1 **Longitudinal analysis of humoral immunity against SARS-CoV-2 Spike in convalescent**
2 **individuals up to 8 months post-symptom onset**
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27 **Key Words:** Coronavirus, COVID-19, SARS-CoV-2, Spike glycoproteins, RBD, Antibodies,
28 Serology, Memory B cells, Humoral responses, ADCC, Neutralization, Convalescent plasma

29 **Abstract**

30 Functional and lasting immune responses to the novel coronavirus (SARS-CoV-2) are currently

31 under intense investigation as antibody titers in plasma have been shown to decline during

32 convalescence. Since the absence of antibodies does not equate to absence of immune memory,

33 we sought to determine the presence of SARS-CoV-2-specific memory B cells in COVID-19

34 convalescent patients. In this study, we report on the evolution of the overall humoral immune

35 responses on 101 blood samples obtained from 32 COVID-19 convalescent patients between 16

36 and 233 days post-symptom onset. Our observations indicate that anti-Spike and anti-RBD IgM

37 in plasma decay rapidly, whereas the reduction of IgG is less prominent. Neutralizing activity in

38 convalescent plasma declines rapidly compared to Fc-effector functions. Concomitantly, the

39 frequencies of RBD-specific IgM+ B cells wane significantly when compared to RBD-specific IgG+

40 B cells, which increase over time, and the number of IgG+ memory B cells which remain stable

41 thereafter for up to 8 months after symptoms onset. With the recent approval of highly effective

42 vaccines for COVID-19, data on the persistence of immune responses are of central importance.

43 Even though overall circulating SARS-CoV-2 Spike-specific antibodies contract over time during

44 convalescence, we demonstrate that RBD-specific B cells increase and persist up to 8 months

45 post symptom onset. We also observe modest increases in RBD-specific IgG+ memory B cells

46 and importantly, detectable IgG and sustained Fc-effector activity in plasma over the 8-month

47 period. Our results add to the current understanding of immune memory following SARS-CoV-2

48 infection, which is critical for the prevention of secondary infections, vaccine efficacy and herd

49 immunity against COVID-19.

50 **Introduction**

51 Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of the

52 ongoing Coronavirus disease 2019 (COVID-19) pandemic, is highly contagious and has infected

53 close to a 100 million people worldwide and caused over 2 million deaths since its discovery. The

54 dynamics and persistence of immune responses in individuals infected with SARS-CoV-2 is

55 currently under needful investigation. Several studies with acute and convalescent COVID-19

56 patients have showed prompt induction of B and T cell responses upon infection, along with the

57 detection of antigen-specific memory B and T cell responses several weeks into convalescence

58 (1-6). Additionally, antibodies induced upon infection have been shown to protect from SARS-

59 CoV-2 reinfection in animal models (7-9). Passive immunization using neutralizing monoclonal

60 antibody treatments decreased viral loads in animal studies and in patients with COVID-19 (10,

61 11). The viral target of neutralizing antibodies is the highly immunogenic trimeric Spike (S)

62 glycoprotein, which facilitates SARS-CoV-2 entry into host cells via its receptor-binding domain

63 (RBD) that interacts with angiotensin-converting enzyme 2 (ACE-2) (12, 13).

64

65 The evolution of overall antibody responses in convalescent individuals is being extensively

66 analysed, with studies showing that Ab titers and neutralization activity against Spike start

67 decreasing during the first weeks after resolution of infection (5, 6, 14-18). Importantly, in addition

68 to neutralizing viral particles, the antiviral activities of SARS-CoV-2-specific antibodies can

69 expand to involve Fc-effector functions, including antibody-dependent cellular cytotoxicity (ADCC)

70 (19, 20). Recent research has highlighted the importance of humoral development and the ability

71 of antibodies to carry out Fc-effector functions in decreasing mortality of patients exhibiting severe

72 disease symptoms (21).

73

74 In this study, we dissect multiple aspects of humoral immunity, including Fc-effector functions and

75 antigen-specific B cells, longitudinally for up to 8 months post symptom onset (PSO) in 32

76 convalescent individuals. Our findings aid in the understanding of durability of COVID-19

77 immunity, which is important in the context of secondary infections, vaccine efficacy and herd

78 immunity.

79

80 **Results**

81 **SARS-CoV-2 RBD-specific and Spike-specific antibody levels in convalescent plasma**

82 **decrease up to 8 months post-symptom onset.**

83 To monitor the evolution of antibody responses longitudinally, we analyzed serological samples

84 from 32 convalescent individuals (Table 1) along with 10 pre-pandemic samples from uninfected

85 individuals as experimental controls. The average age of the donors was 47 years old (range: 20-

86 65 years), and samples were from 17 males and 15 females. Convalescent patients were sampled

87 at four longitudinal time points between 16 and 233 days PSO: 6 weeks (16-95 days; median: 43

88 days), 11 weeks (48-127 days; median: 77 days), 21 weeks (116-171 days; median: 145 days),

89 and 31 weeks (201-233 days; median: 218 days). Participants were tested positive for SARS-

90 CoV-2 infection by reverse transcription PCR (RT-PCR) on nasopharyngeal swab specimens.

91 Convalescent participants were enrolled following two negative RT-PCR tests and a complete

92 resolution of symptoms for at least 14 days before blood sampling.

93

94 We began by evaluating the presence of RBD-specific IgG, IgM, and IgA antibodies by using a

95 previously published enzyme-linked immunosorbent assay (ELISA) against the SARS-CoV-2

96 RBD antigen (16). In agreement with recent reports showing the waning of antibody levels in

97 longitudinal convalescent plasma over time (14-17), we observed that total RBD-specific

98 immunoglobulin (Ig) levels, comprising of IgG, IgM, and IgA, gradually decreased between 6 and

99 31 weeks after the onset of symptoms (Figure 1A). However, the percentage of convalescent

100 individuals presenting detectable RBD-specific Ig levels remained stable, with a consistent

101 seropositivity rate above 90% throughout the sampling time frame. Notably, 100% of the donors

4

102 still had detectable IgG at the last time point, while IgM and IgA diminished more rapidly, with

103 85% and 69% of the donors having undetectable IgM and IgA levels, respectively, 31 weeks PSO

104 (Figure 1B-D; Figure S1A). Since the RBD ELISA is limited to detect antibodies targeting only one

105 domain of the Spike, we developed a high-throughput cell-based ELISA methodology to screen

106 for antibodies recognizing the native full-length S protein on the cell surface. HOS cells stably

107 expressing the SARS-CoV-2 S glycoproteins were incubated with plasma samples, followed by

108 the addition of secondary antibodies recognizing IgG, IgM, and/or IgA. We observed that 100%

109 of the donors still had detectable S-specific total Ig and IgG in their plasma at 31 weeks PSO

110 whereas, only 38% and 54% of the plasma samples tested positive for the presence of Spike-

111 specific IgM and IgA, respectively (Figure 1 E-H; Figure S1B). We confirmed this observation

112 using a recently characterized flow-cytometry based assay (22) determining antibody binding to

113 the full-length S protein on the surface of 293T cells (Figure S2A). The data obtained with both

114 the cell-based ELISA and flow-cytometry techniques correlated significantly (r = 0.8120;

115 p<0.0001) (Figure S2B).

116

117 **Neutralizing and Fc-effector activities of antibodies present in convalescent plasma**

118 **decrease at different rates over time.**

119 Recent studies have shown the importance of neutralizing antibodies in reducing viral load and

120 preventing infection in animal models (10, 23, 24). Neutralizing monoclonal antibody cocktails

121 also reduced viral load in COVID-19 patients (11). Neutralizing activity is often considered as a

122 determining factor in convalescent plasma therapy, although its relative importance compared to

123 Fc-effector activity is still unknown (25-27). Thus, we measured the capacity of convalescent

124 plasma to neutralize pseudoviral particles carrying the SARS-CoV-2 Spike protein over time.

125 Neutralizing antibody titers (ID_{50}) were detected in 63% of the donors at 6 weeks PSO, while none

126 of the uninfected controls had detectable neutralizing activity. Titers declined from 155.6 at 6

127 weeks to 60.0 at 31 weeks PSO, respectively, with 77% of donors having undetectable

128   neutralization activity in their plasma at the last time point (Figure 2A). Since the depletion of IgM

129   from plasma has been associated with loss of viral neutralization capacity (28, 29) and IgA has

130   also been shown to dominate the early neutralizing response (30, 31), the sharp decline in

131   neutralization activity seen in this study is corroborated by the striking decrease in anti-Spike and

132   anti-RBD IgM and IgA levels (Figure 1B, D, F, H; Figure S1A, B).

133

134   Fc-mediated effector functions of antibodies can contribute to the efficacy of immune response

135   against SARS-CoV-2. Recent studies have examined Fc-mediated effector functions of

136   antibodies elicited upon SARS-CoV-2 infection (19, 32). Both the presence of IgG and their Fc-

137   mediated effector activities have been linked to reduced severity of disease (21). Herein, we

138   assessed the ability of plasma from convalescent donors to trigger ADCC responses over time.

139   We developed a new ADCC assay using a human T-lymphoid cell line resistant to NK cell-

140   mediated cell lysis (CEM.NKr) and stably expressing the full-length S protein on the cell surface

141   as target cells. PBMCs from healthy individuals were used as effector cells. ADCC activity was

142   measured by the loss of Spike-expressing GFP+ target cells (Figure S3). We observed that ADCC

143   activity of convalescent plasma decreased gradually between 6 weeks and 31 weeks PSO (Figure

144   2B). However, this decline was modest when compared to the decrease in neutralization activity

145   of plasma (Figure S1C) and 85% of the donors' plasma still elicited substantial ADCC activity at

146   the latest study time point. The presence of Fc-mediated antibody effector functions up to 8

147   months PSO is corroborated with the presence of significant IgG levels.

148

149   **RBD-specific memory B cells develop and remain stable up to 8 months post-symptom**

150   **onset.**

151   Recent studies on convalescent COVID-19 patients have indicated persistent antigen-specific

152   memory B cell responses despite waning antibody levels (5, 6). To monitor the circulating B cell

153   compartment in our cohort of convalescent individuals, antigen-specific B cells were characterized

154   by flow cytometry (identified as CD19+ CD20+). To identify distinct RBD-specific B cells, we used

155   double discrimination with two recombinant RBD protein preparations labelled with fluorochromes

156   Alexa-Fluor 594 and Alexa-Fluor 488, respectively. Detection of this double positive population

157   was specific since it was not detected in PBMCs from uninfected individuals (Figure S4A, B).

