

1 **SARS-CoV-2-specific memory B cells can persist in the elderly despite loss of neutralising**
2 **antibodies**

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24 Abstract

25 Memory B cells (MBC) can provide a recall response able to supplement waning antibodies
26 with an affinity-matured response better able to neutralise variant viruses. We studied a
27 cohort of vulnerable elderly care home residents and younger staff, a high proportion of
28 whom had lost neutralising antibodies (nAb), to investigate their reserve immunity from
29 SARS-CoV-2-specific MBC. Class-switched spike and RBD-tetramer-binding MBC with a
30 classical phenotype persisted five months post-mild/asymptomatic SARS-CoV-2 infection,
31 irrespective of age. Spike/RBD-specific MBC remained detectable in the majority who had lost
32 nAb, although at lower frequencies and with a reduced IgG/IgA isotype ratio. Functional
33 spike/S1/RBD-specific recall was also detectable by ELISpot in some who had lost nAb, but
34 was significantly impaired in the elderly, particularly to RBD. Our findings demonstrate
35 persistence of SARS-CoV-2-specific MBC beyond loss of nAb, but highlight the need for careful
36 monitoring of functional defects in RBD-specific B cell immunity in the elderly.

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39 **One sentence summary:** Circulating class-switched spike and RBD-specific memory B cells can
40 outlast detectable neutralising antibodies but are functionally constrained in the elderly.

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44 Introduction

45 The human coronavirus SARS-CoV-2 has had a particularly devastating impact on the elderly,
46 who are at much greater risk of morbidity and mortality.^{1,2} Understanding the nature of a
47 successful immune response in those who have avoided these outcomes and cleared SARS-
48 CoV-2 after a mild infection, despite advanced age, is key to protecting this vulnerable group
49 in the future. Whether older survivors of SARS-CoV-2 infection are able to mount robust and
50 durable responses with the potential to provide long-term protection from reinfection, and
51 from emerging viral variants, remains to be understood. Insights into the strengths and
52 limitations of the immune response in those who have had a successful outcome of natural
53 infection can inform the future optimisation of vaccines. It is also crucial to understand the
54 nature of the immune protection afforded to previously infected individuals whilst they await
55 vaccination, especially with the ongoing delays in rollout and the lag in provision to low and
56 middle-income countries.

57

58 Antibodies, in particular the neutralising fraction, provide a vital frontline defence to achieve
59 protective immunity against viruses. An initial waning of antibody titres is typically seen after
60 resolution of an acute viral infection.^{3,4} In the case of some viruses, long-lived plasma cells are
61 then able to maintain antibodies for decades.⁵⁻⁷ By contrast, in the months following infection
62 with other viruses, including human coronaviruses like SARS-CoV-2, neutralising antibodies
63 continue to wane and can drop below the threshold of detection in a proportion of
64 individuals.^{3,8-13} Even if antibodies are maintained, they may fail to provide sufficient
65 functional flexibility to cross-recognise viral variants.¹⁴⁻¹⁶ However, antibody responses of
66 inadequate titre or unable to cross-recognise variants can be compensated by a second line
67 of defence provided by antigen-specific memory B cells (MBC), that are poised to react rapidly
68 upon pathogen re-encounter.¹⁷⁻¹⁹ Not only can MBC provide a faster response on re-exposure
69 to the virus, they are also able to diversify in the face of a mutating virus, resulting in more
70 potent, affinity-matured antibody response and enhanced resistance to viral mutations.^{9,20}

71

72 In this study, we therefore analysed whether MBC develop in elderly subjects following the
73 resolution of SARS-CoV-2 infection and whether they can maintain functionality once
74 neutralising antibodies (nAb) have waned. To address these questions, we studied elderly

75 residents that had recovered from SARS-CoV-2 infection following outbreaks in three care
76 homes in the UK, who had mild or asymptomatic infection, a substantial proportion of whom
77 lost detectable nAb by five months after outbreak resolution. MBC were compared between
78 the elderly care home residents and younger staff to assess the impact of ageing. We
79 identified MBC specific for SARS-CoV-2 spike and RBD that persisted when serum nAb had
80 completely waned. Their frequency, phenotype, isotype and function were analysed
81 according to age and/or nAb loss, to inform the assessment and boosting of durable immunity
82 in the elderly.

83

84 Results

85 *SARS-CoV2 spike and RBD-specific memory B cells can persist after loss of neutralising* 86 *antibodies*

87 To study the role of MBC, we obtained PBMC from a subset (n=32) of a large cohort who
88 survived COVID-19 with mild/asymptomatic infection after outbreaks in three care homes in
89 April 2020 (Methods and Table S1).^{21,22} The care home cohort subset was selected to have a
90 wide range of nAb titres detectable against live virus at the first sampling timepoint (T1, May
91 2020, Fig.1). By end September 2020 (T2, five months), 32% of all participants sampled had
92 stable or increasing nAb to live virus. In contrast, 22% had declining titres, and 38% had lost
93 detectable nAb (Fig.1a,b).

94

95 To compare MBC frequencies in those who had maintained or lost nAb, we stained PBMC
96 with SARS-CoV-2 spike trimer tetramers, made by pre-incubating recombinant biotinylated
97 trimeric spike protein with fluorescently-conjugated streptavidin.¹⁵ Dual staining with spike
98 tetramers with two distinct fluorochromes was used to enhance the discrimination of true
99 antigen-specific MBC (Fig.1c), as described previously.²³⁻²⁵ Frequencies of antigen-specific
100 responses were calculated within the memory fraction of B cells (CD19⁺CD20⁺ excluding IgD⁺,
101 CD38^{hi} and CD21⁺CD27⁻ naïve fractions, gating strategy in Fig.S1a, as previously described²⁶).
102 A threshold for background non-specific staining was set at mean+ 2SD of staining seen in an
103 uninfected control cohort derived from the same care homes (seronegative at both time
104 points, Table S1) and from pre-pandemic healthy donor samples (Fig.S1b).

105

106 Spike-specific MBC were detectable in 28 of the 32 tested 5 months post-infection (Fig.1d).
107 The frequency of spike-specific MBC was reduced in those who had lost nAb compared to
108 those in whom they were still detectable (Fig.1e). Of note, however, most of those (85%) who
109 had lost detectable nAb still had some persistent spike-specific MBC, a comparable
110 proportion to that in the group maintaining nAb (Fig.1f). The frequency of spike-specific MBC
111 correlated significantly with the strength of the nAb response (nAb titre to live virus) at 5
112 months (Fig.1g); however, there was partial discordance due to detection of spike-specific
113 MBC in most individuals with no nAb (dotted box, Fig.1g).

114

115 Next, we analysed the MBC response specifically directed against RBD since this is the region
116 within spike which many SARS-CoV-2-specific nAb target.^{15,27-29} RBD-specific MBC were
117 identified by gating on dual spike tetramer-staining populations that also stained with a
118 tetramer formed from recombinant biotinylated RBD protein pre-incubated with
119 fluorescently-conjugated streptavidin (Fig.1h). RBD-specific responses were detectable in 26
120 of the 28 with sufficient magnitude spike-specific MBC responses (>20 dual-spike+ cells) to
121 allow analysis of the RBD-co-staining cells (Fig.1i). The frequency of RBD-specific MBC was
122 significantly reduced in the group who had lost nAb compared to those with stable (or waning
123 but still detectable) nAb (Fig.1i). However, as noted with spike-specific MBC, some RBD-
124 specific MBC remained detectable in most of the cohort, irrespective of whether or not they
125 had lost nAb (Fig.1j). Overall, the magnitude of RBD-specific MBC correlated with nAb titres,
126 although again there was some discordance due to RBD MBC in those who had lost nAb
127 (dotted box in Fig.1k). Importantly, both the RBD positive and RBD negative components of
128 the spike-specific B cell response significantly correlated with nAb titres, though slightly more
129 robustly for the RBD positive subset (Fig.1k, Fig.S1c). This highlights the importance of the
130 RBD as the major target for neutralising antibodies, whilst also underscoring the contribution
131 of antibodies targeting regions outside of the RBD (for example the NTD of the spike
132 protein^{15,29-31}) to the neutralising antibody response, at the 5 month timepoint in this cohort.

