Enhanced FcγRIII/CD16 activation by discrete ligands as independent correlates of disease severity in COVID-19 patients

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One sentence summary

Severity of disease in COVID-19 patients is associated with enhanced CD16 activation by afucosylated S-specific IgG and CD16-reactive circulating IgG-complexes.

Abstract

A dysregulated immune response with high levels of SARS-CoV-2 specific IgG antibodies is a common and distinctive feature of severe or critical COVID-19. Although a robust IgG response is typically considered to be beneficial, an overshooting activation mediated by immune receptors recognizing the Fc part of IgG (FcγRs) is thought to be detrimental and associated with immunopathology. However, direct evidence of FcγR driven immunopathology in COVID-19 is still sparse. Here, we used a cell-based FcγR activation reporter system to systematically analyze SARS-CoV-2 specific IgG responses and IgG-mediated FcγRIII (CD16) activation profiles in COVID-19 patient cohorts categorized by severity of disease. We found that increased CD16 activation by SARS-CoV-2 specific IgG is associated with a known pro-inflammatory IgG modification, namely afucosylation. Further, we identified CD16-reactive soluble IgG immune complexes (sICs) to be present in the serum of COVID-19 patients and show that the resulting CD16 activation by sICs is strongly related to disease severity. Our results provide evidence that CD16 activation by pro-inflammatory SARS-CoV-2 specific IgG together with circulating sICs is a major contributor to COVID-19 immunopathology. These findings highlight the importance of FcγR driven immunopathology in COVID-19. Further, our data highly warrant the development of targeted intervention strategies against IgG driven immunopathology following SARS-CoV-2 infection.

58 **Introduction**

59 Since the emergence of SARS-CoV-2 in late December 2019[1], more than 178 million
60 laboratory confirmed infections (as of June 21, 2021) have been reported, with cases
61 continuously rising[2]. Accordingly, rapid insights into the disease manifestations and
62 pathogenesis have been globally obtained. A hallmark of the coronavirus disease 2019
63 (COVID-19) is a respiratory infection which can progress to an acute respiratory distress
64 syndrome (ARDS). Next to asymptomatic infections, COVID-19 symptoms differ widely
65 according to the disease process and may comprise fever, coughing, pneumonia, dyspnea,
66 hypoxia and lymphopenia[3]. While fever and coughing are common symptoms, pneumonia,
67 hypoxia, dyspnea, certain organ manifestations and lymphopenia indicate critical or fatal
68 infections. Pronounced dyspnea can eventually progress to ARDS, a severe complication
69 frequently observed in critically ill patients[4]. Although disease severity and in particular
70 breathing difficulties are related to viral load[5], age[6-8] and underlying medical conditions[7,8], the
71 kinetics of respiratory failure strongly suggest an essential role of the host immune
72 response[3,7]. Typically, aggravation occurs between 9-12 days after symptom onset[8] and
73 correlates with high levels of SARS-CoV-2 specific IgG antibodies and systemic effects of
74 pro-inflammatory cytokines such as IL-6 and TNFα[3,9,10]. This cytokine release, primarily the
75 result of macrophage and T helper (T_H) cell activation[11], includes pattern recognition receptor
76 (PRR) signaling in the context of innate immunity but also Fcγ receptor (FcγR) activation[12].
77 Triggered by immune complexes (antibody-antigen complex), the cytokine release following
78 FcγR activation represents a potent defense mechanism against invading pathogens. A
79 prototypical activating FcγR in this regard is FcγRIII (CD16) expressed by NK cells and
80 monocyte-derived macrophages (CD16A)[13] or neutrophils (CD16B, 98% sequence identical
81 ectodomains). Specifically, CD16 is able to recognize circulating soluble immune complexes
82 (sICs) as they are formed in certain autoimmune diseases such as lupus[14-17] and viral
83 infections[18]. Overstimulation of activating FcγRs in these cases is associated with disease
84 severity[18-20] and thus an FcγR-driven overshooting inflammatory response[12] might be an
85 explanation for the pronounced immunopathology observed during severe courses of COVID-
86 19[21]. Consistently, hyper-inflammation in SARS-CoV-1 and MERS infected patients has been
87 previously proposed as a possible pathogenic factor[22] and could be demonstrated in mice and
88 macaques infected with SARS-CoV-1[23,24]. Furthermore, N297-dependent glycan-
89 modifications such as afucosylation within the constant region of SARS-CoV-2 specific IgG
90 antibodies enhance binding to FcγRs, in turn driving inflammation. Enhanced FcγR activation