158   RBD-specific B cells were detected 6 weeks PSO with a modest increase in mean frequency up

159   to 31 weeks PSO (0.038% to 0.051%) (Figure 3B). Total RBD-specific B cells were evaluated to

160   distinguish surface Ig isotypes and we observed IgG+, IgM+ and IgA+ cells in 100%, 92% and

161   83% of donors at 6 weeks PSO, respectively. Strikingly, the frequency of RBD-specific IgM+ B

162   cells decreased significantly, with 46% of the donors having undetectable IgM+ B cells 31 weeks

163   PSO (Figure 3C; Figure S1D). Conversely, the frequency of RBD-specific IgG+ B cells

164   significantly increased between 6 and 21 weeks PSO and remained stable up to 8 months PSO

165   (Figure 3D); the detection of RBD-specific IgA+ B cells also persisted in 77% of the donors tested

166   (Figure 3E; Figure S1D). Furthermore, the total RBD-specific B cells were evaluated to distinguish

167   memory and naïve B cells (identified by using CD27 and CD21 markers; Figure S4C, D).

168   Importantly, total RBD-specific memory B cells were detected in 100% of the donors and the

169   mean frequency remained stable between 6 and 31 weeks PSO (0.020% to 0.026%), while RBD-

170   specific naïve B cells were observed in lower proportions and modestly decreased over time

171   (Figure 3F, G). Interestingly, the proportion of donors positive for RBD-specific naïve B cells was

172   reduced by half between the first and last time point as also observed for RBD-specific IgM+ B

173   cells which is consistent with the decline in IgM levels observed between these groups. IgG+

174   RBD-specific memory B cells were detected in 100% of the donors and the frequency of this

175   population modestly increased up to 31 weeks PSO (Figure 3H). Meanwhile, the frequencies of

176   IgA+ RBD-specific memory B cells were low but stable over the 8-month period (Figure 3G, I).

177   **Evaluation of the relationship between different aspects of humoral immunity reveals the**

178   **importance of IgG responses.**

179   To examine interrelations between serological, immunological, and functional parameters

180   assessed in this study, we performed comprehensive sets of correlation analyses. Firstly, at each

181   time point, the strongest immune response clusters were comprised of total Ig and IgG binding

182   levels and thus, increased ADCC activity (Figure 4A, left panel). In contrast, weaker immune

183   response clusters in plasma were formed by IgA and IgM levels and subsequently, neutralization

184   capacities. Overall, the responses decreased over the course of the 8-month period (Figure 4B).

185   The inverse was observed with antigen-specific B cell frequencies (Figure 4B). Apart from naïve

186   B cells and the IgM fraction thereof, total RBD-specific B cells increased over the course of 8

187   months. At each time point, total IgG+ B cells and memory B cells (IgA+ and IgG+) constituted

188   the most prominent responses (Figure 4A, right panel).

189

190   Additionally, at the earliest time point convalescent plasma was collected after symptom onset (6

191   weeks PSO), we observed a vast network of strong positive correlations among   B cell

192   populations, antibody levels, and antibody-mediated functional parameters (Figure 5B).

193   Intriguingly, this was followed by a striking disconnect of associations between B cell frequencies

194   and serological parameters at 11, 21, and 31 weeks PSO (Figure 5C-E). This is caused by the

195   concomitant diminution of antibody levels, yet stabilization of antigen-specific B cells observed at

196   these time points, suggestive of decreased antibody production by B cells after resolution of

197   infection or the gradual replacement of Ig-secreting short-lived plasma cell by memory B cells.

198   When considering the collective datasets, prominent features were two subsequently dividing

199   positive correlation clusters, the first among total and IgG+ RBD-specific B cells and the second

200   among ADCC with total Ig and IgG binding responses. Furthermore, days PSO inversely

201   correlated with neutralization as well as IgM and IgA-specific responses (Figure 5A and S5).

Discussion

202

203 A better understanding of the type and longevity of immune responses following viral infections

204 are critical to reveal immune mechanisms involved in protection from re-infection and protection

205 by vaccination. Data regarding these important issues continues to be gathered for SARS-CoV-

206 2. In this study, we have contributed to the current understanding by reporting on the evolution of

207 the overall humoral immune responses in 101 blood samples obtained from 32 COVID-19

208 convalescent patients between 16 and 233 days PSO. Overall, we observed that IgM levels and

209 the neutralizing capacity of plasma decreases rapidly, whereas IgG and Fc-effector activity are

210 more sustained (Figure 6). The antibody kinetics we observed herein are typical of those seen for

211 other human coronavirus infections, with antibodies peaking 2-4 weeks PSO followed by a

212 contraction phase (33). Furthermore, we show that COVID-19 patients generate RBD-specific

213 memory B cells and IgG+ B memory cells that persist for over 8 months. Similarly, recent studies

214 on the durability of SARS-CoV-2 immune responses have shown that although antibody levels

215 decrease, Spike-specific IgG+ memory B cell responses are generated and maintained (1, 5, 6).

216 Additionally, studies demonstrated enhanced cellular immunity that protects non-human primates

217 from SARS-CoV-2 reinfection in the context of waning neutralizing antibodies (8). Thus, the

218 decline of antibody levels does not negate the protective potential because of the importance of

219 cellular responses against SARS-CoV-2 infection. This understanding is corroborated with a

220 recent risk assessment carried out in a cohort of 43,000 convalescent individuals demonstrating

221 that immunity elicited upon natural SARS-CoV-2 infection protects against reinfection with an

222 efficacy of >90% for at least 7 months (34).

223

224 Fc-mediated effector activity of antibodies has been recently shown to correlate with reduced

225 disease severity and mortality (21). Antibodies capable of mediating Fc-dependant functions,

226 such as antibody-dependent phagocytosis and ADCC, have been isolated from convalescent

227 donors (35). Importantly, Fc-mediated effector activity was shown to protect from SARS-CoV-2

9

228   infection in adapted mice and hamster models (9, 19, 32). Thus, our observations of the

229   persistence of ADCC responses in convalescent plasma up to 8 months PSO suggest that Fc-

230   mediated effector functions could play a vital role in protection from reinfection. Nevertheless, the

231   heterogeneity of immune responses between individuals is and will be an important factor when

232   evaluating the efficacy of immune responses upon re-exposure to SARS-CoV-2.

233

234   A recent study comparing humoral immunity generated by mRNA vaccinees (mRNA-1273 or

235   BNT162b2) and individuals recovered from natural infection observed similarities in antibody

236   binding titers and plasma neutralization capacity (36). Furthermore, this study also observed

237   similar frequencies of RBD-specific memory B cells between vaccinees and infected individuals.

238   Therefore, our results on the persistence of RBD-specific memory B cells up to 8 months after

239   natural infection is reassuring with regards to long-term vaccine efficacy.

240 **Material and Methods**

241 **Ethics Statement**

242 All work was conducted in accordance with the Declaration of Helsinki in terms of informed

243 consent and approval by an appropriate institutional board. Convalescent plasmas were obtained

244 from donors who consented to participate in this research project at CHUM (19.381). The donors

245 met all donor eligibility criteria: previous confirmed COVID-19 infection and complete resolution

246 of symptoms for at least 14 days.

247

248 **Plasma and antibodies**

249 Plasma from SARS-CoV-2-infected and pre-pandemic uninfected donors were collected, heat-

250 inactivated for 1h at 56°C and stored at -80°C until ready to use in subsequent experiments.

251 Plasma from uninfected donors were used as negative controls and used to calculate the

252 seropositivity threshold in our ELISA, cell-based ELISA, and flow cytometry assays. The RBD-

253 specific monoclonal antibody CR3022 was used as a positive control in our ELISAs, cell-based

254 ELISAs, and flow cytometry assays and was previously described (14, 16, 22). Horseradish

255 peroxidase (HRP)-conjugated antibodies able to detect all Ig isotypes (anti-human IgM+IgG+IgA;

256 Jackson ImmunoResearch Laboratories, Inc.) or specific for the Fc region of human IgG

257 (Invitrogen), the Fc region of human IgM (Jackson ImmunoResearch Laboratories, inc.) or the Fc

258 region of human IgA (Jackson ImmunoResearch Laboratories, inc) were used as secondary

259 antibodies to detect antibody binding in ELISA and cell-based ELISA experiments. Alexa Fluor-

260 647-conjugated goat anti-human Abs able to detect all Ig isotypes (anti-human IgM+IgG+IgA;

261 Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies to detect

262 plasma binding in flow cytometry experiments.

263

264 **Cell lines**

265   293T human embryonic kidney cells (obtained from ATCC) were maintained at 37°C under 5%

266   CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Wisent) containing 5% fetal bovine serum

267   (VWR) and 100 µg/ml penicillin-streptomycin (Wisent). 293T-ACE2 and 293T-SARS-CoV-2 Spike

268   cell lines were previously reported (16). For the generation of HOS and CEM.NKr CCR5+ cells

269   stably expressing the SARS-CoV-2 Spike glycoproteins, transgenic lentiviruses were produced in

270   293T using a third-generation lentiviral vector system. Briefly, 293T cells were co-transfected with

271   two packaging plasmids (pLP1 and pLP2), an envelope plasmid (pSVCMV-IN-VSV-G) and a

272   lentiviral transfer plasmid coding for a GFP-tagged SARS-CoV-2 Spike (pLV-SARS-CoV-2 S C-

273   GFPSpark tag) (Sino Biological). Supernatant containing lentiviral particles was used to transduce

274   HOS and CEM.NKr CCR5+ cells in presence of 5µg/mL polybrene. The HOS and CEM.NKr

275   CCR5+ cells stably expressing SARS-CoV-2 Spike (GFP+) were sorted by flow cytometry.

276

277   **Protein expression and purification**

278   FreeStyle 293F cells (Invitrogen) were grown in FreeStyle 293F medium (Invitrogen) to a density

279   of 1×10^6 cells/mL at 37°C with 8% CO_2 with regular agitation (150 rpm). Cells were transfected

280   with a plasmid coding for SARS-CoV-2 S RBD using ExpiFectamine 293 transfection reagent, as

281   directed by the manufacturer (Invitrogen). One week later, cells were pelleted and discarded.

282   Supernatants were filtered using a 0.22 µm filter (Thermo Fisher Scientific). The recombinant

283   RBD proteins were purified by nickel affinity columns, as directed by the manufacturer

284   (Invitrogen). The RBD preparations were dialyzed against phosphate-buffered saline (PBS) and

285   stored in aliquots at -80°C until further use. To assess purity, recombinant proteins were loaded

286   on SDS-PAGE gels and stained with Coomassie Blue.