133

134 These data therefore revealed the persistence of detectable, albeit reduced, MBC specific for
135 both spike and RBD in most people whose nAb titres against live virus had fallen below the
136 threshold of detection. Thus, loss of detectable nAb 5 months after asymptomatic/mild

137 infection is frequently compensated by the presence of a memory response primed to
138 respond upon re-exposure.

139

140

141 *Comparable persistence of spike and RBD-specific MBC in elderly care home residents and*
142 *younger staff*

143 The care home cohort was constructed to sample two comparator groups: elderly residents
144 (median age 86yrs, range 66-96) and a control group of younger staff (median age 56yrs,
145 range 41-65). Five months after asymptomatic/mild infection, similar proportions of staff and
146 residents had lost detectable nAb (Fig.2a), and those who maintained them had similar titres
147 (Fig.2b). We postulated that there may, nevertheless, be a defect in the maintenance of
148 spike/RBD-specific MBC in the elderly compared to younger age group. However, spike-
149 specific MBC were maintained at similar frequencies and in comparable proportions of the
150 elderly residents and younger staff (Fig.2c,d). There were no clear trends for spike-specific
151 MBC to decrease with increasing age, even in residents in their nineties (Fig.2e).

152

153 Similarly, RBD-specific MBC were equally well-maintained in the residents and staff (Fig.2f,g),
154 with no decline in their frequencies (as a fraction of total MBC) with increasing age (Fig.2h).
155 RBD-specific MBC comprised a variable proportion of the total spike-specific MBC response
156 (4.6 to 41.0%; median 24.0%), the remainder representing B cells targeting non-RBD regions
157 of spike. The proportions of RBD and non-RBD-binding spike-specific MBC again showed no
158 changes with age (Fig.2i).

159

160

161 *Skewed isotype of spike/RBD-specific B cells associates with loss of neutralising antibodies*

162 Having identified and quantified antigen-specific B cells with tetramer staining, we were able
163 to apply high-dimensional multiparameter flow cytometry to phenotype these low frequency
164 populations without any *in vitro* manipulation. We investigated the immunoglobulin isotype,
165 memory phenotype, homing markers and transcription factor usage of spike and RBD-specific
166 B cells, and global B cells (Fig.3).

167

168 The vast majority of SARS-CoV-2 MBC expressed IgG, with a similar isotype distribution
169 observed between spike and RBD-specific MBC (Fig.3a,b,c). However, individuals with
170 persistent nAb had a higher frequency of IgG isotype expressing spike- and RBD- specific MBC
171 than their counterparts who had lost nAb (Fig.3b,c), indicating the establishment of a robust,
172 class-switched memory response in these individuals. In contrast, individuals whose nAb had
173 waned below detectable limits had lost more IgG, and had a relative preservation of IgA class-
174 switched spike- and RBD- specific MBC (Fig.3b,c). Elderly residents similarly showed a trend
175 towards less IgG on their spike-specific MBC but, overall, no significant skewing of their
176 immunoglobulin class-switching compared to younger staff (Fig.3b,c). Global B cells showed
177 the same pattern of expression of different immunoglobulin isotypes on their surface in SARS-
178 CoV-2 resolved donors as in uninfected controls, with roughly equal proportions of IgG and
179 IgA and less than 15% IgM (Fig.S2a).

180

181 MBC subsets were examined using the combination of CD27 and CD21. The majority of spike
182 and RBD-specific B cells had a classical resting memory phenotype (CD27⁺CD21⁺),
183 characteristic of functional responses and comparable to the global MBC compartment, in
184 both the elderly resident and staff groups (Fig.3d,e,f, Fig.S2b). ‘Double negative B cells’ have
185 been associated with B cell dysfunction in ageing,³²⁻³⁴ and the ‘DN2’ subset with an
186 extrafollicular short-lived plasmablast response in the acute phase of a cohort with severe
187 COVID-19.³⁵ However, at the five month timepoint following mild/asymptomatic infection in
188 our cohort, neither the elderly nor those who had lost nAb showed any expansion of CD27⁻
189 CD21⁻ B cells (Fig.3e,f) or the DN2 subset (CD27⁻CD21⁻CXCR5^{lo}CD11c^{hi}, Fig.S2c). Instead, there
190 was a selective enrichment of the activated MBC subset (CD27⁺CD21⁻, previously described
191 to be expanded in HIV and Ebola infection or after vaccination³⁶⁻³⁸) in the RBD-binding
192 fraction in elderly residents, with the same trend in those who had lost nAb (Fig.3g). Those
193 who had lost nAb also had reduced expression of the B cell homing molecules CXCR3 and
194 CXCR5 on spike-specific and global MBC (non-significant trend and significant respectively,
195 Fig.S2d,ef). T-bet, a transcription factor critical for acute antiviral function in B cells but
196 associated with dysfunction in chronic infections and autoimmunity,³⁹⁻⁴² also tended to be
197 expressed at lower levels in the spike-specific MBC of those losing nAb (Fig.S2e,f).

198

199 Taken together, the isotype and memory phenotype of global and antigen-specific B cells was
200 largely preserved in the elderly care home population, apart from an increase in spike-specific
201 activated MBC. Individuals who maintained nAb had predominantly IgG-expressing antigen-
202 specific MBC. In contrast, in those who had lost nAb by 5 months, whether staff or residents,
203 residual antigen-specific B cells showed preferential preservation of IgA.

204
205

206 *Elderly residents maintain functional spike/RBD-specific B cells but at reduced frequency*
207 *compared to younger care home staff.*

208 Having found that antigen-specific MBC could persist following complete waning of
209 circulating nAb, we wanted to confirm their potential for functional recall upon re-
210 encountering SARS-CoV-2. We therefore used cultured B cell ELISpots to examine the capacity
211 of persistent SARS-CoV-2-specific MBC to differentiate into plasmablasts capable of secreting
212 IgG capable of binding recombinant trimeric spike, S1 or RBD proteins.

213

214 ELISpots were performed using PBMC from 24 seropositive care home residents and staff,
215 with the threshold for detection set at the highest observed value in an uninfected controls
216 group (five seronegative care home residents and five pre-pandemic controls). Only
217 individuals with responses detectable in a control total IgG well were included in analysis.
218 Where responses were too numerous to count (TNTC), the highest number of spot-forming
219 cells (SFCs) observed in the maximal response to the respective protein was used (Fig.S3a).

220

221 Functional recall responses to SARS-CoV-2 trimeric spike protein were observed in 21 of the
222 24 seropositive individuals tested, with ELISpots tending to be positive in more of those who
223 had maintained nAb (Fig.4a). However, the majority of those who had lost detectable nAb
224 still had a spike-specific response by ELISpot, with no significant difference in their magnitude
225 compared to the nAb group (Fig.4a). ELISpots showed similar results for IgG binding S1 and
226 RBD, with a trend to a lower proportion of positive results in those who had lost nAb but no
227 significant difference in the magnitude of B cell recall responses in those maintaining serum
228 nAb or not (Fig.4b,c).