91 by low-fucosylated anti-SARS-CoV-2-S IgG leading to excessive alveolar macrophage
92 activation has specifically been shown to drive severe COVID-19 disease progression[25].
93 Therefore we aimed to delineate the contribution of IgG-mediated effector functions
94 regarding COVID-19 severity in patient cohorts with various severity of SARS-CoV-2
95 infection. We monitored antigen-specific IgG responses in patients with mild, critical and
96 severe COVID-19 disease with regard to titer, kinetics and CD16 activation. This revealed a
97 marked correlation between CD16 activation by patient IgG and severity of disease.
98 Additionally, we identified circulating sICs to be abundantly present in the serum of critically
99 and severely diseased patients, but not in the serum of those with a mild disease. As sIC-
100 mediated CD16 activation independently correlates with disease severity, we provide
101 evidence that also sIC formation is indicative of disease progression. Our findings enable new
102 avenues of intervention against COVID-19 and highly warrant further investigation into the
103 origin and composition of sICs in COVID-19.
104
105
106 **Results**
107 **Patients and clinical information.**
108 We retrospectively analyzed serial serum samples collected for routine diagnostic testing from
109 41 patients hospitalized at our tertiary care center between March and June 2020 with SARS-
110 CoV-2 infection confirmed by real-time PCR. Based on the clinical course, we categorized
111 patients as either severely diseased (hospitalized with COVID-19 related pneumonia) versus
112 critically diseased (COVID-19 related pneumonia and eventually in need of invasive
113 mechanical ventilation). In total, 27 patients with critical and 14 with severe courses of
114 disease were grouped into separate cohorts (Table 1). Most patients were older than 60 years
115 with an overall mean age of 68 years (63 years and 76 years in the critically and severely
116 diseased patients respectively).
117 The majority of patients in both groups had comorbidities of different origin with
118 cardiovascular diseases including hypertension representing the most frequent pathology
119 (35/41, 85%). Similar to previous reports, high Interleukin 6 (IL-6) and C-reactive protein
120 (CRP) levels were associated with severity of disease (Ø IL-6: 1452.1 pg/ml in the critical
121 group vs 46.1 pg/ml in the severe group and Ø CRP: 162.2 mg/l vs 65.3 mg/l Ø13-25 days
122 post symptom onset respectively). Similarly, procalcitonin, a biomarker of microbial
123 coinfection, was significantly higher in critically diseased patients (Ø value 9.9 ng/ml vs 0.17
124 ng/ml). Bacterial superinfection represented a further complication in 39% of the patients and