287

288   **Enzyme-Linked Immunosorbent Assay (ELISA)**

289   The SARS-CoV-2 RBD ELISA assay used was recently described (16). Briefly, recombinant

290   SARS-CoV-2 S RBD proteins (2.5 µg/ml), or bovine serum albumin (BSA) (2.5 µg/ml) as a

12

291 negative control, were prepared in PBS and adsorbed to plates (MaxiSorp Nunc) overnight at

292 4°C. Coated wells were subsequently blocked with blocking buffer (Tris-buffered saline [TBS]

293 containing 0.1% Tween20 and 2% BSA) for 1h at room temperature. Wells were then washed

294 four times with washing buffer (Tris-buffered saline [TBS] containing 0.1% Tween20). CR3022

295 mAb (50ng/ml) or a 1/250 dilution of plasma from SARS-CoV-2-infected or uninfected donors

296 were prepared in a diluted solution of blocking buffer (0.1 % BSA) and incubated with the RBD-

297 coated wells for 90 minutes at room temperature. Plates were washed four times with washing

298 buffer followed by incubation with secondary Abs (diluted in blocking buffer,0.4% BSA) for 1h at

299 room temperature, followed by four washes. HRP enzyme activity was determined after the

300 addition of a 1:1 mix of Western Lightning oxidizing and luminol reagents (Perkin Elmer Life

301 Sciences). Light emission was measured with a LB942 TriStar luminometer (Berthold

302 Technologies). Signal obtained with BSA was subtracted for each plasma and was then

303 normalized to the signal obtained with CR3022 mAb present in each plate. The seropositivity

304 threshold was established using the following formula: mean of all COVID-19 negative plasma +

305 (3 standard deviation of the mean of all COVID-19 negative plasma).

306

307 **Cell-Based ELISA**

308 Detection of the trimeric SARS-CoV-2 Spike at the surface of HOS cells was performed by cell-

309 based enzyme-linked immunosorbent assay (ELISA). Briefly, parental HOS cells or HOS-Spike

310 cells were seeded in 384-well plates (2.8×10^4 cells per well) overnight. Cells were blocked with

311 blocking buffer (washing buffer [1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris (pH 7.5), and 140 mM

312 NaCl] supplemented with 10mg/mL non-fat dry milk and 5mM Tris [pH 8.0]) for 30min. CR3022

313 mAb (1 µg/ml) or plasma from SARS-CoV-2-infected or uninfected donors (at a dilution of 1/250)

314 were prepared in blocking buffer and incubated with the cells for 1h at room temperature.

315 Respective HRP-conjugated secondary antibodies were then incubated with the samples for 45

316 min at room temperature. For all conditions, cells were washed 6 times with blocking buffer and

13

317   6 times with washing buffer. HRP enzyme activity was determined after the addition of a 1:1 mix

318   of Western Lightning oxidizing and luminol reagents (PerkinElmer Life Sciences). Light emission

319   was measured with an LB 942 TriStar luminometer (Berthold Technologies). Signal obtained with

320   parental HOS was subtracted for each plasma and was then normalized to the signal obtained

321   with CR3022 mAb present in each plate. The seropositivity threshold was established using the

322   following formula: mean of all COVID-19 negative plasma + (3 standard deviation of the mean of

323   all COVID-19 negative plasma).

324

Cell surface staining and flow cytometry analysis

326   293T and 293T-Spike cells were mixed at a 1:1 ratio and stained with the anti-RBD CR3022

327   monoclonal Ab (5 μg/ml) or plasma (1:250 dilution). AlexaFluor-647-conjugated goat anti-human

328   IgM+IgG+IgA Abs (1:800 dilution) were used as secondary antibodies. The percentage of

329   transduced cells (GFP+ cells) was determined by gating the living cell population based on

330   viability dye staining (Aqua Vivid, Invitrogen). Samples were acquired on a LSRII cytometer (BD

331   Biosciences) and data analysis was performed using FlowJo v10.7.1 (Tree Star). The

332   seropositivity threshold was established using the following formula: mean of all COVID-19

333   negative plasma + (3 standard deviation of the mean of all COVID-19 negative plasma).

334

ADCC assay

336   For evaluation of anti-SARS-CoV-2 antibody-dependent cellular cytotoxicity (ADCC), parental

337   CEM.NKr CCR5+ cells were mixed at a 1:1 ratio with CEM.NKr. Spike cells. These cells were

338   stained for viability (AquaVivid; Thermo Fisher Scientific, Waltham, MA, USA) and cellular dyes

339   (cell proliferation dye eFluor670; Thermo Fisher Scientific) and subsequently used as target cells.

340   Overnight rested PBMCs were stained with another cellular marker (cell proliferation dye

341   eFluor450; Thermo Fisher Scientific) and used as effector cells. Stained target and effector cells

342   were mixed at a ratio of 1:10 in 96-well V-bottom plates. Plasma from COVID+ or COVID-

14

343 individuals (1/500 dilution) or monoclonal antibody CR3022 (1 µg/mL) were added to the

344 appropriate wells. The plates were subsequently centrifuged for 1 min at 300xg, and incubated at

345 37°C, 5% CO_2 for 5 hours before being fixed in a 2% PBS-formaldehyde solution. ADCC activity

346 was calculated using the formula: [(% of GFP+ cells in Targets plus Effectors)-(% of GFP+ cells

347 in Targets plus Effectors plus plasma/antibody)]/(% of GFP+ cells in Targets) x 100 by gating on

348 transduced live target cells. All samples were acquired on an LSRII cytometer (BD Biosciences)

349 and data analysis performed using FlowJo v10.7.1 (Tree Star). The specificity threshold was

350 established using the following formula: mean of all COVID-19 negative plasma + (3 standard

351 deviation of the mean of all COVID-19 negative plasma)

352

353 **Virus neutralization assay**

354 293T-ACE2 target cells were infected with single-round luciferase-expressing SARS-CoV-2

355 pseudoparticles in presence of convalescent plasma. Briefly, 293T cells were transfected by the

356 calcium phosphate method with the lentiviral vector pNL4.3 R-E- Luc (NIH AIDS Reagent

357 Program) and a plasmid encoding for SARSCoV-2 Spike at a ratio of 5:4. Two days post-

358 transfection, cell supernatants were harvested and stored at -80°C until use. 293T-ACE2 target

359 cells were seeded at a density of 1×10^4 cells/well in 96-well luminometer-compatible tissue culture

360 plates (Perkin Elmer) 24h before infection. Recombinant viruses in a final volume of 100 µL were

361 incubated with the indicated plasma dilutions (1/50; 1/250; 1/1250; 1/6250; 1/31250) for 1h at

362 37°C and were then added to the target cells followed by incubation for 48h at 37°C; cells were

363 lysed by the addition of 30 µL of passive lysis buffer (Promega) followed by one freeze-thaw cycle.

364 An LB942 TriStar luminometer (Berthold Technologies) was used to measure the luciferase

365 activity of each well after the addition of 100 µL of luciferin buffer (15mM $MgSO_4$, 15mM KPO_4

366 [pH 7.8], 1mM ATP, and 1mM dithiothreitol) and 50 µL of 1mM d-luciferin potassium salt (Thermo

367 Fisher Scientific). The neutralization half-maximal inhibitory dilution (ID_{50}) represents the plasma

368 dilution to inhibit 50% of the infection of 293T-ACE2 cells by SARS-CoV-2 pseudoviruses.

15

369

370 **Detection of antigen-specific B cells**

371 To detect SARS-CoV-2-specific B cells, we conjugated recombinant RBD proteins with Alexa

372 Fluor 488 or Alexa Fluor 594 (Thermo Fisher Scientific) according to the manufacturer's protocol.

373 Approximately 10 x 10^6 frozen PBMC from 13 convalescent donors were prepared in Falcon®

374 5ml-round bottom polystyrene tubes at a final concentration of 14 x 10^6 cells/mL in RPMI 1640

375 medium (Gibco by Life Technologies, #11875-093) supplemented with 10% of fetal bovine serum

376 (Seradigm, #1500-500), Penicillin- Streptomycin (Gibco by Life Technologies, #15140122) and

377 HEPES (Gibco by Life Technologies, #15630-080). After a rest of 2h at 37°C and 5% CO_2, cells

378 were stained using Aquavivid viability marker (Gibco by Life Technologies) in DPBS (Gibco by

379 Life Technologies, #14190-144) at 4°C for 20min. The detection of SARS-CoV-2-antigen specific

380 B cells was done by adding the RBD probes to the following antibody cocktail: IgM BUV737 (Clone

381 UCH-B1, #748928), CD24 BUV805 (Clone ML5, #742010), IgG BV421 (Clone G18-145,

382 #562581), CD3 BV480 (Clone UCHT1, #), CD56 BV480 (Clone NCAM16.2, #566124), CD14

383 BV480 (Clone NCAM16.2, #746304), CD16 BV480 (Clone 3G8, #566108), CD20 BV711 (Clone

384 2H7, #563126), CD21 BV786 (Clone B-LY4, #740969), HLA DR BB700 (Clone G46-6, #566480),

385 CD27 APC R700 (Clone M-T271, #565116) all from BD Biosciences; CD19 BV650 (Clone

386 SJ25C1, #363026) from Biolegend and IgA PE (Clone IS11-8E10 ,#130-113-476) from Miltenyi.

387 Staining was performed at 4°C for 30min and cells were fixed using 2% paraformaldehyde at 4°C

388 for 15min. Stained PBMC samples were acquired on Symphony cytometer (BD Biosciences) and

389 analyzed using FlowJo v10.7.1 (TreeStar). In each experiment, PBMC from unexposed donors

390 (total of n=9) were included to ensure consistent specificity of the assay.

391

392 **Statistical analyses**

393 Statistics were analyzed using GraphPad Prism version 9.0.0 (GraphPad, San Diego, CA). Every

394 dataset was tested for statistical normality and this information was used to apply the appropriate

395   (parametric or nonparametric) statistical test. P values < 0.05 were considered significant;

396   significance values are indicated as $*$ p < 0.05, $**$ p < 0.01, $***$ p < 0.001, $****$ p < 0.0001.

397   Multiplicity adjustments of p values were performed with the Benjamini-Hochberg method in R

398   and R Studio (37, 38) using the data.table and tidyverse packages.

399

Software scripts and visualization

401   Normalized heatmaps were generated using the complexheatmap, tidyverse, and viridis

402   packages in R and RStudio (37, 38). Normalizations were done per parameter. IDs were grouped

403   and clustered separately according to time point. Correlograms were generated using the corrplot

404   and RColorBrewer packages in program R and RStudio using hierarchical clustering according

405   to the first principal component (FPC). Circular barplots were generated in R and RStudio using

406   the tidyverse package with averaged, normalized data per parameter and time point. Edge

407   bundling graphs were generated in undirected mode in R and RStudio using ggraph, igraph,

408   tidyverse, and RColorBrewer packages. Edges are only shown if p < 0.05, and nodes are sized

409   according to the connecting edges' r values. Nodes are color-coded according to groups of

410   parameters. Area graphs were generated for the display of normalized time series. The plots were

411   created in RawGraphs using DensityDesign interpolation and vertically un-centered values (39).

