B cell populations, and samples selected for
398 comparative analyses need to be carefully matched to account for rapid changes in the
399 abundance of activated B cell populations over time. For example, this may explain the
400 discrepancy between this study and the observation by Sokal *et al.* that T-bet expression in
401 spike-specific B cells was higher in individuals with severe COVID-19 than individual with non-
402 severe COVID-19 (8). In the Sokal *et al.* study, samples from severe cases were collected at an

403 earlier time point than those from non-severe cases (median, 18.8 days versus 35.5 days post-
404 symptom onset), which could have influenced these results (8).

405

406 Thus far, no differences have been observed in serum anti-spike antibody titers, *in vitro* serum
407 neutralizing activity, or percentages of spike-specific memory B cells between non-severe and
408 severe COVID-19 patients in the months after infection (18). It remains to be determined
409 whether the durability of B cell memory differs between individuals who experienced non-severe
410 or severe disease. We speculate that individuals who recovered from non-severe disease
411 develop a higher quality memory B cell response that may be longer-lived or may give rise to a
412 larger population of antibody-secreting cells upon re-exposure. An alternative interpretation of
413 the paucity of T-bet⁺ spike-specific memory B cells in patients with severe disease could be that
414 a robust T-bet⁺ B cell response protects against severe COVID-19. Although patients who died
415 from COVID-19 show defects in the development of B cell responses (24, 44), it is likely that the
416 expansion of a population of T-bet⁺ spike-specific memory B cells is a consequence of the
417 immune environment during disease, not a factor contributing to survival. We believe these cells
418 are a sign of a balanced immune response that contributes to viral clearance through the
419 production of neutralizing antibodies and leads to the development of durable B cell memory,
420 but that T-bet⁺ memory B cells do not play a direct role in protection against severe disease.

421

422 In conclusion, we have shown that the memory B cell response against SARS-CoV-2 spike
423 protein develops differently in patients with non-severe disease compared those with severe
424 disease, with more spike-specific B cells that express a B-cell marker profile associated with
425 durable immunity, characterized by expression of T-bet, FcRL5, and CD11c, and low expression
426 of CD21 in individuals who recovered from non-severe COVID-19. Although antibody titers or
427 percentage of spike-specific memory B cells up to one year are similar or higher in individuals
428 who recovered from severe disease as compared to non-severe cases (8, 18), the increased

429 percentage of B cells associated with long-lived immunity in non-severe COVID-19 patients may
430 have consequences for long-term immunity against SARS-CoV-2 re-infection or severity of the
431 resulting disease. T-bet⁺ spike-specific B cells nearly disappeared from the circulation five
432 months post-symptom onset, consistent with loss of these cells from the periphery or migration
433 into tissues. These data aid in the understanding of naturally acquired B cell responses against
434 SARS-CoV-2 and help characterize the B cell populations that may be responsible for durable,
435 long-lived immunity.
436

437 **METHODS**

438 **Isolation of plasma and peripheral blood mononuclear cells**

439 Blood from recovered COVID-19 patients was processed within four hours of blood draw.
440 Plasma was separated from cells by centrifugation at $250 \times g$ for 5 min at room temperature
441 (RT). Plasma was depleted of platelets by centrifugation at $2,000 \times g$ for 15 min at 4°C and was
442 stored at -20°C . Peripheral blood mononuclear cells (PBMCs) were isolated from the cellular
443 fraction as described below. PBMCs used as negative controls were collected in 2018 and early
444 2019 by isolation from buffy coats (Interstate Blood Bank, Memphis, TN) obtained the day
445 following blood draw. Buffy coats were stored and shipped at room temperature (RT) and
446 processed immediately upon arrival. Cells were diluted approximately $4 \times$ in PBS with 2 mM
447 EDTA. PBMCs were layered on Ficoll-Paque (GE Healthcare, cat. no. 17144002) and spun at
448 $760 \times g$ for 20 min at RT to pellet erythrocytes and separate leukocytes. The leukocytes were
449 then washed in PBS with 2 mM EDTA, centrifuged at $425 \times g$ for 15 min at RT, followed by
450 centrifugation at $250 \times g$ for 10 min at RT for all subsequent wash steps necessary to remove all
451 platelets, determined by cloudiness of the supernatant. Next, PBMCs were resuspended in cold
452 IMDM/GlutaMAX (Gibco, cat. no. 31980030) supplemented with 10% heat-inactivated, USA-
453 sourced, fetal bovine serum (FBS) and 5% cell culture grade DMSO, counted, and
454 cryopreserved in the liquid nitrogen vapor phase.

455

456 **Spike and RBD protein production and tetramer synthesis**

457 Recombinant SARS-CoV-2 HexaPro spike and RBD were produced from Addgene plasmid
458 #154754 (50) and BEI plasmid NR-52309, respectively, for use in ELISA experiments. DNA
459 plasmids were purified from DH5alpha Competent Cell (Zymo, cat. no. T3007) cultures using a
460 ZymoPURE II Plasmid Maxiprep (Zymo, cat. no. D4203). 25 μg plasmid was used to transfect
461 25 ml Expi293F (Thermo, cat. no. A14635) culture per manufacturer's instructions. Culture
462 supernatants were collected 5 days post-transfection. His-tagged S and RBD were purified