125 was only slightly more frequent in patients with critical disease (11/27, 41% vs 5/14, 33%).
126 More than half of the patients (59%) were treated with hydroxycloroquine/Kaletra®, (18/27,
127 67% in the critical group vs 6/14, 43% in the severe group). Notably, at the time of serum
128 acquisition, only one patient received steroid treatment, which was given due to underlying
129 chronic obstructive pulmonary disease. Finally, mortality rate was 37% (10/27) in critically
130 and 7% (1/14) in severely diseased patients.
131
132 **Kinetics of IgG antibody responses following symptom onset across severe and critical**
133 **courses of disease.**
134 It has been proposed that elevated SARS-CoV-2 antibody titers are associated with disease
135 severity[10] and may play a role not only in the clearance but also in the pathogenesis of SARS-
136 CoV-2 infection[26]. We therefore initially analyzed the levels and kinetics of SARS-CoV-2
137 specific IgG in serial serum samples from patients hospitalized with critical (n=27) or severe
138 (n=14) illness, a setting we also used in the following experiments. A total of 125 (critically
139 diseased) and 79 (severely diseased) serum samples, obtained from the aforementioned
140 patients at different time points within 6-25 days following symptom onset were analyzed by
141 commercially available S1- and N- specific ELISA-based assays. Assay specificity was
142 confirmed analyzing healthy donor (HD) serum samples (n=30) as negative control (Figure 1-
143 figure supplement 1 A, B). Most patients developed detectable SARS-CoV-2 specific IgG
144 responses within 9-14 days after symptom onset. SARS-CoV-2 specific IgG gradually
145 increased over time in both severely and critically diseased patients reaching a plateau at 18-
146 20 days after symptom onset (Figure 1 A, B). Varying antibody response kinetics were
147 observed for each individual patient (Figure 1-figure supplement 2 A-D) with anti-N IgG
148 titers rising significantly earlier than anti-S1 IgG (12.5 days ± 3.3 days vs 10.6 ± 3.8; p=
149 0.0091). A trend towards earlier seroconversion for anti-S1 IgG could be observed in
150 critically diseased patients (mean time of seroconversion 11.4 ± 3.0 days in critically diseased
151 patients vs 12.9 ± 3.8 days for severely diseased patients; p = 0.24), whereas time of
152 seroconversion for anti-N IgG was similar in both groups (10.1 ± 3.2 and 10.4 ± 4.2 days for
153 critically and severely diseased patients, respectively; p = 0.83). S1- and N-specific IgG levels
154 at plateau did not significantly differ between the two groups. No significant difference
155 between deceased and discharged patients was measured 13-25 days after symptom onset
156 (Figure 1- figure supplement 1 C, D, E). Next, we evaluated and compared the neutralizing
157 capacity of SARS-CoV-2 IgG in either critically versus severely diseased patients in a plaque-
158 reduction assay (Figure 1 C). All patients mounted a robust neutralizing antibody response

159 (91% ± 10.5 % neutralization at a 1:64 serum dilution), with peaking titers at 18-20 days
160 following symptom onset. Of note, two critically diseased patients developed a neutralizing
161 response already at 6-8 days after symptom onset. In summary, we observed only minor
162 differences in cohort wide kinetics of S1- or N- specific IgG levels between patients
163 hospitalized with severe or critical clinical courses indicating that antibody levels per se did
164 not correlate with severity of disease in our study.
165
166 **Patients with severe COVID 19 show enhanced CD16 activation by S-specific IgG**
167 **antibodies.**
168 FcγRIII (CD16) activation initiates multiple protective effector functions such as antibody-
169 dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells as well as antibody-
170 dependent cytokine and chemokine secretion by NK cells and macrophages[12,27]. However,
171 excessive FcγR stimulation can have severe adverse effects such as elevated cytokine release
172 as observed in autoimmune diseases or viral infections[12]. Therefore, we hypothesized that an
173 exaggerated FcγR mediated activation triggered by SARS-CoV-2 specific IgG might
174 contribute to the exacerbation of COVID-19 in severely compared to critically diseased
175 patients. To address this, we analyzed the ability of SARS-CoV-2 specific antibodies to
176 activate CD16 (158V) using a previously established cell-based reporter system[28] (Figure 2-
177 figure supplement 1A). Considering the typically late time point of health deterioration, we
178 performed an analysis of CD16 activation triggered by SARS-CoV-2 specific IgG on serum
179 samples obtained 13-25 days following symptom onset (Figure 2). Sera were analyzed at a
180 1:500 dilution to stay within the dynamic range of detection (Figure 2- figure supplement 2).
181 Depending on the availability of sample material 2-8 samples/patient/time-point were
182 included in this analysis. If available in sufficient quantity, sera were reanalyzed.
183 Reproducibility was tested using available serum surplus (Figure 2- figure supplement 3).
184 Sera from 28 patients with mild SARS-CoV-2 infection and 30 healthy blood donors were
185 included for reference. Semi-quantitative assessment of IgG titers using antigen-specific
186 ELISA revealed comparable levels between critically and severely diseased patient cohorts
187 (Figure 2 A, B, C). In contrast, S- (p=0.0147) and RBD-specific (p=0.0120) but not N-
188 specific IgG-mediated CD16 activation was significantly increased in critically compared to
189 severely diseased patients (Figure 2 D-F). Furthermore, normalizing CD16 activation to
190 antigen-specific IgG titers, revealed significantly stronger CD16 activation by S- (p=0.0033)
191 and N-specific (p=0.006) IgG compared to mildly diseased patients (Figure 2 G-I).
192 Intriguingly, we observed a heterogeneous CD16 activation pattern characterized by either