229

230 The magnitude of RBD recall response assessed by ELISpot showed a significant correlation
231 with both spike and RBD MBC detection by tetramer staining (Fig.4d,e). However, there was
232 some discordance due to individuals who had tetramer-binding spike or RBD B cells that did
233 not produce detectable IgG by ELISpot (dotted boxes, Fig.4d,e), mainly in those who had lost
234 nAb. Importantly, these data revealed that circulating antigen-specific B cells can be detected
235 in the absence of functional recall.

236
237 Next, we compared functional responses to all three proteins for each individual, ranked
238 according to nAb status and age. Individuals with strong recall to spike (as measured by
239 ELISpot) tended to also have strong responses to S1 and RBD, whereas others had weak
240 responses to all three antigens (Fig.4f). Functional MBC recall responses decreased with
241 increasing age in both the groups, regardless of maintenance of serum nAb (Fig.4f). Thus,
242 elderly residents had significantly lower ELISpot MBC responses against spike, S1, and
243 particularly RBD, than the younger staff group (Fig.4g,h,i). Focusing on elderly residents who
244 had lost nAb, we found that none of these individuals sustained MBC capable of functional
245 recall to RBD (Fig.4j).

246
247 Overall, the measurement of nAb against live virus combined with assessment of spike and
248 RBD-specific MBC by tetramer staining and functional ELISpot provided complementary
249 insights into B cell immunity (Fig.4k). A substantial proportion of those who had lost
250 neutralising activity against live virus, maintained spike and RBD-specific MBC detectable
251 with one or both assays, regardless of age. However, some of those with persistent antigen-
252 specific MBC could not mount a detectable functional response, particularly the elderly who
253 had lost nAb (Fig.4k).

254

255 Discussion

256 In this study we sampled a cohort of very elderly residents and younger staff who developed
257 mild/asymptomatic SARS-CoV-2 infection during care home outbreaks, a high proportion of
258 whom had lost nAb by five months. This allowed us to dissect the potential for B cell memory
259 to persist beyond serum nAb, providing a back-up reserve to humoral immunity. We

260 demonstrated that the majority of the cohort maintained detectable frequencies of spike and
261 RBD-specific MBC by flow cytometry, even where they had lost circulating antibodies capable
262 of live virus neutralisation. Tetramer staining allowed accurate *ex vivo* quantification and
263 characterisation of antigen-specific MBC, revealing that individuals who had lost nAb had
264 lower frequencies of spike and RBD-specific MBC, with a preserved classical memory
265 phenotype but class-switching skewed away from IgG towards IgA. Elderly and younger
266 recovered individuals infected in the same care home outbreaks maintained similar
267 frequencies of spike and RBD-specific tetramer-staining B cells, with comparable phenotypes
268 and isotypes. However functional assessment using ELISpot assays demonstrated that the
269 persisting spike, and particularly RBD-specific, MBC had reduced potential for antibody
270 production in the elderly.

271
272 The success of an infection or vaccine in inducing durable humoral immunity is dependent on
273 the generation of long-lived plasma cells and MBC.^{17–19} The longevity of the plasma cell
274 response, capable of sustaining antibodies, varies widely following different viral infections.^{5–}
275 ⁷ A recent study has demonstrated the presence of bone marrow plasma cells secreting IgG
276 against SARS-CoV-2 spike protein in fifteen of nineteen individuals examined seven months
277 post infection,⁴³ in line with the durability of some antibodies in the first year after mild
278 infection. Nevertheless, many studies have also highlighted the potential for neutralising
279 antibodies to SARS-CoV-2 to wane to a point where there is an, as yet ill-defined, risk of re-
280 infection.^{44–46} Our study deliberately focuses on the role of MBC in those with waning or
281 undetectable nAb to live virus, despite persistence of binding antibodies. MBC, previously
282 identified in younger COVID-19 cohorts,^{11,26,47,48} can provide a crucial back-up by responding
283 quickly to pathogen re-encounter or vaccination to form new plasmablasts, producing potent
284 affinity-matured antibodies with more flexible recognition of viral variants;^{9,20} this is
285 consistent with the enhanced nAb response described following vaccination of previously
286 SARS-CoV-2 infected healthcare workers.⁴⁹ Our demonstration that B cells of relevant
287 specificities can still be detected even when nAb titres are waning or completely abrogated
288 provides some reassurance that a memory response remains intact in the elderly. Future
289 large-scale studies are needed to assess whether B cell memory serves as an independent
290 correlate of protection or whether reliance on MBC to mount a new response in the absence

291 of existing antibodies provides a critical window of opportunity for a virus that replicates as
292 rapidly as SARS-CoV-2.

293

294 One strategy to combat antibodies that are waning or unable to cross-recognise emerging
295 variants is the use of booster vaccines. Our finding that the elderly have impaired
296 differentiation of their persistent spike/RBD-specific MBC into antibody producing cells
297 detected by ELISpot assays provides biological rationale for a potential need for more
298 frequent booster vaccination in this high-risk group. The frequency, phenotype and class-
299 switching of antigen-specific B cells did not reveal obvious changes in the elderly group to
300 account for this functional defect, other than an increase in the CD27⁺CD21⁻ subset. The
301 activated CD27⁺CD21⁻ subset of MBC has recently been noted to remain expanded in some
302 resolved COVID-19 patients,⁵⁰ consistent with emerging literature supporting the possibility
303 of prolonged antigen persistence, exemplified by a recent study detecting SARS-CoV-2 in the
304 small bowel four months after asymptomatic infection.⁹ Our finding of more antigen-specific
305 CD27⁺CD21⁻ MBC in the older age group raises the possibility there is more prolonged antigen
306 persistence and resultant B cell activation following SARS-CoV-2 infection in the elderly.
307 However, the ageing immune system is characterised by a tendency to low-level chronic
308 inflammation,^{51,52} which could also contribute to prolonged activation of SARS-CoV-2 MBC.
309 Analogous to our findings in elderly care home residents, both older subjects and those with
310 HIV have been found to have persistent circulating MBC but defective plasmablast formation,
311 resulting in reduced influenza vaccine-induced antibodies.^{53,54} Such age-related defects in B
312 cell responses to vaccination have been attributed to a combination of B cell intrinsic
313 senescence and defective T follicular helper cells (Tfh) in germinal centres.⁵⁵⁻⁵⁷

314

315 A caveat to our study is that we were only able to study circulating B cells, whilst additional
316 recall responses may be compartmentalised within the mucosa. A recent study suggested
317 mild infection can stimulate mucosal SARS-CoV-2-specific IgA secretion in the absence of
318 circulating antibodies.⁵⁸ The bias towards the retention of IgA⁺ spike/RBD-specific MBC in
319 those who had lost all detectable serum nAb to live virus could therefore be reflective of a
320 stronger mucosal response in these individuals. An increase in mucosal-homing IgA responses
321 has been described as a feature of the ageing immune response,⁵⁹ consistent with the older
322 composition of our cohort. Alternatively, the relative preservation of IgA rather than IgG

323 spike/RBD-specific MBC in those with the fastest waning nAb may simply reflect the recent
324 observations that IgA dominates the early nAb response to SARS-CoV-2 infection,⁵⁶ and may
325 not decline as fast as the IgG response.^{9,60} Since our ELISpot assays did not measure the
326 function of IgA isotype B cells, we may have under-estimated the full extent of residual SARS-
327 CoV-2-specific responses, particularly in those with a more IgA-skewed response. In addition,
328 several studies have shown that the magnitude of the MBC response to SARS-CoV-2 continues
329 to increase beyond six months,^{9,23,50,61} again implying that we may have under-estimated the
330 extent of recall potential in our cohort at five months. Future studies should also examine the
331 preservation of non-spike-specific MBC with the potential to produce antibodies mediating
332 antiviral effects beyond neutralisation, since other viral proteins (ORF3a, membrane and
333 nucleocapsid) can play a dominant role in triggering antibody-dependent NK cell activation.⁶²