463 using HiTrap chelating high performance columns (Cytiva, cat. no. 17-0408-01) charged with
464 Ni^{2+} , washed with PBS (pH 7.2), and eluted with 0.3 M imidazole in PBS (pH
465 7.2). The imidazole was removed from the eluted protein buffer using a HiPrep desalting column
466 (Cytiva, cat. no. 17-5087-01). The protein was concentrated using a 30 kDa MWCO Amicon
467 ultra-15 centrifugal filter (Sigma, cat. no. UFC903024), and stored at -70°C . For the production
468 of biotinylated S and RBD protein used for the synthesis of antigen tetramers, Addgene plasmid
469 #154754 and BEI plasmid NR-52309 were altered to include a biotinylation site. The modified
470 plasmids have been deposited to Addgene: #166856 and #166857 for spike and RBD,
471 respectively. Cells were co-transfected with either plasmid #166856 or #166857 and a plasmid
472 encoding BirA ligase (Addgene #32408) at a 4:1 ratio (m/m). 100 mM D-biotin (Sigma, cat. no.
473 B4639) was added immediately post-transfection to a final concentration of 10 mM. Spike and
474 RBD tetramers were synthesized by incubating biotinylated protein with fluorophore-conjugated
475 streptavidin overnight at 4°C (streptavidin-PE, Tonbo, cat. no. 50-4317-U100, streptavidin-APC,
476 Tonbo, cat. no. 20-4317-U100, streptavidin-BV421, BioLegend, cat. no. 405226) at a molar ratio
477 of 5:1, respectively, to generate three individual tetramers: spike-PE, spike-APC, and RBD-
478 BV421.

479

480 **Protein gels**

481 As a quality control step, purified spike and RBD proteins were visualized on a polyacrylamide
482 gel (**Figure S8**). 800 ng of protein was loaded on a 4 – 12% Bis-Tris gel (Thermo, cat. no.
483 NP0321BOX) after it was mixed with 2 \times Laemmli loading buffer and incubated at 85°C for 5
484 min. The gel was run with MOPS running buffer (Thermo, cat. no. NP0001) at 200 V for 50 min
485 after which the proteins were stained using Imperial Protein Stain (Thermo, cat. no. PI24615)
486 per manufacturer's instructions.

487

488 **B cell isolation and staining**

489 Cryopreserved PBMCs were thawed in a water bath set at 37°C and immediately mixed with
490 pre-warmed thawing medium (IMDM/GlutaMAX supplemented with 10% heat inactivated FBS
491 and 0.01% Universal Nuclease (Thermo, cat. no. 88700). After brief centrifugation (250 × g, 5
492 min) at RT, the cell pellet was resuspended in warm thawing medium and viable cells were
493 counted using trypan blue on a Cellometer Mini (Nexcelom) automated cell counter. Next, the
494 PBMCs were pelleted (250 × g, 5 min, RT), resuspended in isolation buffer (PBS supplemented
495 with 2% heat inactivated FBS and 1 mM EDTA) at 50 million live cells/ml, and filtered through a
496 35 µm sterile filter cap (Corning, cat. no. 352235) to break apart any aggregated cells. B cells
497 were isolated using the EasySep Human B Cell Isolation Kit (StemCell, cat. no. 17954)
498 according to manufacturer's instructions. After washing with cold PBS (250 × g, 5 min, RT), the
499 isolated B cells were incubated with 1 µl Zombie UV Fixable Viability kit (Biolegend, cat. no.
500 423107) per 1 ml cell suspension for 30 min on ice. Cells were subsequently washed with cold
501 PBS with 1% bovine serum albumin (BSA), followed by an incubation at 4°C for 30 min with an
502 antigen tetramer cocktail (**Table S2**). Next, the cells were washed again with cold PBS with 1%
503 BSA and incubated at 4°C for 30 min with a B cell surface marker antibody cocktail (**Table S2**),
504 after which the cells were washed again with cold PBS with 1% BSA and resuspended in 1 ml
505 Transcription Factor Fix/Perm Concentrate (Tonbo, part of cat. no. TNB-0607-KIT) diluted with 3
506 parts Transcription Factor Fix/Perm Diluent (Tonbo) and incubated at 4°C for 1 hour. After the
507 incubation, the cells were washed twice with 3 ml of 1× Flow Cytometry Perm Buffer (Tonbo)
508 and resuspended in intracellular marker antibody cocktail (**Table S2**) diluted in 1× Flow
509 Cytometry Perm Buffer. After an incubation at 4°C for 30 min., the cells were washed twice with
510 3 ml 1× Flow Cytometry Perm Buffer and once with 3 ml cold PBS + 1% BSA. The cells were
511 then fixed by adding 4% formaldehyde at a 1:1 ratio to sample (v/v), washed with 3 ml cold PBS
512 + 1% BSA, and resuspended to 20 – 30 million cells/ml in PBS with 1% BSA, and filtered into a
513 FACS tube through a 35 µm sterile filter cap. Cells were analyzed by flow cytometry
514 immediately following fixation.

515

516 **Flow cytometry analysis**

517 B cells were analyzed on a Cytex Aurora spectral flow cytometer equipped with five lasers.
518 SpectroFlo QC Beads (Cytex, cat. no. SKU N7-97355) were run prior to each experiment for
519 performance tracking. Quality control and LJ tracking reports were used to ensure machine
520 performance and settings between different runs were comparable. B cells isolated from control
521 PBMCs collected pre-pandemic were used for the compensation of the live/dead stain and for
522 the unstained control. UltraComp eBeads (Thermo, cat. no. 01-2222-41) were used for
523 compensation of all other single stain fluorophores. FlowJo was used for gating and quantifying
524 cell frequencies. The cytometry analysis software OMIQ was used for the integration and
525 dimension reduction analysis. In short, Uniform Manifold Approximation and Projection (UMAPs)
526 were created by first pre-gating single/live/CD19⁺/CD20⁺/CD38^{lo} cells. Features used for
527 UMAP projection included mean fluorescence intensity of staining markers, excluding live/dead,
528 RBD, spike1, and spike2 with default parameters (neighbors = 15, metric = Euclidean, random
529 seed = 9889) and included all COVID-19 samples used in this study for initial projection.
530 Subsequently, files were subsampled to include an equal representation of cells from each
531 donor and concatenated within each group. For the projection of B cell subsets, Ig isotypes, and
532 antigen-specific B cells onto the UMAP, gates were manually set to identify populations of
533 interest using two-dimensional displays, which were then overlaid onto the UMAP projection.