193 high or low CD16-activating sera irrespective of the clinical manifestation (Figure 2 D-F).
194 Overall, a significant positive correlation could be determined between anti-SARS-CoV-2
195 antigen IgG titers and CD16 activation (Figure 2-figure supplement 4). Our data document a
196 sustained CD16 activation by SARS-CoV-2 specific antibodies particularly in patients
197 suffering from critical COVID-19 disease. Based on these results we confirmed the notion
198 that elevated FcγRIII activation by S- and or RBD-specific IgG might contribute to disease
199 severity of COVID-19.
200
201 **Enhanced Fcγ-afucosylation of S-specific IgG in critically and severely diseased patients**
202 **results in increased CD16 activation.**
203 We speculated that differences in Fcγ mediated effector functions might contribute to disease
204 severity of COVID-19 and thus compared CD16 high- versus CD16 low-activating patient
205 sera regarding their SARS-CoV-2 specific IgG core fucosylation. Inspired by previous
206 findings[29-31] we focused on determining IgG core fucosylation of S- and N- specific SARS-
207 CoV2 IgG. To determine IgG core fucosylation we adapted a lectin-based ELISA preceded by
208 antigen-specific antibody purification from immobilized SARS-CoV-2-antigen. Analysis of
209 anti-S and anti-N IgG core fucosylation was performed on serum pools containing five sera of
210 either critically or severely diseased patients obtained 13-25 days post symptom onset. Given
211 the aforementioned heterogeneity in CD16-activation, we analyzed pools of 5 sera of either
212 critically or severely diseased patients characterized by either high or low CD16-activation.To
213 stay within the dynamic detection range, relative fucosylation was analyzed at a dilution of
214 1:4 (Figure 3). When analyzing serum pools from critically and severely diseased patients we
215 determined a significantly lower level of core fucosylation among the high CD16 activators
216 (Figure 3, plain-colored bars) compared to the low CD16 activators (Figure 3, shaded bars).
217 This applied for both the S- and N-specific antibodies. These results are in line with
218 previously published findings regarding the effect of Fcγ-afucosylation on CD16 effector
219 functions[29,32] and recapitulate similar findings in the context of COVID-19[30,31]. However, we
220 did not observe significant differences between critically and severely diseased patients.
221
222 **COVID-19 disease severity correlates with an increase in CD16-reactive soluble IgG**
223 **complexes.**
224 Aside from afucosylation, it has been proposed that uncleared antigen-antibody immune
225 complexes (ICs) might further exacerbate inflammation, explaining complications observed in
226 COVID-19 such as cytokine storm, systemic vasculitis, microvascular thrombosis and organ