412

17

431   **Figure Captions**

432

433   **Figure 1. Decline of RBD- and Spike-specific antibodies in longitudinal convalescent**

434   **plasma.**

435   (**A-D**) Indirect ELISA was performed using recombinant SARS-CoV-2 RBD protein and incubation

436   with COVID-19+ plasma samples recovered between 6 and 31 weeks post-symptom onset. Anti-

437   RBD antibody binding was detected using HRP-conjugated (**A**) anti-human IgM+IgG+IgA (**B**) anti-

438   human IgM , (**C**) anti-human IgG, or (**D**) anti-human IgA. Relative light unit (RLU) values obtained

439   with BSA (negative control) were subtracted and further normalized to the signal obtained with

440   the anti-RBD CR3022 mAb present in each plate. (**E-H**) Cell-based ELISA was performed using

441   HOS cells expressing full-length SARS-CoV-2 Spike and incubation with COVID-19+ plasma

442   samples recovered between 6 and 31 weeks post-symptom onset. Anti-Spike antibody binding

443   was detected using HRP-conjugated (**E**) anti-human IgM+IgG+IgA (**F**) anti-human IgM, (**G**) anti-

444   human IgG, or (**H**) anti-human IgA. RLU values obtained with parental HOS (negative control)

445   were subtracted and further normalized to the signal obtained with the CR3022 mAb present in

446   each plate. (**Left panels**) Each curve represents the normalized RLUs obtained with the plasma

447   of one donor at every donation as a function of the days after symptom onset. (**Right panels**)

448   Plasma samples were grouped in different timepoints post-symptom onset (6, 11, 21 and 31

449   weeks). Undetectable measures are represented as white symbols, and limits of detection are

450   plotted. Error bars indicate means ± SEM. Statistical significance was tested using repeated

451   measures one-way ANOVA with a Holm-Sidak post-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;

452   **** $P < 0.0001$).

453

454   **Figure 2. Neutralization and Fc-effector function activities in convalescent plasma**

455   **decrease over time.**

456 (**A**) Pseudoviral particles coding for the luciferase reporter gene and bearing the SARS-CoV-2 S

457 glycoproteins were used to infect 293T-ACE2 cells. Neutralizing activity was measured by

458 incubating pseudoviruses with serial dilutions of COVID-19+ plasma samples recovered between

459 6 and 31 weeks post-symptom onset at 37°C for 1 h prior to infection of 293T-ACE2 cells.

460 Neutralization half maximal inhibitory serum dilution (ID_{50}) values were determined using a

461 normalized non-linear regression using GraphPad Prism software. (**B**) CEM.NKr parental cells

462 were mixed at a 1:1 ratio with CEM.NKr-Spike cells and were used as target cells. PBMCs from

463 uninfected donors were used as effector cells in a FACS-based ADCC assay. The graphs shown

464 represent the percentages of ADCC obtained in the presence of COVID-19+ plasma samples

465 recovered between 6 and 31 weeks post-symptom onset. (**Left panels**) Each curve represents

466 (**A**) the neutralization ID_{50} or (**B**) the percentages of ADCC obtained with the plasma of one donor

467 at every donation as a function of the days after symptom onset. (**Right panels**) Plasma samples

468 were grouped in different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Undetectable

469 measures are represented as white symbols, and limits of detection are plotted. Error bars

470 indicate means ± SEM. Statistical significance was tested using repeated measures one-way

471 ANOVA with a Holm-Sidak post-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

472

473 **Figure 3. RBD-specific memory B cells develop and persist up to 8 months post-symptom**

474 **onset.**

475 (**A**) Flow cytometry plots of staining with fluorescent SARS-CoV-2 RBD probes on CD19+ CD20+

476 HLA-DR+ B cells. Samples from one representative convalescent donor are shown for 4 different

477 timepoints post-symptom onset (6, 11, 21 and 31 weeks). All percentages shown represent the

478 frequency of RBD-specific B cells on the total CD19+ CD20+ B cell population. (**B-G**)

479 Characterization of RBD-specific B cells was performed on longitudinal PBMC samples obtained

480 from COVID-19+ convalescent individuals between 6 and 31 weeks post-symptom onset. (**B**)

481 Total RBD-specific B cells were segregated by subsets based on cell surface expression of (**C**)

482   IgM, (**D**) IgG or (**E**) IgA BCR isotypes. Frequency of (**F**) total memory, (**G**) naïve, (**H**) IgG+ memory

483   and (**I**) IgA+ memory B cells were determined based on CD21 and CD27 expression. (**Left**

484   **panels**) Each curve represents the frequency of a B cell subset on the total B cell population

485   obtained with PBMCs from one donor at every donation as a function of the days after symptom

486   onset. (**Right panels**) PBMC samples were grouped in different timepoints post-symptom onset

487   (6, 11, 21 and 31 weeks). Error bars indicate means ± SEM. Statistical significance was tested

488   using repeated measures one-way ANOVA with a Holm-Sidak post-test (* $P < 0.05$; ns,

489   nonsignificant)

490

491   **Figure 4. Longitudinal patterns of B cell levels and humoral immune responses.**

492   (**A**) (**Left panel**) Heatmap of humoral immune responses normalized per parameter. Columns

493   represent immune response parameters clustered based on similarity and grouped according to

494   the provided color code. Rows represent IDs grouped according to study time point. IDs are

495   clustered according to their immune response profiles within each time point. (**Right panel**)

496   Heatmap of B cell levels with similar display as in (**left panel**). (**B**) Circular bar plots represent

497   averaged values of parameters at each time point.

498

499   **Figure 5. Longitudinal plasticity and separation of B cell and humoral correlation clusters.**

500   Edge bundling correlation plots where red and blue edges represent positive and negative

501   correlations between connected parameters, respectively. Only significant correlations ($p < 0.05$)

502   are displayed. Nodes are color-coded based on the grouping of parameters according to the

503   legend at the bottom. Node size corresponds to the degree of relatedness of correlations. Edge

504   bundling plots are shown for correlation analyses using all data points (**A**) and datasets of

505   individual time points, i.e., at 6 weeks (**B**), 11 weeks (**C**), 21 weeks (**D**), and 31 weeks (**E**).

506

507 **Figure 6. Evolution of humoral immune responses and B cell levels in SARS-CoV-2**

508 **convalescent individuals over time.**

509 Area plots showing time series of selected, humoral immune responses and B cell levels by

510 interpolation of normalized, averaged values per parameter and time point. The timeline is shown

511 at the bottom with ticks indicating study time points.

512

513 **Supplemental Figure 1. Anti-SARS-CoV-2 IgM and IgA levels decline faster than IgG in the**

514 **convalescence phase.**

515 (**A**) The graph shown represents the mean values for anti-RBD ELISA (from Figure 1A-D) at

516 different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (**B**) The

517 graph shown represents the mean values for anti-Spike cell-based ELISA (from Figure 1E-H) at

518 different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (**C**) The

519 graph shown represents the mean values for neutralization and ADCC responses (from Figure

520 2) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (**D**) The

521 graph shown represents the mean values for RBD-specific B cell frequencies (from Figure 3B-

522 E) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint.

523

524 **Supplemental Figure 2. Detection of antibodies against SARS-CoV-2 Spike by flow**

525 **cytometry correlates with anti-Spike detection by cell-based ELISA.**

526 (**A**) Cell-surface staining of 293T cells stably expressing full-length SARS-CoV-2 Spike using

527 samples from COVID-19+ convalescent donors at different times after symptoms onset (6, 11, 21

528 and 31 weeks). The graphs shown represent the median fluorescence intensities (MFI) obtained

529 on the GFP+ population. MFIs values obtained with parental 293T (GFP-) were subtracted. (**Left**

530 **panel**) Each curve represents the MFIs obtained with the plasma of one donor at every donation

531 as a function of the days after symptom onset. (**Right panel**) Plasma samples were grouped in

22

532   different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Undetectable measures are

533   represented as white symbols, and limits of detection are plotted. Error bars indicate means ±

534   SEM. (**B**) The levels of anti-Spike total Ig quantified by flow cytometry were correlated with the

535   level of anti-Spike total Ig quantified by cell-based ELISA. Statistical significance was tested using

536   (**A**) a repeated measures one-way ANOVA with a Holm-Sidak post-test or (**B**) a Spearman

537   correlation rank test (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$).

538

539   **Supplemental Figure 3. Gating strategy for ADCC measurements.**

540   Target cells were identified according to cell morphology by light-scatter parameters (first column)

541   and excluding doublets cells (second column). Cells were then gated on eFluor670+ cells

542   (excluding the effector cells labeled with eFluor450; third column). Finally, the percentage of

543   GFP+ target cells was used to calculate ADCC activity (last column). Examples of gating using

544   (**A**) parental CEM.NKr or (**B**) a 1:1 ratio mix of CEM.NKr and CEM.NKr.Spike as target cells in

545   absence or (**C**) in presence of effector cells. (**D**) ADCC assay performed in the presence of plasma

546   samples from one representative convalescent donor at 4 different timepoints post-symptom

547   onset (6, 11, 21 and 31 weeks).

548

549   **Supplemental Figure 4. Gating strategy for SARS-CoV-2-specific B cell characterization.**

550   (**A-B**) Representative flow cytometry gates to identify RBD-specific B cells from PBMCs of (**A**)

551   uninfected and (**B**) convalescent donor. (**C-D**) Flow cytometry gates used to differentiate RBD-

552   specific B cell subtypes using isotypic and maturation cell surface markers on samples obtained

553   (**C**) 6 weeks and (**D**) 31 weeks post-symptom onset. After identification of isotypic subtypes, RBD-

554   specific naïve and memory B cells were characterized based on surface expression of CD21 and

555   CD27. The different RBD-specific B cell subpopulations were superimposed on total

556   CD19+/CD20+/HLA-DR+ B cells (grey). Legend: IgM+ and naïve IgM+ B cells, blue; IgG+ and

557   memory IgG+ B cells, red; IgA+ and memory IgA+ B cells, orange.