534

535 **Detection of spike protein and receptor binding domain using an enzyme-linked** 536 **immunosorbent assay**

537 96-well plates (Corning, cat. no. 07-200-721) were coated with 50 µl of SARS-CoV-2 spike or
538 RBD at a concentration of 2 µg per ml in PBS and incubated overnight at 4°C. An additional
539 plate was coated with goat anti-human IgG (Sigma, cat. no. I2136-1ML) at 4 µg/ml and IgM
540 (Sigma, cat. no. I1636-2ML) at 8 µg/ml, and served as a positive control. Plates were washed

541 twice using a gentle stream of deionized water from a faucet and subsequently incubated with
542 200 µl blocking buffer (PBS with 0.01% Tween-20 (Fisher, cat. no. BP337-100) and 3% non-fat
543 dry milk (SACO)) for 1 hour at RT. Three-fold serial dilutions of 20 µg/ml human IgG or IgM
544 were prepared in dilution buffer (PBS with 0.01% Tween-20 and 1% non-fat dry milk) and were
545 added to the IgG or IgM-coated and blocked plates in 100 µl total volume. Plasma samples
546 were heat inactivated prior to handling by incubating at 56°C for 30 min. Plasma samples were
547 then diluted in dilution buffer starting at a 1:50 dilution, with an additional 7 two-fold serial
548 dilutions. The diluted plasma samples, 27 from convalescent patients exposed to SARS-CoV-2
549 and 1 from a non-infected human control, were added to the plates in a total volume of 200 µl.
550 After a two-hour incubation at RT, the plates were washed once with 150 µl of PBST by
551 removing the samples and adding PBST by pipetting and subsequently washed 6 times using a
552 gentle stream of deionized water from a faucet. Next, 100 µl anti-human IgG or IgM -
553 horseradish peroxidase (HRP) conjugates (BioLegend), diluted 1:2500 and 1:5000 in dilution
554 buffer, respectively, was added and the plates were incubated for 1 hour at RT. After four
555 subsequent washes with deionized water 100 µl TMB (Thermo, cat. no. PI34024) was added
556 and the plates were incubated in the dark at RT. The oxidation of TMB was stopped by adding
557 100 µl 0.18 M H₂SO₄ when the wells containing the most diluted IgG/IgM standard started to
558 color. The absorbance was measured at 450 nm using a Synergy H4 Hybrid Plate Reader
559 (BioTek). The average background signal of wells incubated without plasma was subtracted
560 from the absorbance values for each sample. All plasma samples were measured in duplicate
561 and the average absorbance reading was used to calculate the area under the curve using
562 GraphPad 9.

563

564 **Statistics**

565 Statistical analyses were performed in GraphPad 9. Differences between two groups were
566 evaluated using a Mann-Whitney U test. Differences in the expression of intracellular and cell

567 surface markers were tested using a Kruskal-Wallis test, followed by comparisons between
568 selected pairs of groups using Dunn's post-hoc test, corrected for multiple comparisons. Non-
569 parametric tests were used because the limited sample size did not allow for reliable evaluation
570 of normal distribution and homoscedasticity.

571

572 **Study approval**

573 Samples and associated clinical data used in this study were received de-identified from the
574 University of Texas Health San Antonio COVID-19 Repository. This repository was reviewed
575 and approved by the University of Texas Health Science Center at San Antonio Institutional
576 Review Board. All study participants provided written informed consent prior to specimen
577 collection for the repository to include collection of associated clinical information and use of left-
578 over clinical specimens for research. The COVID-19 Repository utilizes an honest broker
579 system to maintain participant confidentiality and release of de-identified data or specimens to
580 recipient investigators.

581 **AUTHOR CONTRIBUTIONS**

582 EMB secured funding for the study. RAR and EMB conceived the research question and
583 designed the study. RAR isolated plasma and PBMCs, and acquired and analyzed flow
584 cytometry data. KC performed and analyzed ELISAs. RAR, SB, and SJG produced S protein
585 and RBD, which were purified by AMC. GC, RET, and TFP provided clinical samples and data.
586 RG provided critical feedback on the manuscript. RAR, SB, and EMB wrote the manuscript with
587 input from all other co-authors.

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595 Coronavirus 2, Wuhan-Hu-1 spike glycoprotein receptor binding domain (RBD), NR-52309.
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597 The plasmid encoding BirA was a kind gift from Dr. Gavin Wright (Addgene plasmid # 32408).
598
599