227 failure[33-35]. However, the presence of circulating, soluble ICs (sICs) in critically or severely
228 diseased patients has not been conclusively shown yet. As extensive FcγR activation by sICs
229 might contribute to the severe systemic inflammatory state occurring in some COVID-19
230 patients with prolonged disease, we surmised that sICs might be a putative explanation for the
231 marked differences in IL-6, PCT and CRP levels between critically and severely diseased
232 patients (Table 1). We thus set out to characterize our patient cohort regarding the presence of
233 sICs in serum samples taken at various time points during disease and after hospitalization. To
234 this end, we deployed a novel cell-based reporter assay developed to quantify CD16 (158V)
235 activation by IgG-containing sICs[17]. As recently shown, the assay does not react to
236 monomeric IgG or small dimeric complexes in solution, but specifically identifies multimeric
237 sICs and has been successfully used to identify sICs in patients with systemic lupus
238 erythematosus (SLE)[16]. Moreover, the assay is sensitive to sICs size with larger complexes
239 leading to stronger receptor activation compared to small complexes. Analysis of serum
240 samples, obtained 13-25 days after symptom onset, revealed the presence of highly CD16-
241 reactive sICs in SARS-CoV-2 infected patients compared to healthy individuals (Figure 4A).
242 Next, we compared sIC-mediated CD16 activation between COVID-19 patients of varying
243 disease severity. While all COVID-19 patient groups tested positive for reactive sICs
244 compared to healthy control (HD) sera, we found that critically diseased patients show a
245 striking increase in reactive sICs compared to patients with severe or mild disease (Figure
246 4B). Only 6 out of 27 patients with critical disease (22%) showed no sIC-mediated CD16
247 activation. As we did not detect reactive sICs in the serum of 47 patients with acute
248 respiratory distress syndrome (ARDS; mean age 57.5 years) in response to infections of
249 different etiology including CMV reactivation, HIV, influenza or TBC infection, we conclude
250 that the formation of reactive sICs is associated with severe SARS-CoV-2 disease (Figure 4-
251 figure supplement 1). Longitudinal analysis of reactive sICs in the serum of critically or
252 severely diseased patients revealed high CD16 activation levels in 4 critically diseased
253 patients already 6 to 8 days after symptom onset (Figure 4C). Of note, 2 of 4 patients with an
254 early increase of circulating reactive sIC eventually died. sIC-mediated CD16 activation
255 persisted in 14 of 19 critically diseased patients at high levels until day 26 after symptom
256 onset. sIC-mediated CD16 activation in severely diseased patients was slightly delayed
257 compared to critically diseased patients and was first detected in 4 patients 9-11 days after
258 symptom onset (Figure 4C). Only 4 of 14 patients with severe disease showed detectable sIC-
259 mediated CD16 activation. To verify that sICs represent the CD16-reactive component in the
260 serum of COVID-19 patients, we analyzed serum-mediated CD16 activation before and after