334

335 In conclusion, by focusing on an elderly cohort with a high proportion of nAb loss, we
336 demonstrated that this waning in the first line of humoral defence can be compensated by
337 the presence of a reserve of adaptive B cell memory in the majority of cases. Our findings
338 highlight the importance of including measures of B cell memory in larger studies of natural
339 infection and vaccination to determine their role as additional correlates of protection. Our
340 data underscore that identifying antigen-specific B cells by tetramer antigen staining is useful
341 for quantitation and thorough *ex vivo* characterisation, but may not necessarily equate with
342 the preservation of a functional response, as also observed in chronic viral infection.^{42,63} The
343 relative preservation of IgA antigen-specific MBC in those with waned serum nAb raises the
344 possibility that mucosal sequestered immunity may outlast that detectable in the circulation.
345 Increased expansion of activated MBC in the elderly highlights the need to investigate
346 whether they are more prone to prolonged stimulation from persistent reservoirs of SARS-
347 CoV-2 antigen. A finding of concern was the lack of detectable functional recall to RBD in
348 elderly donors who had lost nAb; given that RBD is the dominant site for nAb this supports
349 the need for additional monitoring and/or booster vaccines to maintain sufficient antibodies
350 to neutralise emerging variants in this highly vulnerable group.

351

352 **Materials and Methods**

353 **Participants**

354 SARS-CoV-2 antigen specific memory B cell (MBC) responses were compared between elderly
355 care home resident and younger staff counterparts exposed to the virus within the same
356 environment. Six care homes reporting SARS-CoV-2 outbreaks to Public Health England (PHE)
357 were recruited to longitudinal SARS-CoV-2 RT-PCR and serological follow-up in April 2020
358 (T0).^{1,21} Serostatus of individuals within these homes at one month and five months after the
359 outbreaks (T1 and T2 respectively) was established using binding and functional assays as
360 previously described.^{21,22} Briefly, a native virus lysate assay (PHE) and/or receptor binding
361 domain assay (RBD, PHE) determined seropositivity, and a live virus neutralising antibody
362 assay to prototype England.2 SARS-CoV-2 virus was used to determine neutralising antibody
363 titres.^{21,22}

364

365 A total of 32 SARS-CoV-2 individuals (22 residents; 10 staff) were recruited, all of whom were
366 seropositive by the binding assays described above at both sampling time points (T1: May
367 2020, T2: September 2020), alongside 11 SARS-CoV-2 seronegative control individuals from
368 three of the care homes. Participants donated 30ml of blood to be processed for peripheral
369 blood mononuclear cells (PBMCs) and serum five months after the initial outbreaks (T2). The
370 investigation protocol was reviewed and approved by the PHE Research Ethics and
371 Governance Group (REGG Ref NR0204). Written information regarding the study was
372 provided to all participants; verbal consent for testing was obtained by care home managers
373 from staff members and residents or their next of kin as appropriate.

374

375 Stored pre-pandemic samples from seven healthy individuals were used as controls,
376 recruited under ethics number 11/LO/0421 approved by the 'South East Coast - Brighton
377 and Sussex Research Ethics Committee.

378

379 **Sample processing and data collection**

380 Venepuncture blood samples collected in lithium heparin coated and serum separation tubes
381 were used for isolation of PBMC and serum respectively. PBMC were isolated by density
382 centrifugation using Pancoll human (PAN-Biotech). Isolated PBMC were frozen in foetal

383 bovine serum (FBS) supplemented with 10% DMSO (Sigma Aldrich). Prior to use samples were
384 thawed and washed in PBS. Serum was collected following centrifugation and stored at -80
385 degrees prior to use.

386

387 Clinical and laboratory data including age, gender, symptom status at T0 and SARS-CoV-2 RT-
388 PCR status at T0 were available for all participants.(Table S1)¹

389

390 **Protein expression and purification**

391 Recombinant spike (S) and spike receptor binding domain (RBD) proteins of SARS-CoV-2 for
392 antigen-specific B cell flow cytometry and ELISpot were expressed and purified as previously
393 described.¹⁵ Briefly, spike glycoprotein trimer (uncleaved spike stabilised in the prefusion
394 conformation (GGGG substitution at furin cleavage site and 2P mutation)⁶⁴ and RBD protein¹²
395 were cloned into a pHlsec vector containing Avi and 6xHis tags. Biotinylated Spike and RBD
396 were expressed in Expi293F cells (Thermofisher Scientific). Supernatants were harvested after
397 7 days and purified. For the production of biotinylated protein, spike and RBD encoding
398 plasmids were co-transfected with BirA and PEI-Max in the presence of 200uM biotin.

399

400 Recombinant S1 protein constructs spanning SARS-CoV-2 residues 1-530 for ELISpot were
401 produced as previously described.^{28,30} Briefly, codon-optimised DNA fragments were cloned
402 into mammalian expression vector pQ-3C-2xStrep to create plasmids, which were then
403 transfected into Expi293F cells growing at 37 in 5% CO2 atmosphere using ExpiFectamine
404 reagent (Thermofisher Scientific). Proteins were purified by strep-tag affinity and
405 subsequently size exclusion chromatography.

406

407 **Flow cytometry**

408 High dimensional multiparameter flow cytometry was used for *ex vivo* identification of spike
409 and RBD- specific B cells. Two panels (surface and intranuclear) of monoclonal antibodies
410 (mAbs) were used to phenotype global and antigen specific subsets (Table S2). Biotinylated
411 tetrameric spike (1ug) and RBD (0.5ug) were fluorochrome linked for flow cytometry by
412 incubating with streptavidin conjugated APC (Prozyme) and PE (Prozyme) (Spike), and BV421
413 (Biolegend) (RBD) for 30 minutes in the dark on ice.

414

415 PBMC were thawed and incubated with Live/Dead fixable dead cell stain (UV, ThermoFisher
416 Scientific) and saturating concentrations of phenotyping mAbs (Table S2) diluted in 50%
417 1xPBS 50% Brilliant Violet Buffer (BD Biosciences). For identification of SARS-CoV-2 antigen
418 specific B cells 1ug per 500ul of stain each of tetrameric Spike-APC and Spike-PE and 0.5ug
419 per 500ul stain of tetrameric RBD-BV421 were added to the cell preparation. Parallel samples
420 stained with an identical panel of mAbs, but excluding the SARS-CoV-2 proteins (fluorescence
421 minus one controls (FMO)) were used as controls for non-specific binding.

422

423 Cells were incubated in the staining solution for 30 minutes at room temperature, washed
424 with PBS, and subsequently fixed with either fixation and permeabilization solution (BD
425 Biosciences) or FoxP3 Buffer Set (BD Biosciences) according to the manufacturer's
426 instructions for surface and intranuclear staining respectively. Saturating concentrations of
427 mAbs diluted in 1xPBS were added following permeabilization for the detection of
428 intranuclear proteins. All samples were acquired on a Fortessa-X20 (BD Biosciences) and
429 analysed using FlowJo (TreeStar).

430

431 B cell subsets were defined as follows: MBC - CD19⁺CD20⁺ excluding IgD⁺, CD38^{hi} and
432 CD21⁺CD27⁻ naïve fractions, (gating strategy in Supp.Fig1a), DN2 - CD19⁺CD20⁺CD38[±]-CD21⁻
433 CD27⁻CD11c^{hi}CXCR5^{lo}. For analysis of RBD- co-staining cells sufficient magnitude spike-specific
434 MBC (≥20 dual-spike+ cells) were required. For phenotypic analysis of spike-specific and RBD-
435 specific cells sufficient magnitude responses (≥50 cells in the relevant parent gate) were
436 required.