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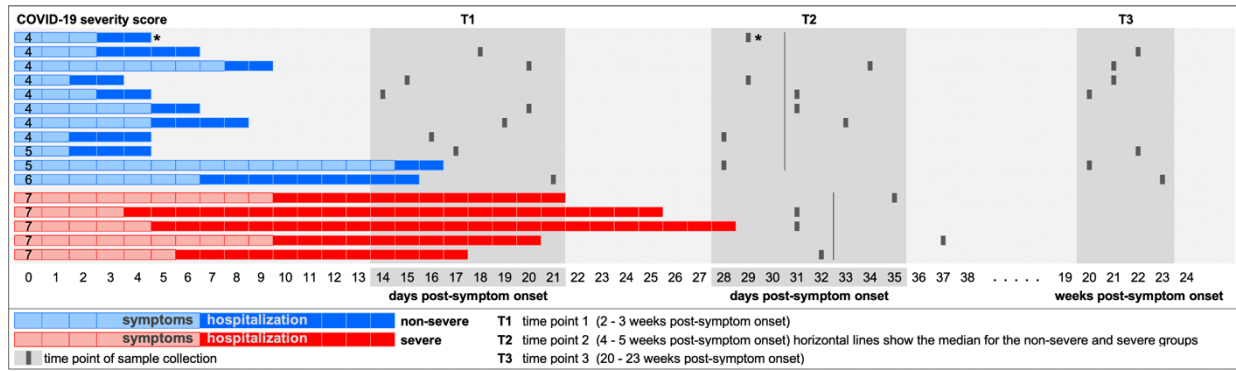
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744

745 **FIGURES**

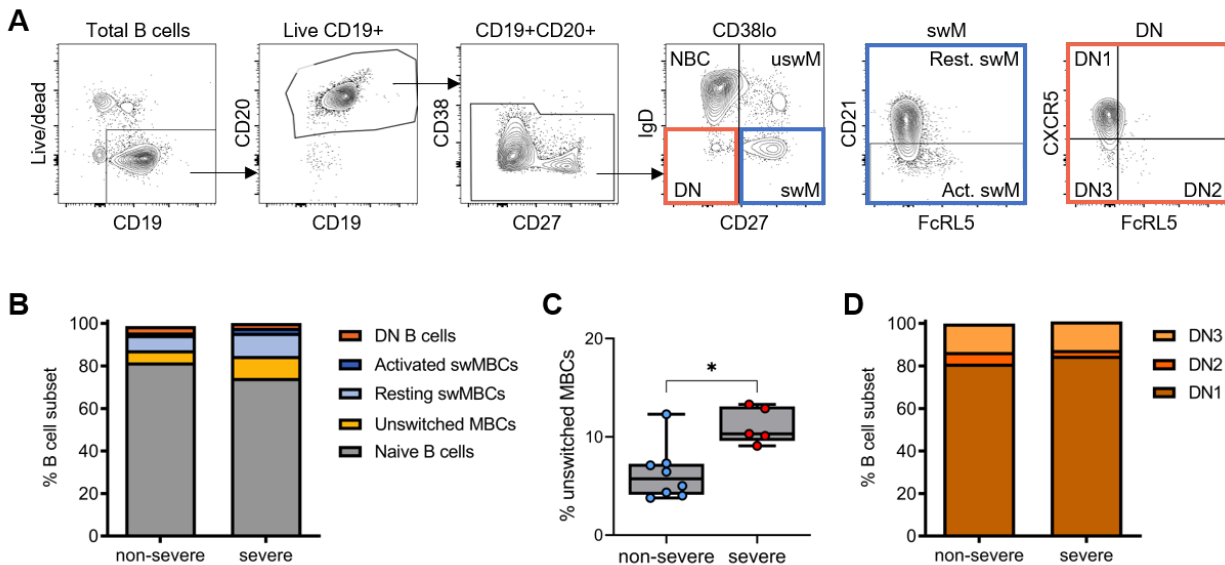


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747 **Figure 1: Timing of sample collection.** Hospitalized COVID-19 patients were recruited for the
 748 study and sampled after hospital discharge. Samples from T2 were used for the comparison of
 749 B cell responses between individuals who recovered from non-severe and severe COVID-19
 750 (**Figures 2 – 4**). All samples at T2 were collected 4 – 5 weeks post-symptom onset (PSO), with
 751 the exception of one severe donor who was sampled just after 5 weeks PSO (37 days).
 752 Longitudinal analysis of samples collected at T1 – T3 could only be performed for individuals
 753 who experienced non-severe disease, because the majority of severe patients were still
 754 hospitalized at T1 and we were unsuccessful in recruiting recovered severe patients for a follow-
 755 up blood draw at T3. The donor marked with * was included in the analysis of B cell subsets
 756 (**Figure 2**) and the percentage of spike-specific B cells (**Figure 3C**), but could not be included in
 757 all subsequent analyses because only a small number of spike-specific B cells (n = 6) was
 758 detected in this sample.