558

559 **Supplemental Figure 5. Correlations between serological, immunological and**

560 **demographic determinants.**

561 Correlograms were generated by plotting together all serological, immunological and

562 demographic data obtained from convalescent patients. Circles are color-coded and sized

563 according to the magnitude of the correlation coefficient (r). Red circles represent positive

564 correlations between two variables and blue circles represent negative correlations. Asterisks

565 indicate statistically significant correlations (*P < 0.05, **P < 0.01, ***P < 0.005). Correlation

566 analysis was done using Spearman correlation rank tests. Parameters are clustered hierarchically

567 according to the first principal component (FPC). Black surrounding boxes indicate adjusted p-

568 values < 0.05 using Benjamini-Hochberg multiplicity correction. Legend: Cb-ELISA = cell-based

569 ELISA, Neut = Neutralization, mem = memory, PSO = post-symptom onset, Sex = Female.

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Table 1. Longitudinal SARS-CoV-2 convalescent cohort

Group	n	Days after onset of symptoms (median; day range)	Age (median; age range)	Sex	
				Male (n)	Female (n)
6 weeks	32	43 (16-95)	47 (20-65)	17	15
11 weeks	28	77 (48-127)	47 (20-65)	16	12
21 weeks	28	145 (116-171)	48 (20-65)	16	12
31 weeks	13	218 (201-233)	46 (20-65)	9	4
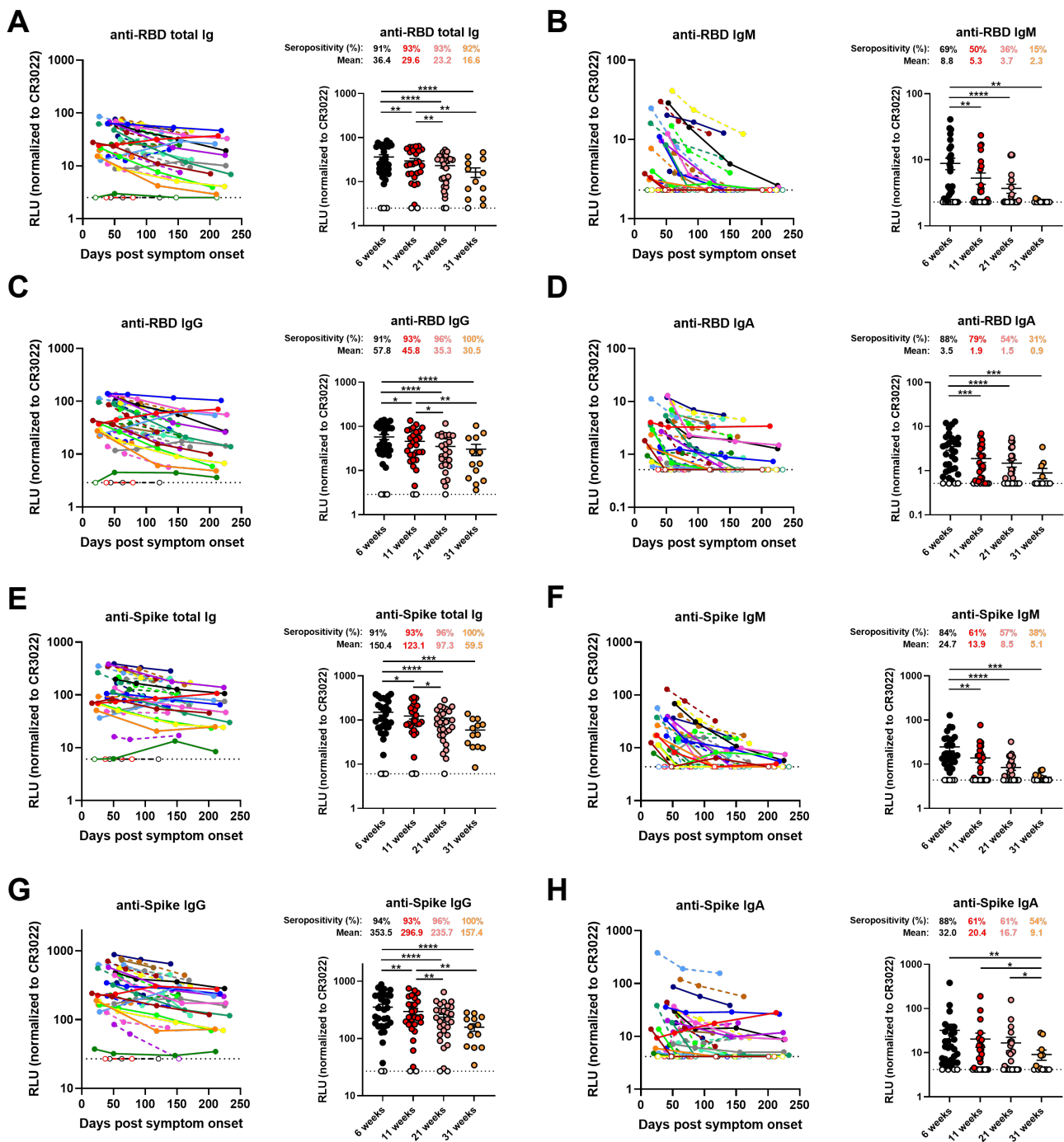
Figure 1. Decline of RBD- and Spike-specific antibodies in longitudinal convalescent plasma.
(A-D) Indirect ELISA was performed using recombinant SARS-CoV-2 RBD protein and incubation with COVID-19+ plasma samples recovered between 6 and 31 weeks post-symptom onset. Anti-RBD antibody binding was detected using HRP-conjugated (A) anti-human IgM+IgG+IgA (B) anti-human IgM , (C) anti-human IgG, or (D) anti-human IgA. Relative light unit (RLU) values obtained with BSA (negative control) were subtracted and further normalized to the signal obtained with the anti-RBD CR3022 mAb present in each plate. (E-H) Cell-based ELISA was performed using HOS cells expressing full-length SARS-CoV-2 Spike and incubation with COVID-19+ plasma samples recovered between 6 and 31 weeks post-symptom onset. Anti-Spike antibody binding was detected using HRP-conjugated (E) anti-human IgM+IgG+IgA (F) anti-human IgM, (G) anti-human IgG, or (H) anti-human IgA. RLU values obtained with parental HOS (negative control) were subtracted and further normalized to the signal obtained with the CR3022 mAb present in each plate. (Left panels) Each curve represents the normalized RLUs obtained with the plasma of one donor at every donation as a function of the days after symptom onset. (Right panels) Plasma samples were grouped in different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Undetectable measures are represented as white symbols, and limits of detection are plotted. Error bars indicate means ± SEM. Statistical significance was tested using repeated measures one-way ANOVA with a Holm-Sidak post-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).
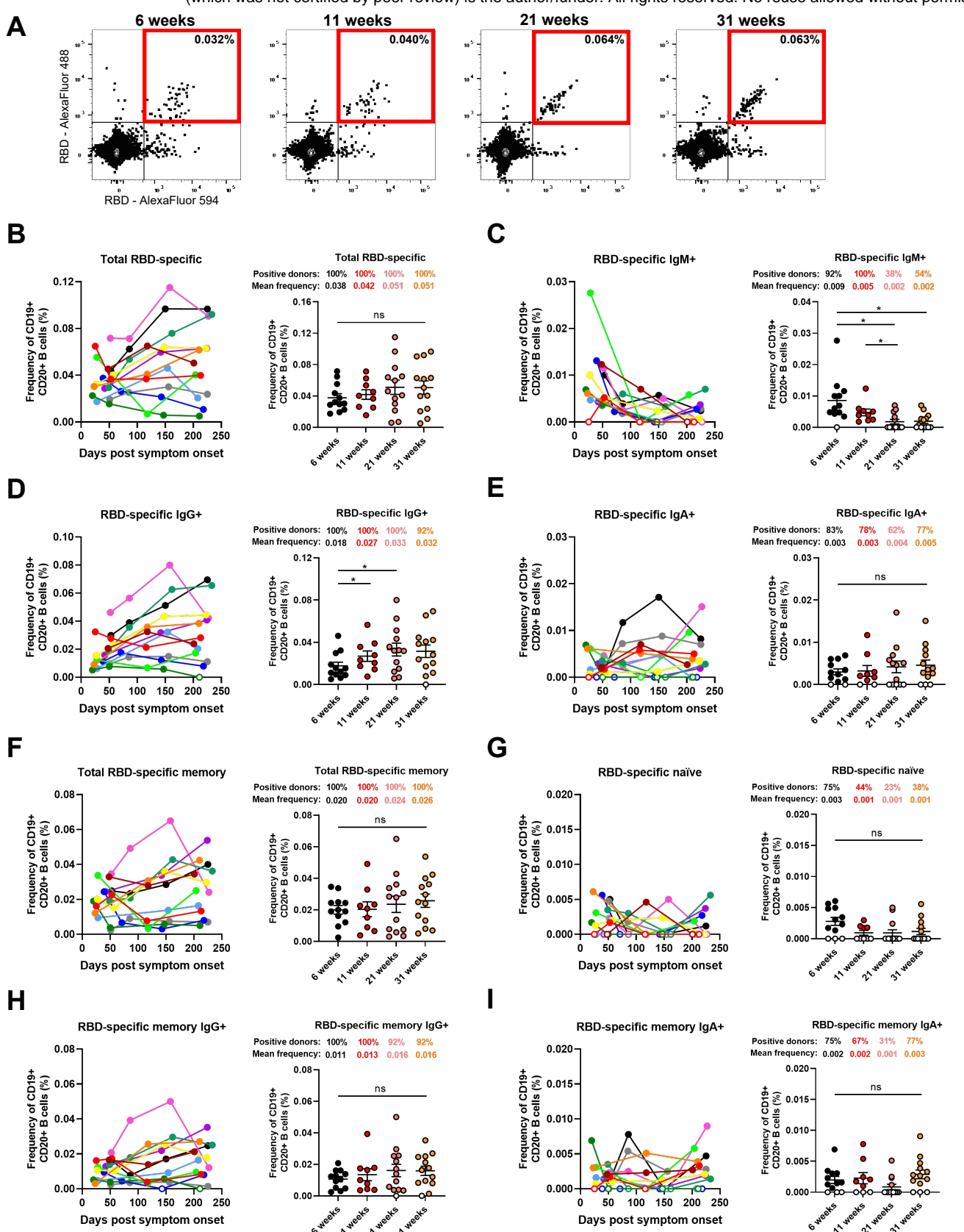
Figure 2. Neutralization and Fc-effector function activities in convalescent plasma decrease over time.
(A) Pseudoviral particles coding for the luciferase reporter gene and bearing the SARS-CoV-2 S glycoproteins were used to infect 293T-ACE2 cells. Neutralizing activity was measured by incubating pseudoviruses with serial dilutions of COVID-19+ plasma samples recovered between 6 and 31 weeks post-symptom onset at 37°C for 1 h prior to infection of 293T-ACE2 cells. Neutralization half maximal inhibitory serum dilution (ID50) values were determined using a normalized non-linear regression using GraphPad Prism software. (B) CEM.NKr parental cells were mixed at a 1:1 ratio with CEM.NKr-Spike cells and were used as target cells. PBMCs from uninfected donors were used as effector cells in a FACS-based ADCC assay. The graphs shown represent the percentages of ADCC obtained in the presence of COVID-19+ plasma samples recovered between 6 and 31 weeks post-symptom onset. (Left panels) Each curve represents (A) the neutralization ID50 or (B) the percentages of ADCC obtained with the plasma of one donor at every donation as a function of the days after symptom onset. (Right panels) Plasma samples were grouped in different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Undetectable measures are represented as white symbols, and limits of detection are plotted. Error bars indicate means ± SEM. Statistical significance was tested using repeated measures one-way ANOVA with a Holm-Sidak post-test (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