261 PEG8000-precipitation. This treatment was previously shown to selectively precipitate large
262 IgG complexes from solution[36]. For this analysis, pools of 8 sera, showing either high (IC+)
263 or no (IC-) CD16 activation, were compared. Sera from healthy donors (HD) served as a
264 negative control. Compatible with the hypothesis of serum-derived sICs driving CD16
265 activation, no activation was observed following incubation with 3.5% PEG8000 (Figure 4-
266 figure supplement 2 A). To ensure, that the treatment did not precipitate monomeric IgG, we
267 tested the depleted sera for remaining S1- and N-specific IgG. As depicted S1- and N-
268 specific IgG could still be detected at high levels in samples treated with 3.5% PEG8000
269 (Figure 4-figure supplement 2 B). When resolving sIC-mediated CD16 activation over the
270 complete time of hospitalization for select patients from which samples at different time
271 points were available, we observed that sIC reactivity follows a similar course as anti-S1 IgG
272 titers in ELISA (Fig. 4-figure supplement 3). While this might imply that sIC formation
273 involves SARS CoV-2 S1 antigen, we were not able to identify any SARS-COV-2-derived
274 antigens in PEG8000-precipitated sICs using tandem mass spectrometry (data not shown). To
275 further exclude the formation of multimeric sICs formed from circulating S1 antigen, we also
276 specifically targeted S1 for precipitation from patient serum using biotinylated S1-specific
277 monoclonal antibodies. However and in line with our previous approach, S1-specific
278 precipitation using streptavidin-sepharose beads and subsequent mass spectrometry analysis
279 for any SARS-CoV-2-specific antigens in sICs remained inconclusive (data not shown).
280 Recently, the role of neutrophil mediated intravascular NETosis was found to play a critical
281 role in thrombose formation and subsequent organ damage observed in severe clinical forms
282 of COVID-19[37]. Since this process could mediate the formation of aggregated IgG as a form
283 of sICs, we next tested whether Benzonase® nuclease treatment of patient serum would
284 dissolve reactive sICs. To this end we tested sera from critically diseased patients or healthy
285 individuals and compared CD16 reactivity before and after nuclease treatment (Figure 4-
286 figure supplement 4). Nuclease activity in diluted human serum was controlled using plasmid
287 DNA for reference. This revealed that nucleic acid was not involved in the formation of
288 CD16-reactive sICs in critically diseased patients. Finally, we tested pooled patient sera for
289 autoantibodies against a panel of prototypical autoantigens associated with autoimmune
290 disease including anti-nuclear autoantibodies (ANA) by indirect immunofluorescence,
291 dsDNA autoantibodies by ELISA and autoantibodies against the extractable nuclear antigens
292 (nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, CENP B, PCNA, nucleosomes,
293 histones, ribosomal P-protein, AMA-M2, DFS70) by dot blot in case SARS-CoV-2 infection
294 triggers autoantibody formation and possible sIC formation. However, no significant

295 autoantibody titers could be detected in any sera pool (data not shown). Although we were not
296 able to identify their origin, our data clearly indicates the presence of circulating sICs in
297 COVID-19 patients with an increase in CD16-reactive sICs corresponding with severity of
298 disease. Accordingly, we conclude that circulating sICs are a hitherto unknown, yet
299 contributing factor to COVID-19 disease severity and, regarding infectious diseases, our
300 findings represent an observation unique to severely diseased COVID-19 patients.
301
302 **Discussion**
303 We collected and analyzed data from 41 COVID-19 patients hospitalized at the University
304 Hospital Freiburg. Patients were categorized by severity of disease into severely (n=14) and
305 critically diseased patients (n=27). Both groups were of comparable average age and had a
306 similar male-to-female ratio. For comparison we also analyzed 28 mildly diseased and 30
307 healthy individuals. As key findings we identify the potential of *de novo* produced SARS-
308 CoV-2 IgG and the presence of soluble immune complexes activating FcγRIII/CD16 as
309 independent risk factors closely associated with severe courses of COVID-19.
310
311 **CD16 activation by SARS-CoV-2 specific IgG is increased in critically diseased patients.**
312 FcγR activation by anti-viral IgG represents a potent defense mechanism, directing the
313 immune response to the site of viral replication[38-41]. However, persistent infection can lead to
314 prolonged stimulation of FcγRs, driving an overshooting and potentially damaging
315 inflammatory response[12,18]. A potential key component in prolonged inflammation following
316 SARS-CoV-2 infection is FcγR mediated cytokine and chemokine release by several immune
317 cells as their activation has been implied in severity of disease[29,31,42]. Along these lines,
318 glycan profiles of anti-SARS-CoV2 IgG have been identified as major correlates of COVID-
319 19 disease progression[29-31]. As these modifications particularly affect CD16 activation, we
320 characterized our patient groups with regard to CD16 activation by S- and N-specific IgG and
321 determined significantly increased CD16 activation by S, N and RBD specific IgG in sera
322 from severely and critically diseased patients compared to mildly diseased patients. We
323 further identified a higher level of afucosylation being indicative of COVID-19 severity,
324 which is in line with previous studies[29-31], and now directly link this general observation to an
325 increased CD16 activation. Taken together, we find that CD16 activation by SARS-CoV-2-
326 specific IgG can be directly correlated to IgG glycan modifications and disease severity.
327