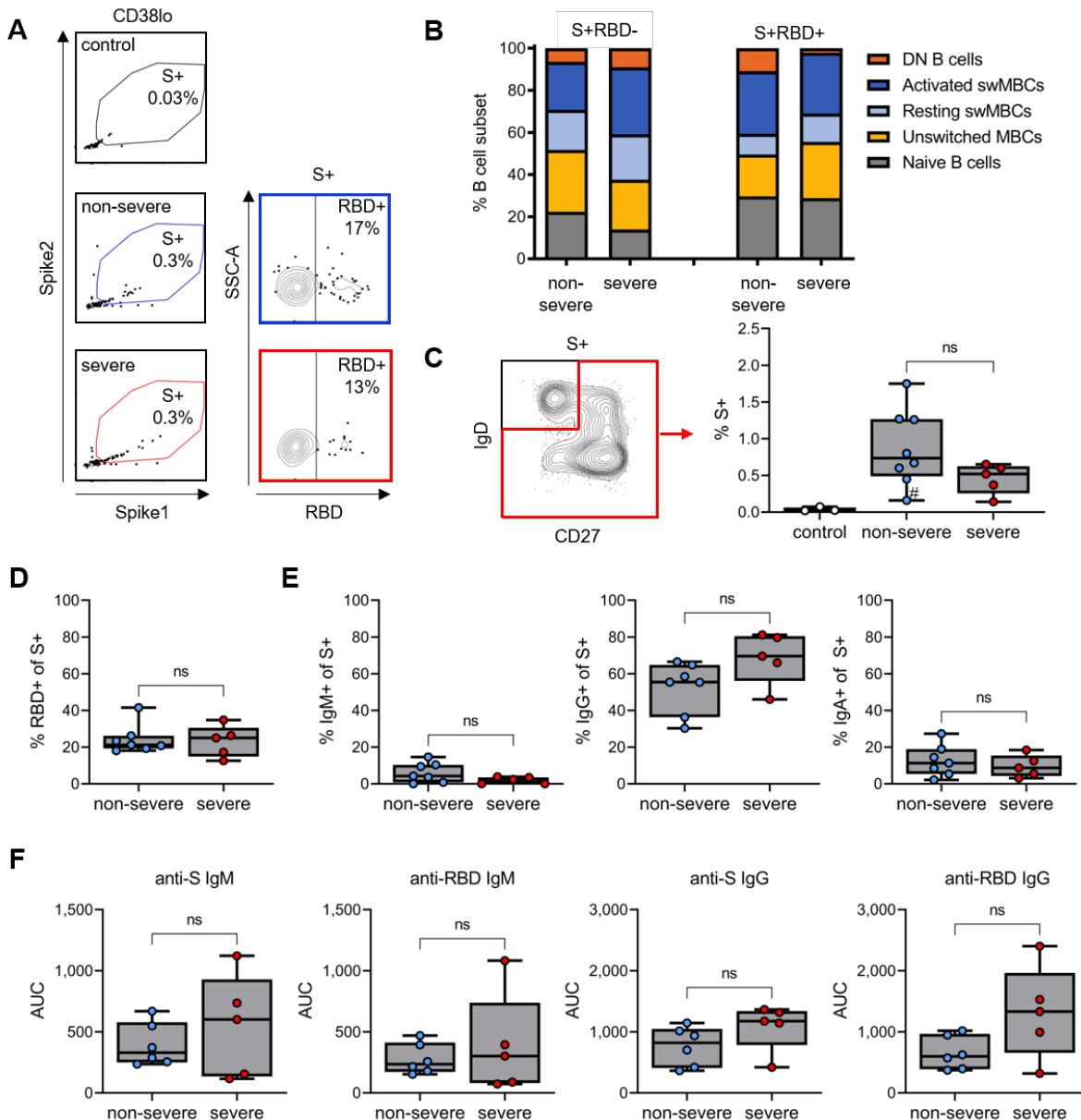
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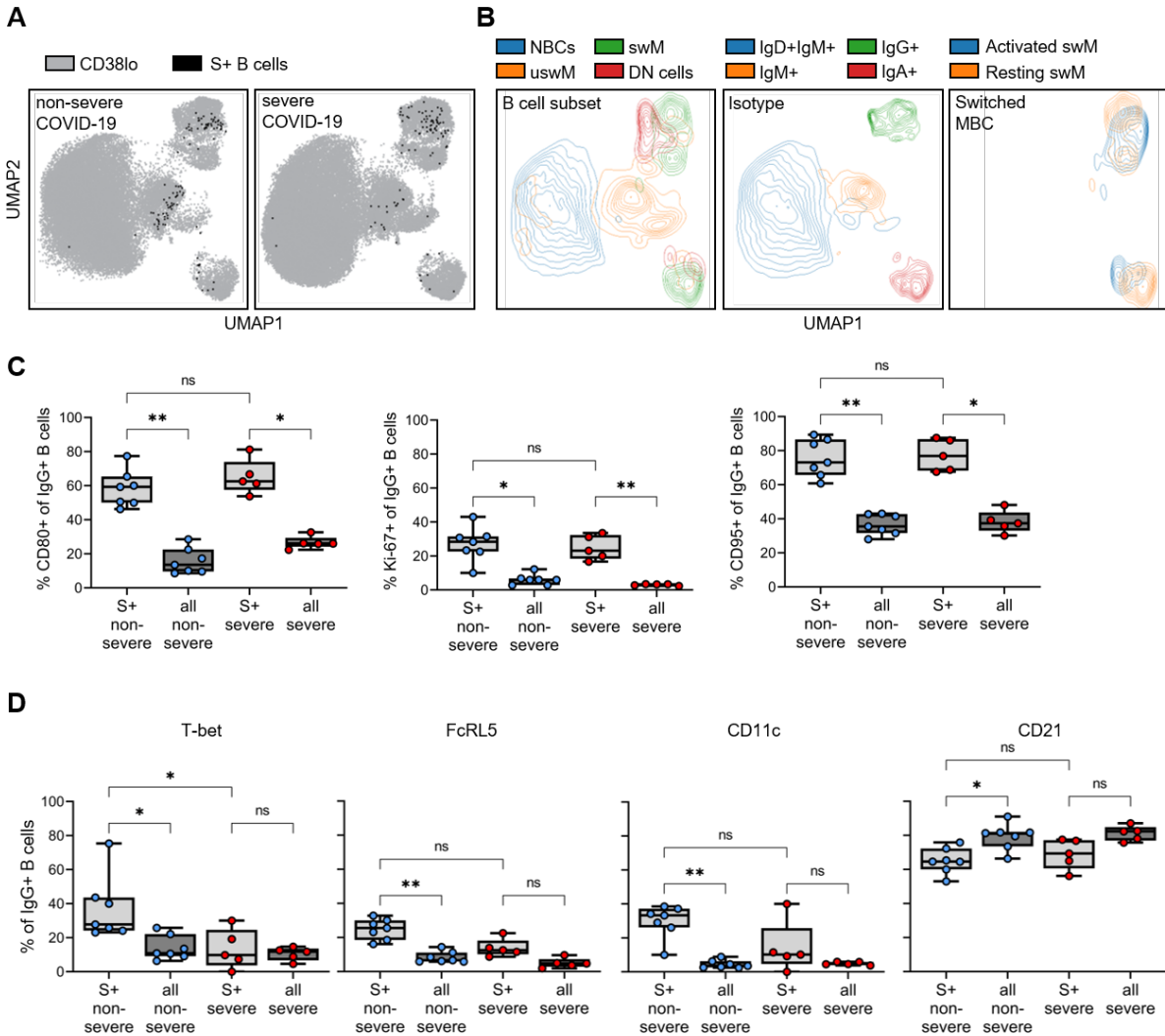
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762 **Figure 2: Distribution of major B cell subsets in recovered COVID-19 patients. A)** Gating
 763 strategy to obtain non-antibody-secreting B cells (CD38^{lo}) that are further divided into naïve,
 764 unswitched memory (uswM), switched memory (swM), and double negative (DN). SwM B cells
 765 were divided into activated and resting populations based on the expression of CD21. DN cells
 766 were divided into DN1 – 3 based on the expression of FcRL5 and CXCR5. **B)** Median
 767 percentage of each B cell subset in samples from individuals who recovered from non-severe
 768 COVID-19 and severe COVID-19. **C)** Percentage of unswitched memory B cells, which was
 769 increased in individuals who recovered from severe disease as compared to those who had
 770 non-severe COVID-19. **D)** Median proportions of the three different DN populations 1 month
 771 after non-severe or severe disease. In panel B – D, results are shown for individuals who
 772 recovered from non-severe COVID-19 (n = 8) and severe COVID-19 (n = 5). See **Figure S1** for
 773 graphs with individual data points for the data shown in panels B and D. * P < 0.05