437

438 **MBC recall response to SARS-CoV-2: ELISpot**

439 To activate MBC differentiation, 1x10⁶ PBMC were stimulated with 1ug/ml R848 (TLR7/8
440 agonist; Resiquimod (Invivogen)) diluted in complete RPMI (cRPMI; RPMI supplemented with
441 10% FBS plus recombinant human IL-2 (20IU/ml; Peprotech), as previously described.^{65,66}
442 Activated cells were incubated for six days with a media change on day three.

443

444 ELISpot plates (Mabtech) were pre-coated with recombinant SARS-CoV-2 trimeric spike
445 (1μg/ml), S1 (1μg/ml) and RBD (10μg/ml) and anti-human IgG (1μg/ml Jackson
446 Immunoresearch) overnight at 4°C. Coated plates were blocked with cRPMI with 10% FBS

447 prior to the addition of cells. Cultured PBMC were added at varying concentrations depending
448 on SARS-CoV-2 antigen and incubated at 37°C at 5% CO₂ for 18 hours: 50,000 cells/ well to
449 detect spike- specific IgG secreting cells; 100,000 cells/well to detect S1 and RBD IgG secreting
450 cells; and 1000 cells/well for detection of total IgG secreting cells. To control for non-specific
451 binding, uncoated control wells were incubated with 100,000 pre-stimulated cells. The
452 following day, ELISpot plates were washed in filtered PBS supplemented with 0.5% Tween 20
453 (Merck) and incubated for four hours in the dark at room temperature with 1ug/ml goat anti-
454 human IgG horse radish peroxidase antibody (Jackson ImmunoResearch). Cells were again
455 washed three times with PBS-Tween 20 (0.5%) and three times with PBS, then developed with
456 3-Amino-9-ethylcarbazole (AEC) substrate (BD Biosciences) according to the manufacturer's
457 instructions. ELISpot plates were washed with ddH₂O before analysis using ViruSpot
458 (Autoimmun Diagnostika). All conditions were performed in duplicate and responses
459 averaged.

460

461 **Data analysis and statistics**

462 Data were analysed using GraphPad Prism. Descriptive statistical analyses were performed.
463 Continuous data that did not follow a normal distribution were described as medians with
464 interquartile ranges and differences compared using the Mann-Whitney U test, Wilcoxon's
465 paired *t*-test or Kruskal Wallis test with Dunn's post hoc test for pairwise multiple
466 comparisons as appropriate. Contingency table analyses were conducted using Fisher's exact
467 test. Correlations for non-parametric data were assessed using Spearman's rank correlation
468 with 95% CI.

469

470 **Supplementary Materials**

471 **Fig. S1. Gating strategy and threshold for detection of spike-specific responses**

472 **Fig. S2. Phenotyping of spike-specific and global MBC**

473 **Fig. S3. Representative ELISpot responses to SARS-CoV-2 proteins**

474 **Table S1. Cohort Characteristics**

475 **Table S2. Antibody list**

476

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677

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699 Conceptualization: AJS, MZ, LEM, MKM; Methodology: AJS, ARB, LM, KJD, SNL, LEM, MKM;

700 Investigation: AJS, ARB, SL, MP, RG, CRS, LEM; Sample and clinical data acquisition: AJS, FA,

701 SNL, JYC; Analysis: AJS, ARB, SL, MP, RG, LEM, MKM; Funding acquisition: SNL, JYC, MZ, MKM;

702 Supervision: SNL, MZ, LEM, MKM; Writing – original draft: AJS, MKM; Writing – review &

703 editing: All authors

704

705 **Competing interests**

706 None declared

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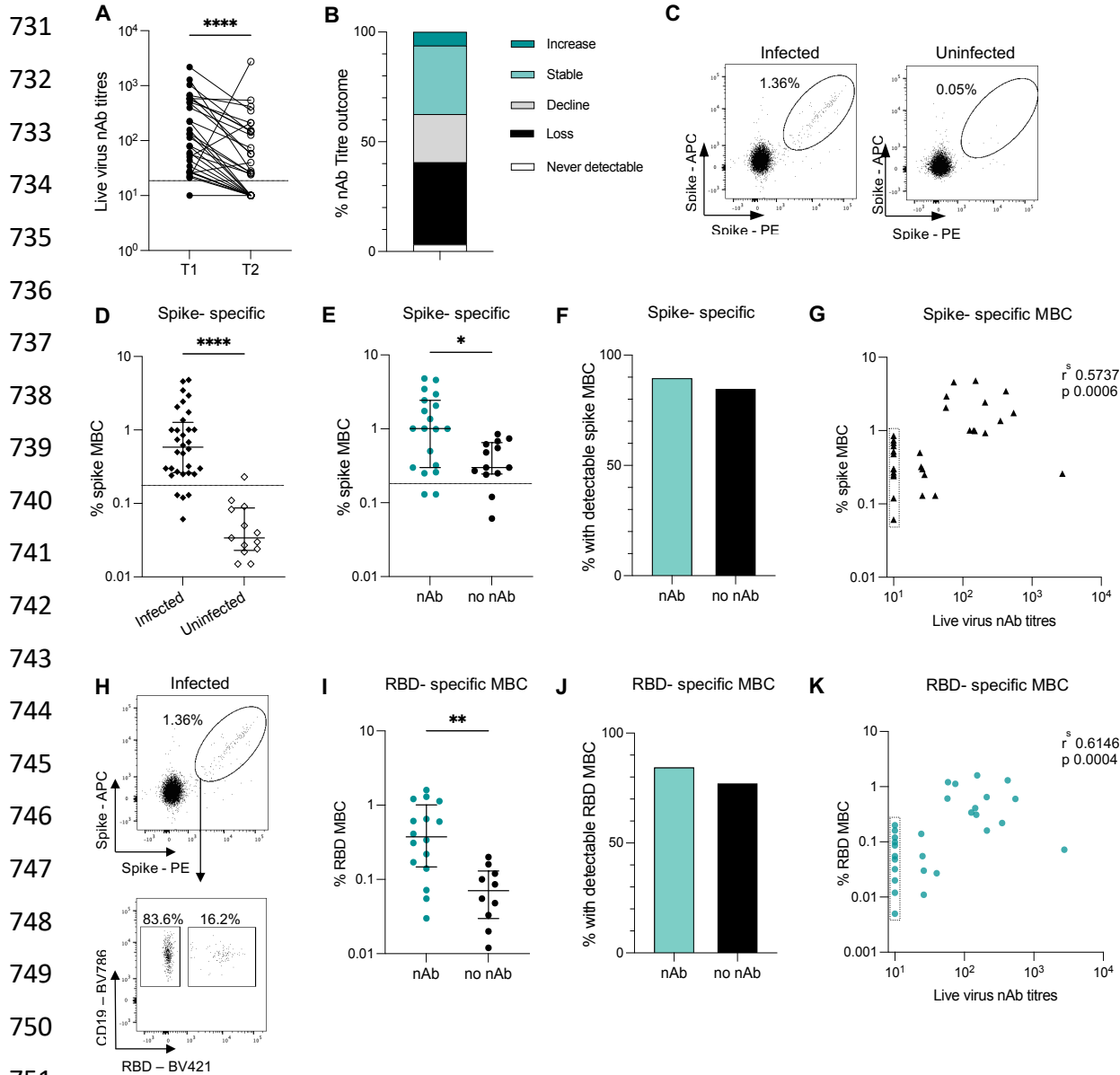
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728 **Figures**

729 **Figure 1.**

730



752 **Spike and RBD-specific memory B cells persist five months post-SARS-CoV-2 infection**
 753 **despite waning neutralising antibodies**

754 **(A)** Paired live virus nAb titres (nAb) at T1 and T2 of individuals infected prior to T1 (n=32).