Figure 3. RBD-specific memory B cells develop and persist up to 8 months post-symptom onset.
(A) Flow cytometry plots of staining with fluorescent SARS-CoV-2 RBD probes on CD19+ CD20+ HLA-DR+ B cells. Samples from one representative convalescent donor are shown for 4 different timepoints post-symptom onset (6, 11, 21 and 31 weeks). All percentages shown represent the frequency of RBD-specific B cells on the total CD19+ CD20+ B cell population. (B-G) Characterization of RBD-specific B cells was performed on longitudinal PBMC samples obtained from COVID-19+ convalescent individuals between 6 and 31 weeks post-symptom onset. (B) Total RBD-specific B cells were segregated by subsets based on cell surface expression of (C) IgM, (D) IgG or (E) IgA BCR isotypes. Frequency of (F) total memory, (G) naïve, (H) IgG+ memory and (I) IgA+ memory B cells were determined based on CD21 and CD27 expression. (Left panels) Each curve represents the frequency of a B cell subset on the total B cell population obtained with PBMCs from one donor at every donation as a function of the days after symptom onset. (Right panels) PBMC samples were grouped in different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Error bars indicate means ± SEM. Statistical significance was tested using repeated measures one-way ANOVA with a Holm-Sidak post-test (* P < 0.05; ns, nonsignificant)
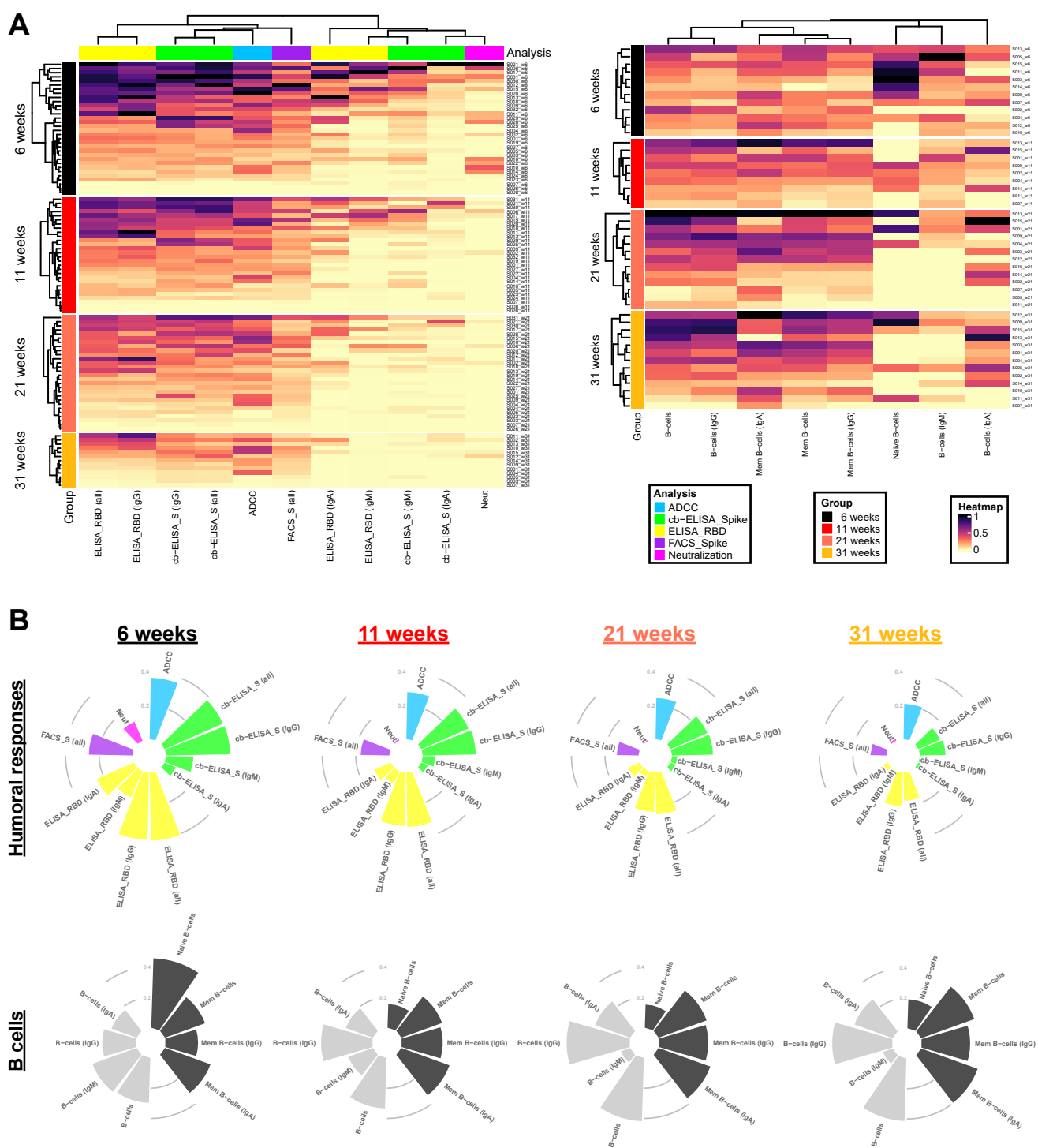
Figure 4. Differential longitudinal patterns of B cell levels and humoral immune responses.
(A) (Left panel) Heatmap of humoral immune responses normalized per parameter. Columns represent immune response parameters clustered based on similarity and grouped according to the provided color code. Rows represent IDs grouped according to study time point. IDs are clustered according to their immune response profiles within each time point. (Right panel) Heatmap of B cell levels with similar display as in (left panel). (B) Circular bar plots represent averaged values of parameters at each time point.
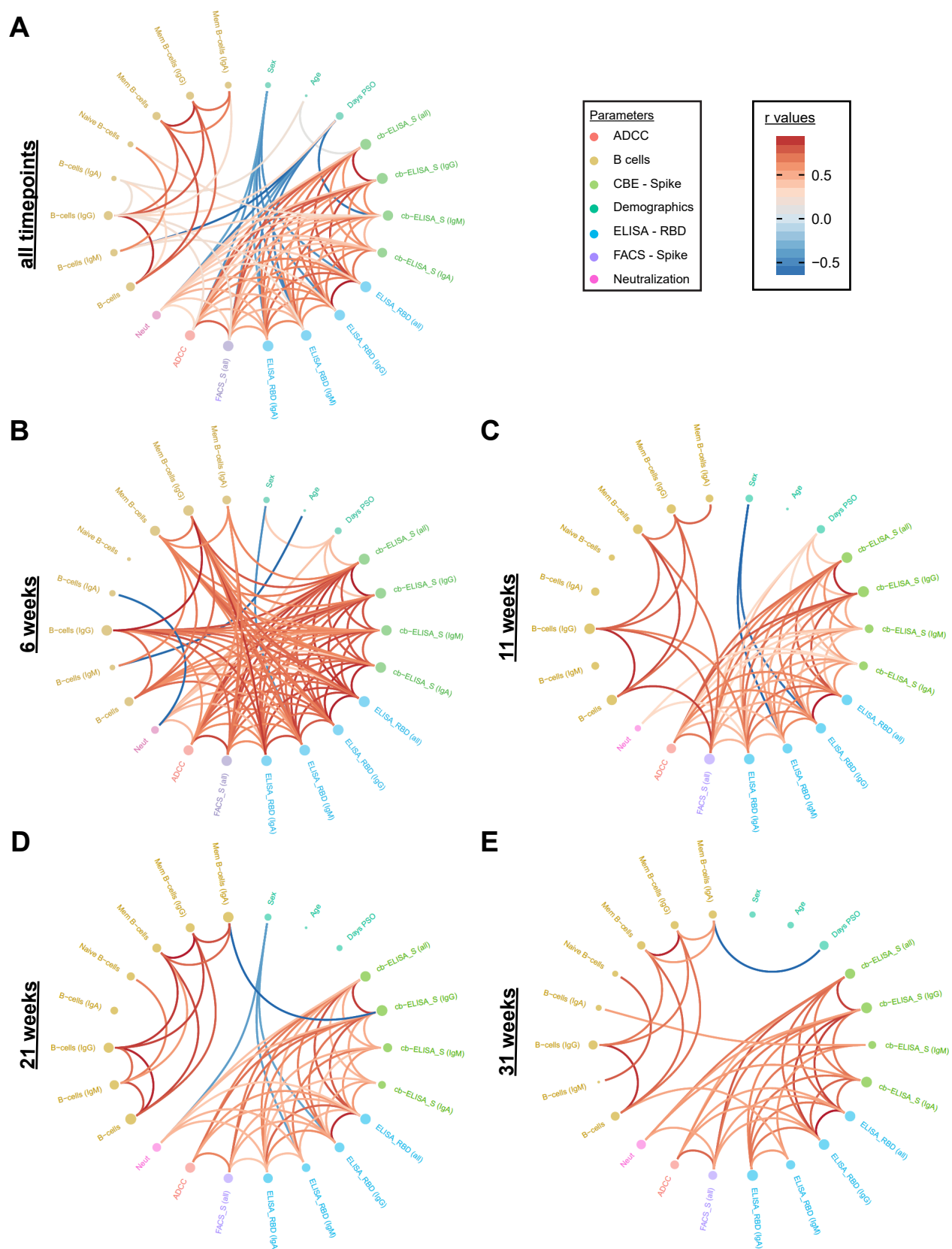
Figure 5. Longitudinal plasticity and separation of B cell and humoral correlation clusters.
Edge bundling correlation plots where red and blue edges represent positive and negative correlations between connected parameters, respectively. Only significant correlations (p < 0.05) are displayed. Nodes are color-coded based on the grouping of parameters according to the legend at the bottom. Node size corresponds to the degree of relatedness of correlations. Edge bundling plots are shown for correlation analyses using all data points (A) and datasets of individual time points, i.e., at 6 weeks (B), 11 weeks (C), 21 weeks (D), and 31 weeks (E).