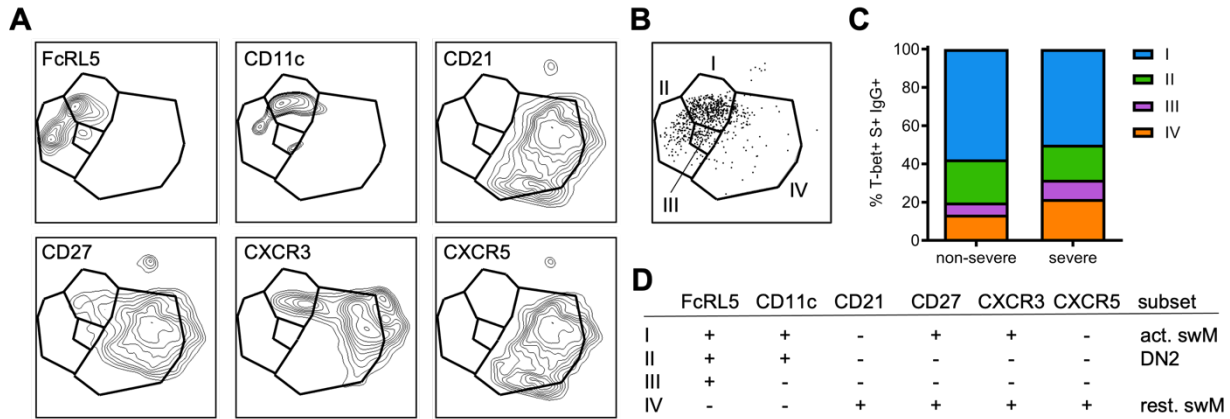
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775 **Figure 3: Detection of spike-specific and RBD-specific B cells and plasma antibody**
 776 **responses. A)** Flow cytometry gating of spike-specific (S+) and RBD-specific (RBD+) B cells.
 777 **B)** Phenotype of non-RBD-specific (S+RBD-) and RBD-specific (S+RBD+) B cells, showing the
 778 median percentage among each group. Graphs with individual data points for each B cell subset
 779 are presented in **Figure S2. C)** Percentage of antigen-experienced spike-specific B cells in
 780 individuals who have never been exposed to SARS-CoV-2 (control) or who recovered from non-
 781 severe or severe disease. **D)** Percentage of spike-specific B cells that recognized RBD. **E)**
 782 Percentage of class-switched spike-specific B cells that express IgM (left), IgG (middle), or IgA
 783 (right). **F)** Plasma IgM (left) and IgG (right) titers against spike and RBD. Note that the range of
 784 the Y axes are different between IgM and IgG. In panel C, results are shown for individuals who
 785 recovered from non-severe COVID-19 (n = 8) and severe COVID-19 (n = 5). Only seven
 786 individuals with non-severe COVID-19 were included in panels B, D, and E, due to the low
 787 number of spike-specific B cells detected in the individual marked with # in panel C.