755 **(B)** Proportion of infected individuals with change in nAb indicated between T1 and T2:

756 increase = ≥ 4 fold rise in nAb; static = >4 fold decrease <4 fold increase; decline ≥ 4 fold

757 decrease; loss = no detectable nAb titres at T2 from detectable nAb at T1; never detectable

758 = absence of nAb titres at T1 and T2 (n=32). **(C)** Representative FACS plots of dual staining

759 with SARS-CoV-2 spike tetramers on MBC (CD3-CD14-CD19+CD20+CD38(+/-) IgD- excluding
760 naïve (CD21+CD27-)) for previously infected (left) and uninfected (right) individuals. **(D, E)**
761 Frequency of dual spike-specific MBC **(D)** in infected (n=32) and uninfected (n=13) and **(E)** in
762 infected individuals with (nAb, n=19) and without (no nAb, n=13) detectable nAb at T2.
763 Dashed lines indicate threshold for spike-specific responses determined by uninfected
764 controls (Supplementary figure 1b). **(F)** Proportion of infected individuals with detectable
765 spike-MBC above the threshold stratified by presence (nAb, n=19) and absence (no nAb,
766 n=13) of detectable nAb at T2. **(G)** Correlation between frequency of spike MBC and live
767 virus nAb titres in infected individuals (n=32). **(H)** Representative FACS plots of dual staining
768 with SARS-CoV-2 spike tetramers on MBC (top panel) and RBD tetramer on dual spike
769 specific cells (lower panel) of an infected individual. Minimum number of cells in spike-
770 specific gate required for RBD probe analysis = 20 **(I)** Frequency of RBD- specific MBC in
771 infected individuals with detectable spike-specific responses stratified by presence (nAb,
772 n=16) and absence (no nAb, n=10) detectable nAb at T2. **(J)** Proportion of infected
773 individuals with detectable RBD MBC stratified by presence (nAb, n=16) and absence (no
774 nAb, n=10) of detectable nAb at T2. **(K)** Correlation between frequency of RBD MBC and live
775 virus nAb titres in all infected individuals (n=29). **(A)** Wilcoxon matched pairs, $p \leq 0.0001$. **(D,**
776 **E, I)** Bars indicate median and interquartile range; Mann Whitney U Test; **(D)** $p \leq 0.0001$, **(E)**
777 $p=0.0114$, **(I)** $p=0.0012$. **(F, J)** Fisher's exact test ; **(F)** $p= 0.6285$, **(J)** $p= 0.6664$. **(G, K)** Dotted
778 box indicates individuals with discordant MBC and nAb response. Spearman's rank
779 correlation. nAb, neutralising antibody; MBC, memory B cell, RBD, receptor binding domain.
780 Analysis of RBD specific MBC only in those with ≥ 20 cells in spike-specific gate.

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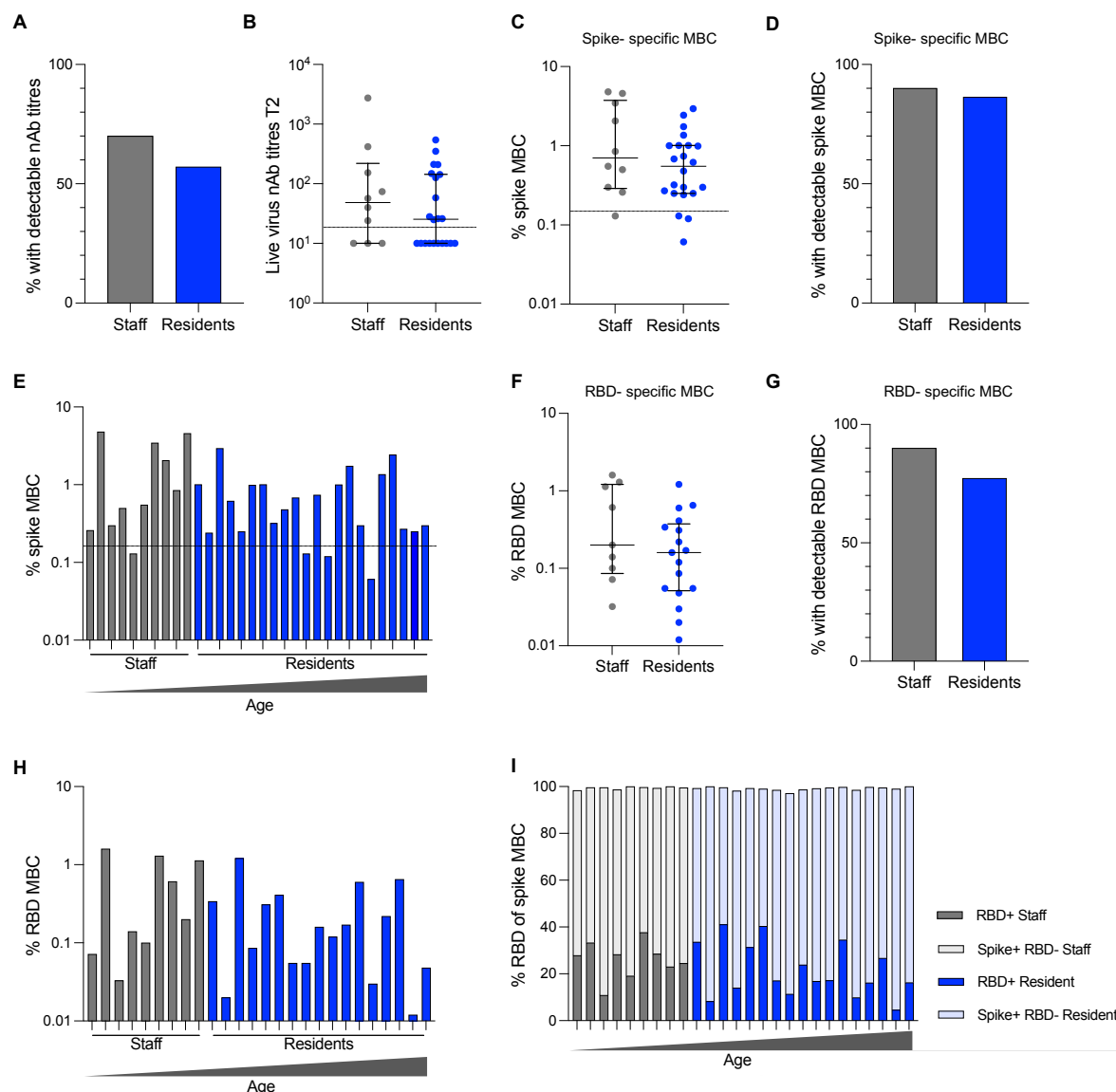
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791 **Figure 2.**

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794 **Comparable persistence of spike and RBD-specific memory B cells in elderly care home**
 795 **residents and younger staff**

796 **(A)** Proportion of staff (n= 10) and residents (n=21) with detectable nAb at T1 who
 797 continued to have detectable nAb at T2. **(B)** nAb titres at T2 for all infected individuals
 798 stratified by staff (n=10) and residents (n=22). Dashed line indicates assay threshold for
 799 detection, undetectable titres assigned a value of 10. **(C)** Frequency of dual spike-specific
 800 MBC in staff (n=10) and residents (n=22). **(D)** Proportion of infected individuals with
 801 detectable spike-MBC stratified by staff (n=10) and resident (n=22) status. **(E)** Frequency of
 802 dual spike-specific MBC for staff (grey) and residents (blue) ordered by age from youngest