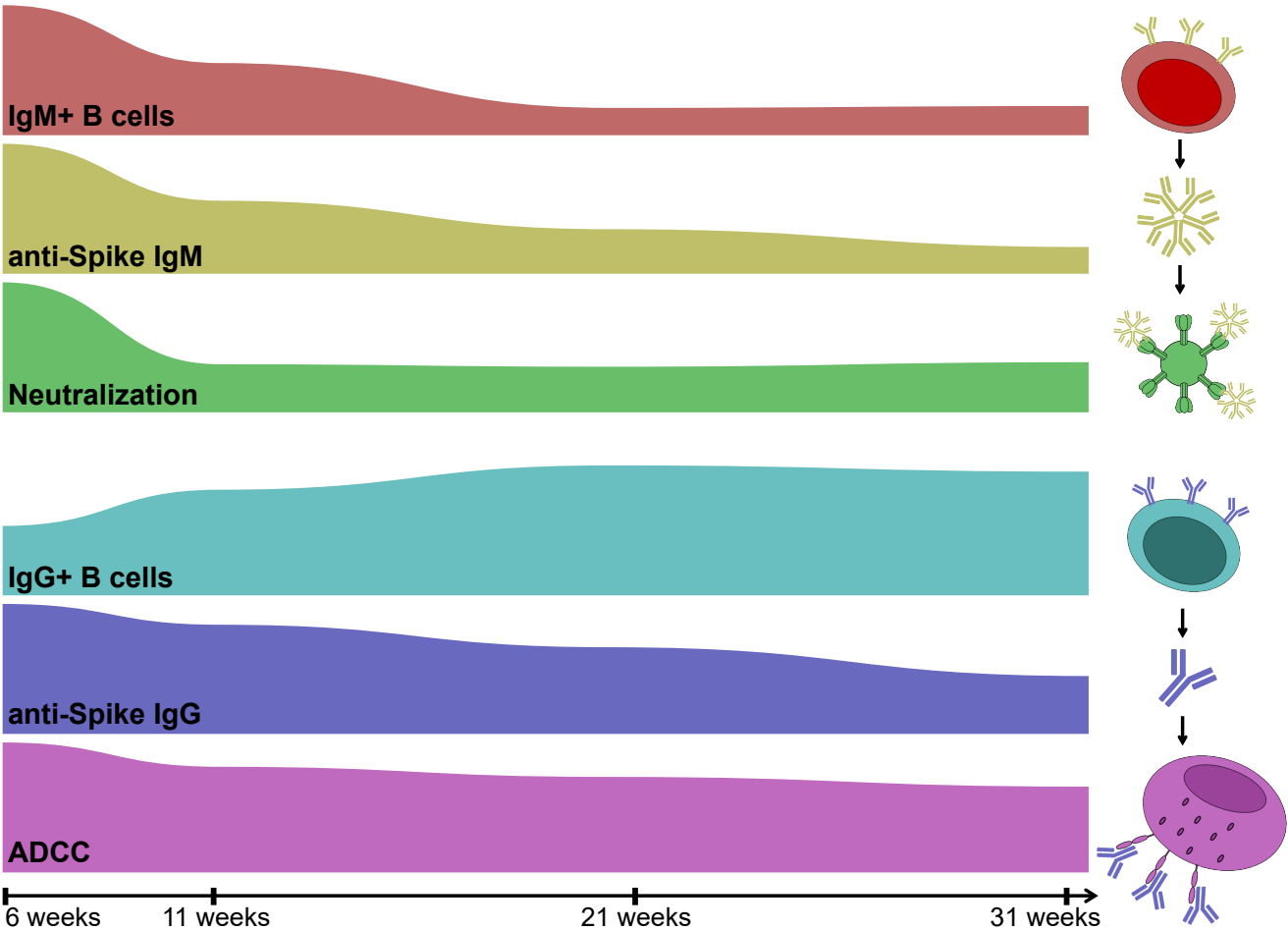
Figure 6. Evolution of humoral immune responses and B cell levels in SARS-CoV-2 convalescent individuals over time.
Area plots showing time series of selected, humoral immune responses and B cell levels by interpolation of normalized, averaged values per parameter and time point. The timeline is shown at the bottom with ticks indicating study time points.
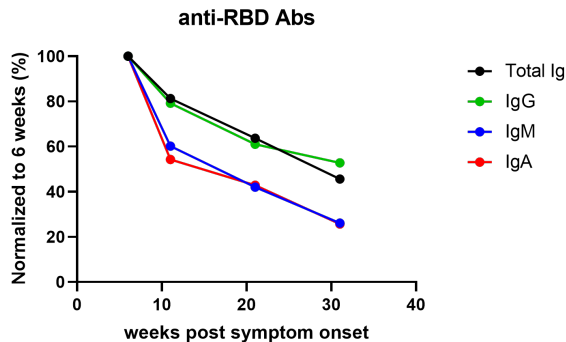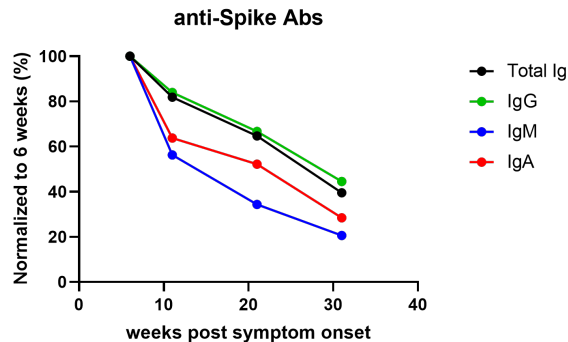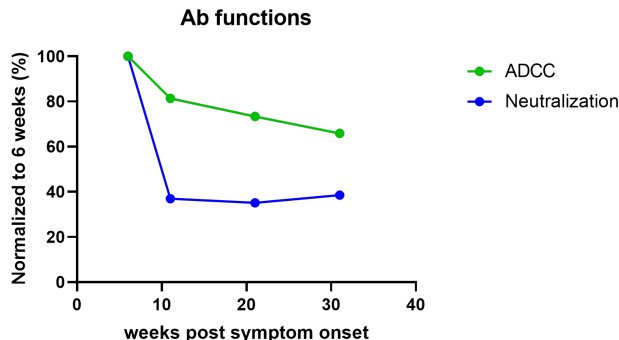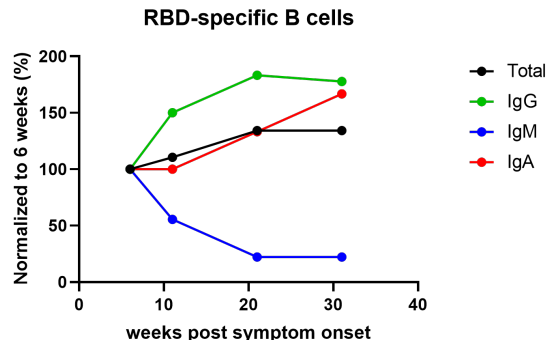
Supplemental Figure 1. Anti-SARS-CoV-2 IgM and IgA levels decline faster than IgG in the convalescence phase.
(A) The graph shown represents the mean values for anti-RBD ELISA (from Figure 1A-D) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (B) The graph shown represents the mean values for anti-Spike cell-based ELISA (from Figure 1E-H) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (C) The graph shown represents the mean values for neutralization and ADCC responses (from Figure 2) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint. (D) The graph shown represents the mean values for RBD-specific B cell frequencies (from Figure 3B-E) at different timepoints (6, 11, 21 and 31 weeks) normalized to the 6 weeks timepoint.
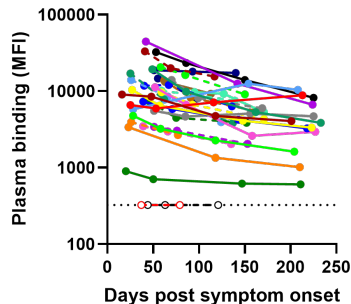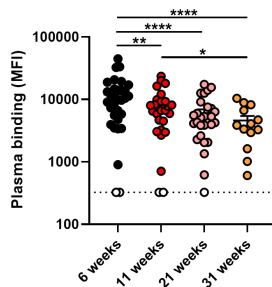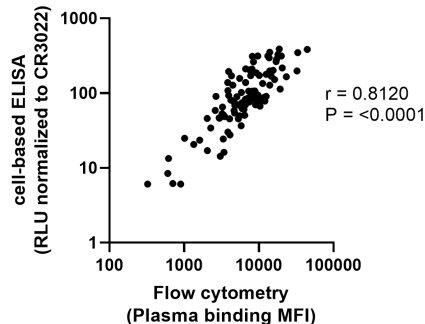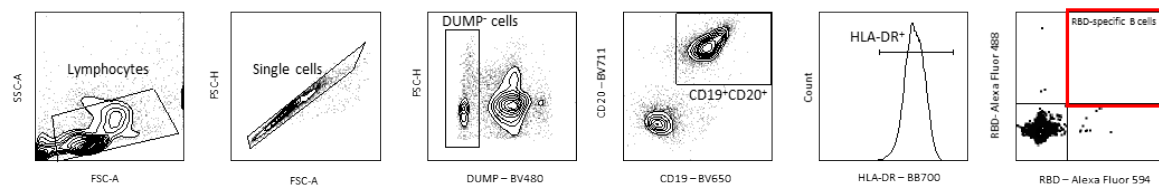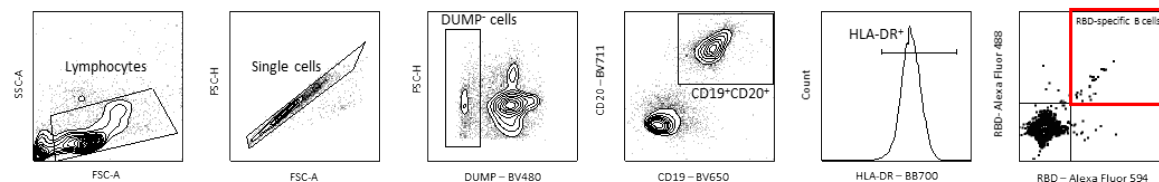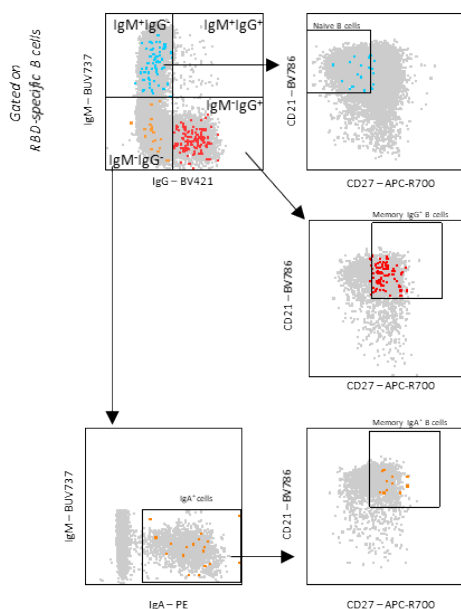
Supplemental Figure 2. Detection of antibodies against SARS-CoV-2 Spike by flow cytometry correlates with anti-Spike detection by cell-based ELISA.
(A) Cell-surface staining of 293T cells stably expressing full-length SARS-CoV-2 Spike using samples from COVID-19+ convalescent donors at different times after symptoms onset (6, 11, 21 and 31 weeks). The graphs shown represent the median fluorescence intensities (MFI) obtained on the GFP+ population. MFIs values obtained with parental 293T (GFP-) were subtracted. (Left panel) Each curve represents the MFIs obtained with the plasma of one donor at every donation as a function of the days after symptom onset. (Right panel) Plasma samples were grouped in different timepoints post-symptom onset (6, 11, 21 and 31 weeks). Undetectable measures are represented as white symbols, and limits of detection are plotted. Error bars indicate means ± SEM. (B) The levels of anti-Spike total Ig quantified by flow cytometry were correlated with the level of anti-Spike total Ig quantified by cell-based ELISA. Statistical significance was tested using (A) a repeated measures one-way ANOVA with a Holm-Sidak post-test or (B) a Spearman correlation rank test (* P < 0.05; ** P < 0.01; **** P < 0.0001).