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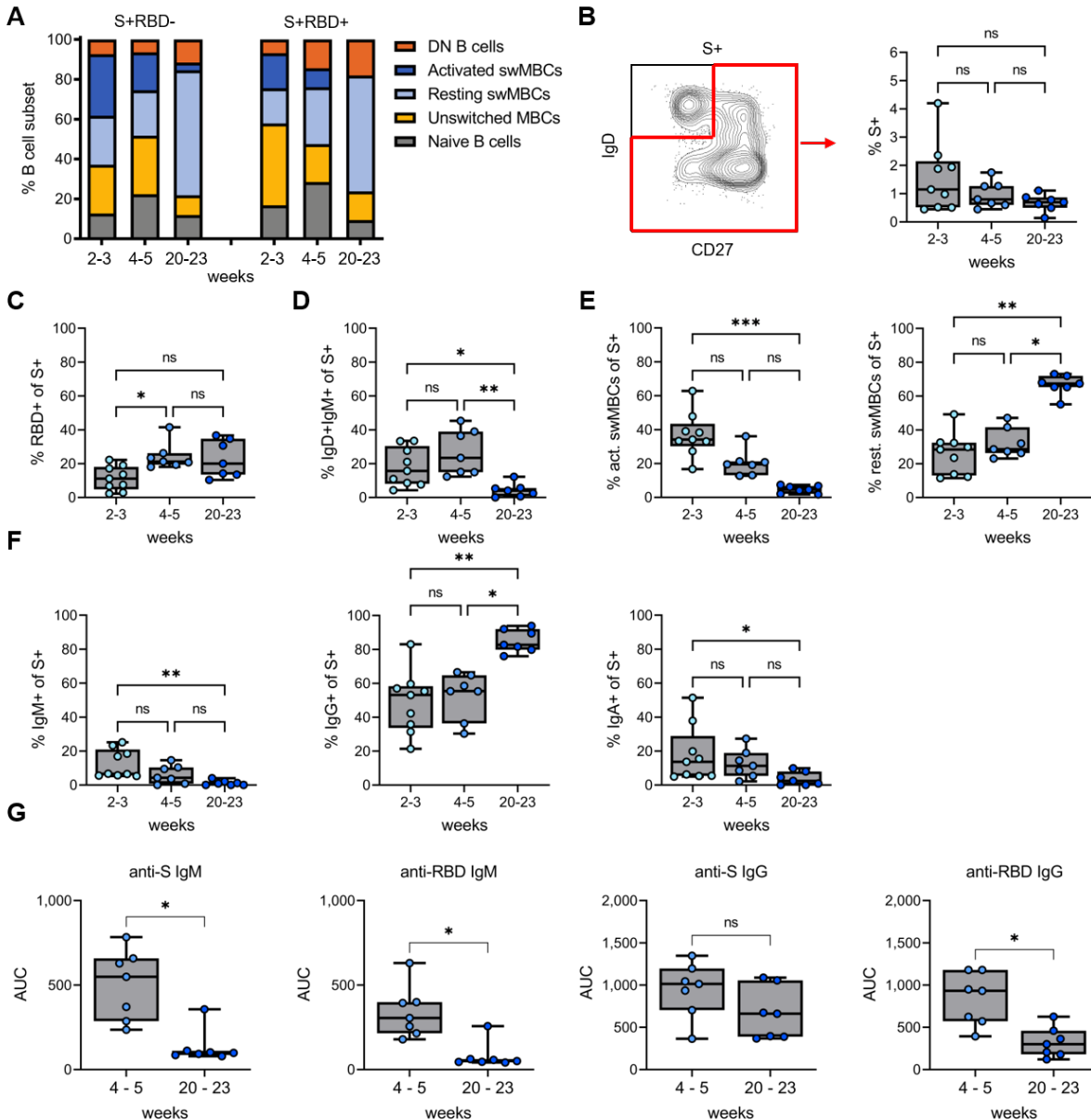
789 **Figure 4: Differences in the percentage spike-specific T-bet⁺ B cells between patients**
 790 **recovered from non-severe and severe COVID-19. A)** Composite UMAP showing the overlay
 791 of spike-specific B cells from individuals who recovered from non-severe (left) or severe (right)
 792 disease onto all B cells from that group. **B)** Overlay of major B cell subsets and isotypes onto
 793 the composite UMAP. **C)** Expression of CD80, Ki-67, and CD95 by spike-specific (S⁺) IgG⁺ B
 794 cells and all IgG⁺ B cells in individuals who experienced non-severe or severe COVID-19. **D)**
 795 Expression of T-bet, FcRL5, CD11c, and CD21 by spike-specific IgG⁺ B cells and all IgG⁺ B
 796 cells in individuals who experienced non-severe or severe COVID-19. Results are shown for
 797 seven individuals who recovered from non-severe COVID-19 and five patients who recovered
 798 from severe COVID-19. * P < 0.05; ** P < 0.01