803 on the left to oldest on the right. **(F)** Frequency of RBD- specific MBC in staff (n=9) and
804 residents (n=17) with detectable spike specific responses. **(G)** Proportion of infected
805 individuals with detectable RBD MBC stratified by staff (n=10) and resident (n=22) status.
806 **(H)** Frequency of RBD-specific MBC for staff (grey) and residents (blue) ordered by age from
807 youngest on the left to oldest on the right. **(I)** Proportion of dual spike specific cells with
808 specificity for RBD (staff= dark grey; residents = dark blue), or non-RBD region (staff = pale
809 grey; residents = pale blue) in staff (n=9) and residents (n=17). **(A, D, G)** Fisher's exact test;
810 **(A)** $p > 0.9999$, **(D)** $p > 0.9999$, **(G)** $p = 0.6367$. **(B, C, F)** Bars indicate median and interquartile
811 range; Mann Whitney U test; **(B)** $p = 0.4367$, **(C)** $p = 0.2552$, **(F)** $p = 0.2359$. **(C, E)** Dashed line
812 indicates threshold for spike-specific responses determined by uninfected controls
813 (Supplementary figure 1b).

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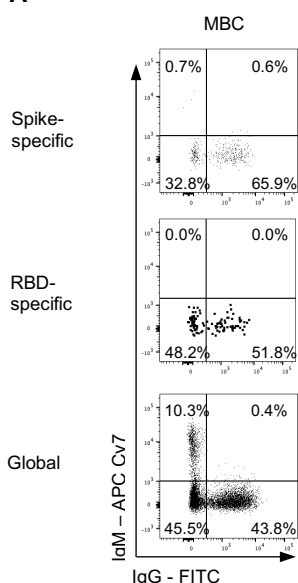
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835 **Figure 3.**

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837 **A**



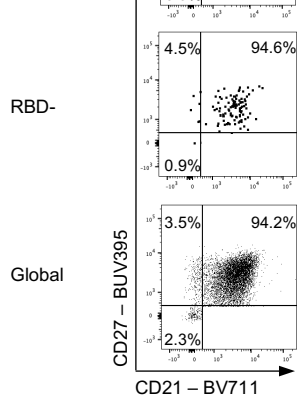
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848 **D**



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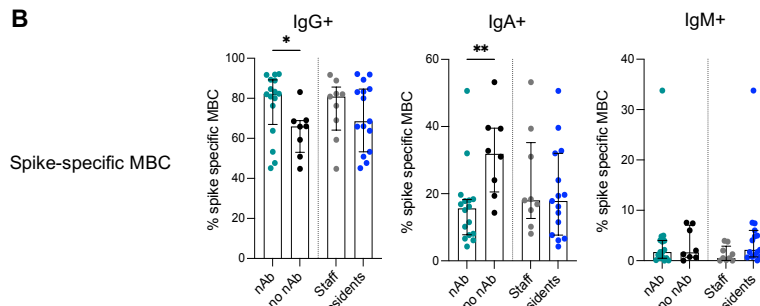
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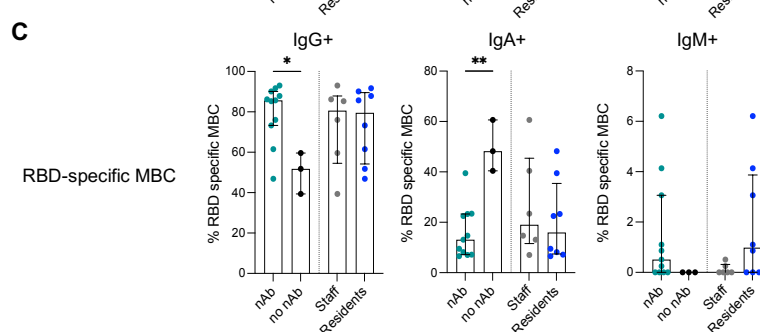
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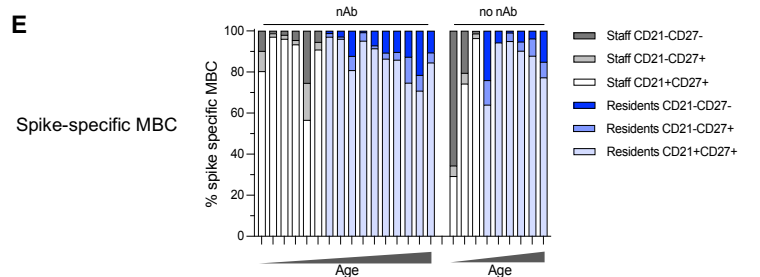
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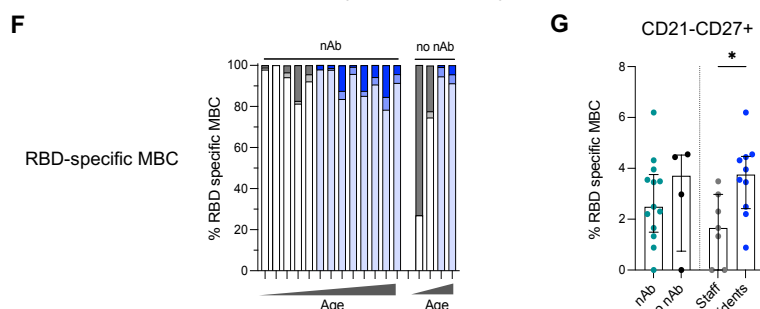
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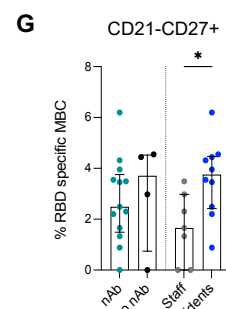
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859 **Preserved memory phenotype but skewed isotype of spike/RBD-specific B cells with loss**
860 **of neutralising antibodies**

861 (A) Representative FACS plots of IgM and IgG on spike-specific (top panel), RBD-specific
862 (middle panel), and global (bottom panel) CD19+CD20+CD38lo/neg IgD- MBC from an
863 infected individual. (B) Frequency of IgG+, IgA+ (denoted by IgD-, IgG-, IgM-) and IgM+ spike-
864 specific MBC stratified by presence (nAb, n=16) and absence (no nAb, n=8) of detectable
865 nAb at T2, and by staff (grey, n=9) and resident (blue, n=15) status. (C) Frequency of IgG+,
866 IgA+ (denoted by IgD-, IgG-, IgM-) and IgM+ RBD-specific MBC stratified by stratified by

867 presence (nAb, n=11) and absence (no nAb, n=3) of detectable nAb at T2, and by staff (grey,
868 n=6) and resident (blue, n=8) status. **(D)** Representative FACS plots of CD21 and CD27 gating
869 on spike-specific (top panel), RBD- specific (middle panel), and global (bottom panel)
870 CD19+CD20+CD38lo/neg IgD-MBC from an infected individual. **(E)** Frequency of CD21-
871 CD27+, CD21+CD27+ and CD21-CD27- MBC subsets of spike- specific MBC stratified by
872 presence (nAb, n=16) and absence (no nAb, n=9) of detectable nAb at T2 ordered by
873 increasing age. **(F)** Frequency of CD21-CD27+, CD21+CD27+ and CD21-CD27- MBC subsets of
874 RBD- specific MBC stratified by presence (nAb, n=13) and absence (no nAb, n=4) of
875 detectable nAb at T2 ordered by increasing age. **(G)** Frequency of CD21-CD27+ RBD- specific
876 MBC stratified by presence (nAb, n=13) and absence (no nAb, n=4) of detectable nAb at T2,
877 and by staff (grey, n=7) and resident (blue, n=10) status. **(B, C, G)** Bars indicate median and
878 interquartile range; Mann Whitney U test; **(B)** IgG p=0.0382; ns, IgA p=0.0045; ns, IgM ns;
879 ns; **(C)** IgG p=0.0220; ns, IgA p=0.0055; ns, IgM ns; ns; **(G)** ns; 0.0180.
880 Analysis of individuals ≥ 50 cells in the relevant parent gate for all phenotypic analysis.