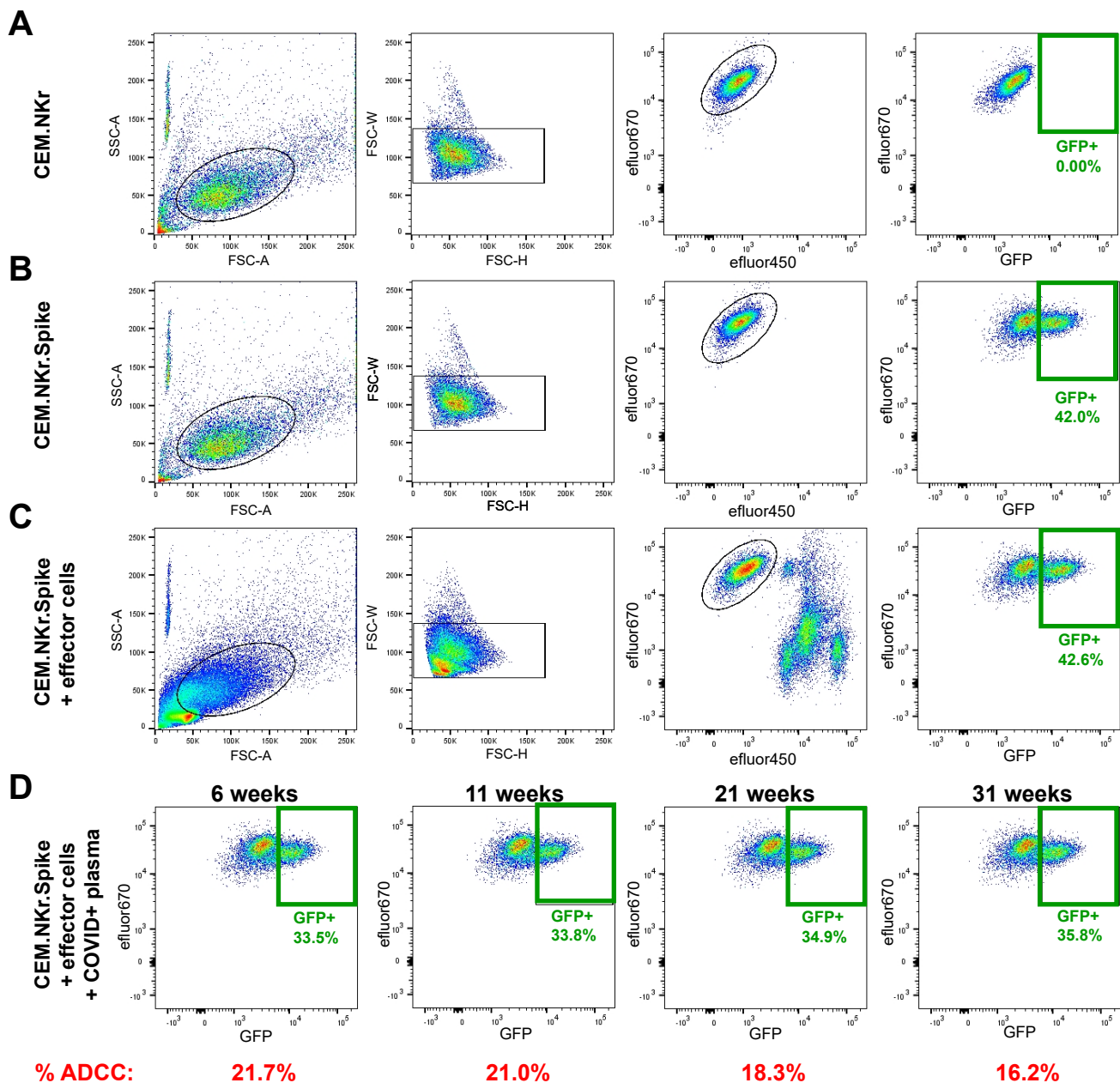
Supplemental Figure 3. Gating strategy for ADCC measurements.
Target cells were identified according to cell morphology by light-scatter parameters (first column) and excluding doublets cells (second column). Cells were then gated on eFluor670+ cells (excluding the effector cells labeled with eFluor450; third column). Finally, the percentage of GFP+ target cells was used to calculate ADCC activity (last column). Examples of gating using (A) parental CEM.NKr or (B) a 1:1 ratio mix of CEM.NKr and CEM.NKr.Spike as target cells in absence or (C) in presence of effector cells. (D) ADCC assay performed in the presence of plasma samples from one representative convalescent donor at 4 different timepoints post-symptom onset (6, 11, 21 and 31 weeks).
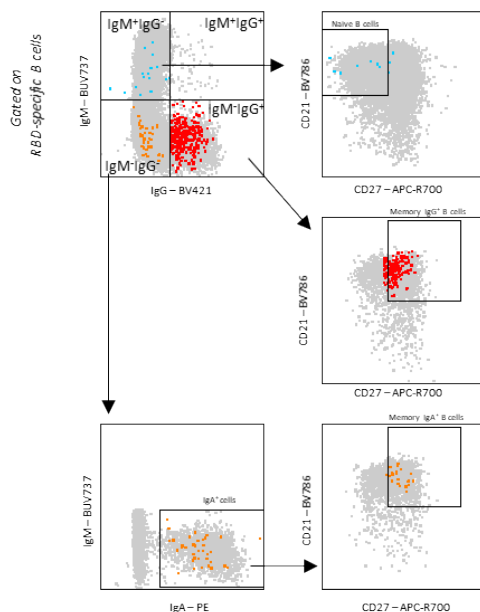
Supplemental Figure 4. Gating strategy for SARS-CoV-2-specific B cell characterization.
(A-B) Representative flow cytometry gates to identify RBD-specific B cells from PBMCs of (A) uninfected and (B) convalescent donor. (C-D) Flow cytometry gates used to differentiate RBD-specific B cell subtypes using isotypic and maturation cell surface markers on samples obtained (C) 6 weeks and (D) 31 weeks post-symptom onset. After identification of isotypic subtypes, RBD-specific naïve and memory B cells were characterized based on surface expression of CD21 and CD27. The different RBD-specific B cell subpopulations were superimposed on total CD19+/CD20+/HLA-DR+ B cells (grey). Legend: IgM+ and naïve IgM+ B cells, blue; IgG+ and memory IgG+ B cells, red; IgA+ and memory IgA+ B cells, orange.
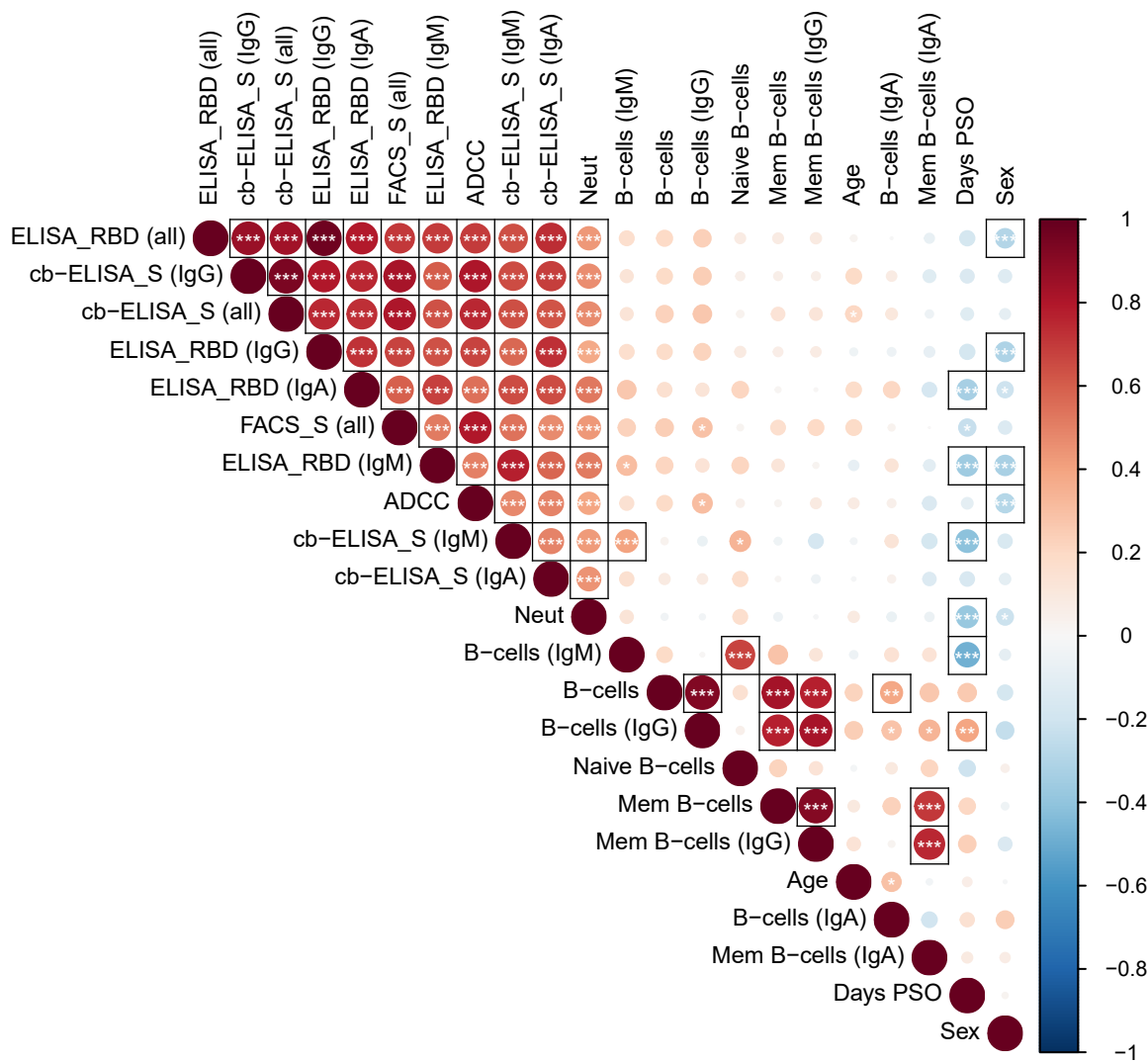
Supplemental Figure 5. Correlations between serological, immunological and demographic determinants. Correlograms were generated by plotting together all serological, immunological and demographic data obtained from convalescent patients. Circles are color-coded and sized according to the magnitude of the correlation coefficient (r). Red circles represent positive correlations between two variables and blue circles represent negative correlations. Asterisks indicate statistically significant correlations (*P < 0.05, **P < 0.01, ***P < 0.005). Correlation analysis was done using Spearman correlation rank tests. Parameters are clustered hierarchically according to the first principal component (FPC). Black surrounding boxes indicate adjusted p-values < 0.05 using Benjamini-Hochberg multiplicity correction. Legend: Cb-ELISA = cell-based ELISA, Neut = Neutralization, mem = memory, PSO = post-symptom onset, Sex = Female.