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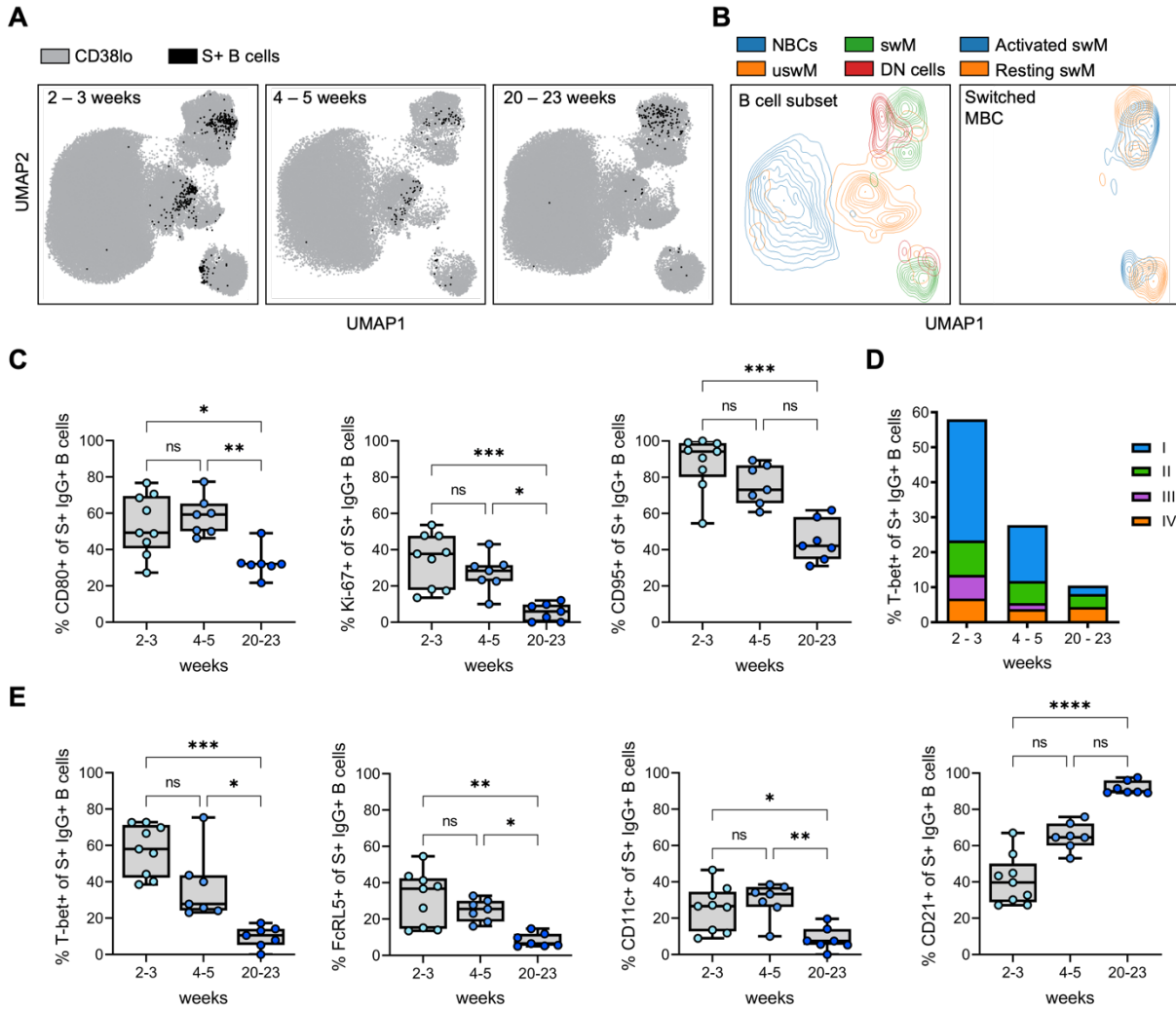
800 **Figure 5: The phenotype of T-bet⁺ IgG⁺ spike-specific B cells.** **A)** UMAPs showing the
 801 expression of individual markers in T-bet⁺ IgG⁺ spike-specific B cells. **B)** Gating of four
 802 populations of T-bet⁺ IgG⁺ B cells based on unique expression profiles of the markers shown in
 803 panel A. **C)** Distribution of the four T-bet⁺ IgG⁺ B cell population among individuals who
 804 recovered from non-severe (n = 7) and severe (n = 5) COVID-19. **D)** Summary of the phenotype
 805 of the four T-bet⁺ IgG⁺ B cell populations identified here and their classification into one of the
 806 major B cell subsets: activated switched memory (act. swM), DN2, or resting switched memory
 807 (rest. swM) B cells.

808



809

810 **Figure 6: Change from activated to resting memory phenotype of IgG⁺ spike-specific B**
 811 **cells at five months post-symptom onset. A)** Phenotype distribution of non-RBD-specific
 812 (S+RBD-) and RBD-specific (S+RBD+) B cells, showing the median percentage among each
 813 group. Graphs with individual data points for each B cell subset are presented in **Figure S6. B)**
 814 Percentage of antigen-experienced spike-specific B cells at 2 – 3, 4 – 5, and 20 – 23 weeks
 815 post-symptom onset. **C)** Percentage of S+RBD+ B cells among antigen-experienced spike-
 816 specific B cells. **D)** Percentage of unswitched memory B cells among antigen-experienced
 817 spike-specific B cells. **E)** Percentage of activated (left) and resting (right) switched memory B
 818 cells among antigen-experienced spike-specific B cells. **F)** Percentage of class-switched spike-
 819 specific B cells that express IgM (left), IgA (middle), or IgG (right). **G)** Plasma IgM (left) and IgG
 820 (right) titers against spike and RBD. In all panels, results are shown for samples collected 2 – 3
 821 (n = 9), 4 – 5 (n = 7), and 20 – 23 (n = 7) weeks post-symptom onset. * P < 0.05; ** P < 0.01; ***
 822 P < 0.001



823

824 **Figure 7: Longitudinal dynamics in the percentage of spike-specific B cells that express**
 825 **T-bet and various surface markers shortly after recovery and five months post-symptom**
 826 **onset. A)** Composite UMAP showing the overlay of spike-specific B cells from individuals who
 827 recovered from non-severe COVID-19 at 2-3 (left), 4-5 (middle), and 20-23 (left) weeks post-
 828 symptom onset. **B)** Overlay of major B cell subsets and activated/resting switched memory B
 829 cells onto the composite UMAP. **C)** Expression of CD80, Ki-67, and CD95 by spike-specific (S+)
 830 IgG⁺ B cells at the three time points. **D)** Distribution of the four T-bet⁺ subsets (as defined in
 831 **Figure 5)** among spike-specific IgG⁺ B cells at the three time points post-symptom onset. **E)**
 832 Expression of T-bet, FcRL5, CD11c, and CD21 by spike-specific IgG⁺ B cells at the three time
 833 points post-symptom onset. In all graphs, results are shown for samples collected 2 – 3 (n = 9),
 834 4 – 5 (n = 7), and 20 – 23 (n = 7) weeks post-symptom onset. * P < 0.05; ** P < 0.01; *** P <
 835 0.001; **** P < 0.0001