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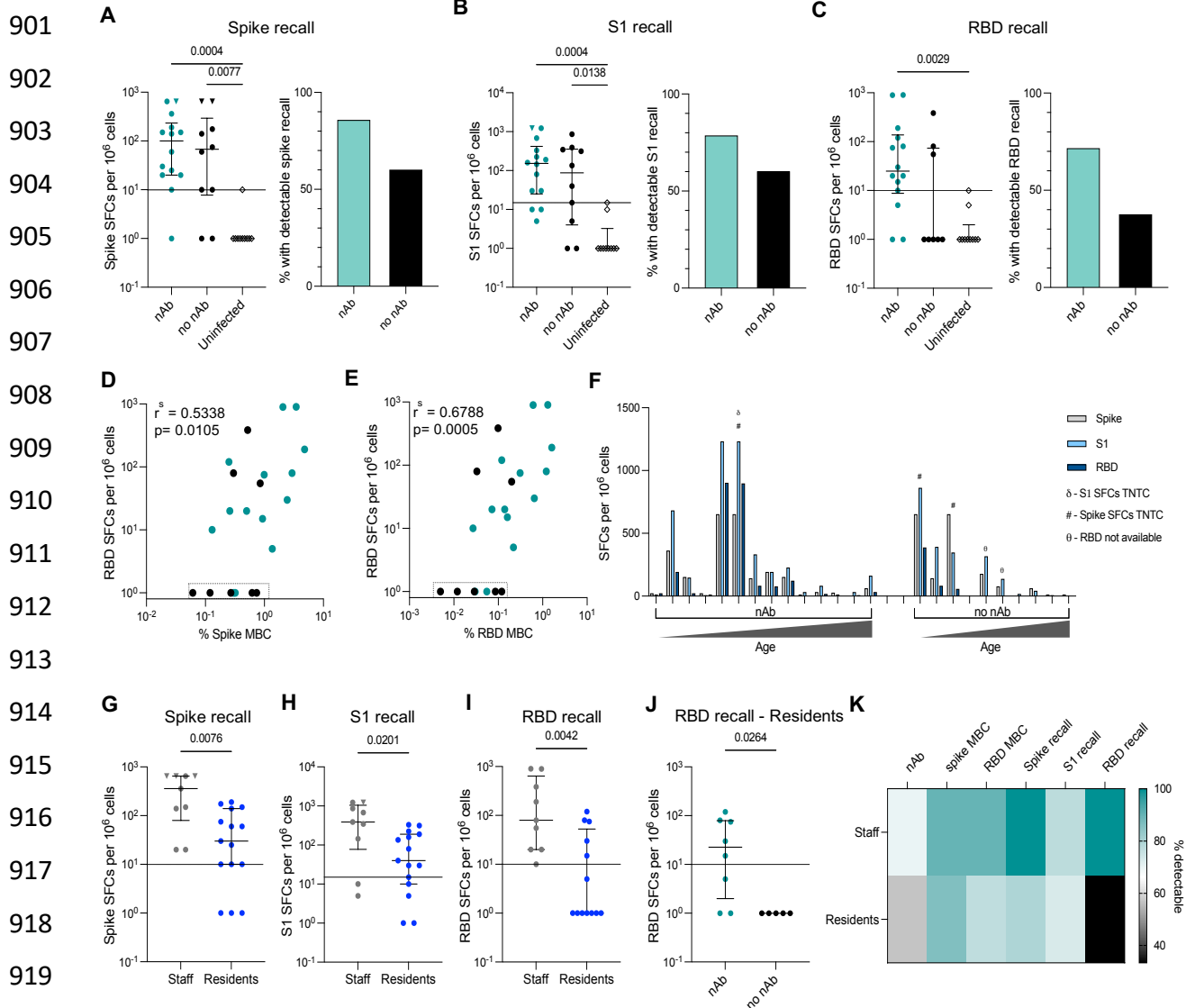
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899 **Figure 4.**

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921 **Elderly maintain some functional spike/RBD-specific B cells at reduced frequency**

922 **compared to younger care home staff**

923 **(A, B, C)** Left panels: SFCs per 1 million PBMC for infected individuals stratified by presence
 924 (nAb) and absence (no nAb) of detectable nAb at T2 and for uninfected controls. Right
 925 panel: proportion of infected individuals stratified by presence (nAb) and absence (no nAb)
 926 of detectable nAb at T2 with detectable recall responses. **(A)** Spike protein (nAb n=14, no
 927 nAb n=10, uninfected n=10), **(B)** S1 protein (nAb n=14, no nAb n=10, uninfected n=10), **(C)**
 928 RBD protein (nAb n=14, no nAb n=8, uninfected n=10). **(D, E)** Correlation between SFCs per
 929 10⁶ PBMC to SARS-CoV-2 RBD protein and **(D)** frequency of spike specific MBC, **(E)** frequency
 930 of RBD positive MBC for those with nAb (green) and without nAb (black). **(F)** SFCs per 1

931 million PBMC to Spike (grey), S1 (pale blue), and RBD (dark blue) per individual stratified by
932 presence (nAb, n=14) and absence (no nAb, n=10) of detectable nAb at T2 ordered by
933 increasing age. **(G, H, I)** SFCs per 1 million PBMC for infected individuals stratified by staff
934 and resident status, **(G)** Spike protein (staff n=9, resident n=15), **(H)** S1 protein (staff n=9,
935 resident n=15, **(I)** RBD protein (staff n=9, resident n=13). **(J)** SFCs per 1 million PBMC to RBD
936 protein for infected residents stratified by presence (nAb, n=8) and absence (no nAb, n=5) of
937 detectable nAb at T2. **(K)** Summary heatmap of proportion of staff and residents with nAb
938 titres detectable at T2, spike- and RBD- specific MBC by flow cytometry, and spike, S1 and
939 RBD recall by ELISpot. **(A, B, C)** Left panels: Bars indicated median and interquartile range,
940 dashed line indicates threshold indicated by seronegative and pre-pandemic controls.
941 Kruskal Wallis multiple comparison ANOVA with Dunn's correction, significance as indicated.
942 **(A, B, C)** Right panels: Fisher's exact test **(A)** p= 0.3413, **(B)** p= 0.3926, **(C)** p= 0.1870. **(D, E)**
943 Dotted box indicates individuals with discordant MBC and ELISpot response. Spearman's
944 rank correlation. **(G, H, I, J)** Bars indicated median and interquartile range, dashed line
945 indicates threshold indicated by seronegative and pre-pandemic controls. Mann Whitney U
946 Test, significance as indicated.
947 **(A, B, G, H)** Inverted triangle: individuals where the responses were TNTC, these individuals
948 have been assigned the maximal response observed. **(F)** δ : SFCs TNTC in response to S1; #:
949 SFCs were too numerous to count in response to Spike. For these individuals values have
950 been assigned the maximum response observed on the plate for analyses. θ : RBD counts
951 unavailable.
952 Individuals with a zero response to any antigen have been assigned a value of 1 to allow
953 plots to be drawn on a logarithmic scale. All statistical analysis performed using original
954 values. SFC, spot forming cells; RBD, Receptor binding domain; MBC, memory B cells, TNTC,
955 too numerous to count.
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