328 **Circulating sICs contribute to COVID-19 disease severity**

329 Based on the herein presented results identifying CD16 activation to be a major correlate of
330 disease severity, we further explored this using an adapted FcγR activation assay optimized to
331 measure soluble immune complexes (sICs)[17]. Indeed, we provide first evidence of such
332 circulating sICs in the serum of COVID-19 patients and experimentally confirm previous
333 hypotheses suggesting immune complexes as potential drivers of disease progression in
334 COVID-19[33-35]. In fundamental contrast to opsonized antigens decorating virus-infected cells,
335 sICs become distributed systemically. Thus constitutive activation of $CD16^+$ monocytes,
336 granulocytes and NK cells could readily explain systemic responses which potentiate local
337 inflammation in virus-infected tissues intensifying organ damage and dysfunction. Although
338 the origin of the circulating immune complexes still remains elusive, we clearly show that the
339 presence of IgG-containing sICs is restricted to SARS-CoV-2 infection and that complexed
340 IgG is directly responsible and sufficient for the observed CD16 activation by patient serum.
341 As we find sIC reactivity to roughly follow SARS-CoV2-S specific IgG responses, we
342 speculated that circulating S or shed S1-antigens might still be involved in sIC formation,
343 albeit in a form we were not able to detect. In support of this hypothesis, it has recently been
344 published that circulating S-antigen can be detected in the plasma of individuals who received
345 two doses of the mRNA-1273 (Moderna) vaccine[43]. S1 shedding was also reported to play a
346 role in virion infectivity as the introduction of a D614G mutation significantly enhanced
347 infectivity due to an increased S-protein density in the virion[44]. Finally, others have identified
348 potential mechanisms leading to S-antigen shedding in viral infection and proposed a link
349 between the presence of circulating S-antigen and disease severity[45]. Alternatively, IgG auto-
350 antibodies of diverse specificity might cause the formation of the CD16 triggering sICs we
351 herein identified. In fact, several studies have described that auto-antibodies can be frequently
352 detected in critically ill COVID-19 patients[46-48]. In particular, very recent work shows that an
353 acute SARS-CoV-2 infection triggers the *de novo* IgG production against multiple
354 autoantigens. Interestingly, 60-80% of all hospitalized COVID-19 patients had anti-cytokine
355 IgG (ACA)[49]. The authors show that ACA levels and specificity change over time during
356 hospitalization, suggesting ACA induction in response to viral infection and inflammation.
357 Further, it has been shown that pre-existing neutralizing anti-type I interferon antibodies,
358 which can be found in about 10% of patients with severe COVID-19 pneumonia, are related
359 to the highest risk of developing life-threatening COVID-19 disease[50]. Therefore, the *de novo*
360 induction of anti-cytokine auto-antibodies in a large proportion of hospitalized COVID-19
361 patients as described by Chang et al[49], might indeed represent the origin of circulating sICs in

362 COVID-19. In such a scenario, immune responses are deviated first by depletion of important
363 cytokines and second through the formation of pathological sICs which trigger immunological
364 damage. We show that critically diseased patients show significantly higher levels of reactive
365 sICs compared to less severely diseased patients. In addition, sIC responses can be found
366 significantly earlier in critically diseased patients, which was associated with a fatal disease
367 outcome. We also find that patients show a wide range of sIC reactivity. According to the
368 Heidelberger-Kendall precipitation curve[51], sIC size is critically dependent on the
369 antigen:antibody stoichiometry. As the used FcγR activation assay is sensitive to sIC size[17], it
370 is highly likely that this also plays a role when measuring COVID-19 patient serum. As some
371 patients show high anti-SARS-CoV-2-S IgG titers in the absence of sICs and vice versa we
372 speculate that sIC size plays a role in CD16 reactivity. Therefore, we propose that in addition
373 to the presence of sICs, the size of sICs plays a role in CD16 driven COVID-19
374 immunopathology.
375 Taken together, we conclude that CD16 activation in COVID-19 disease is governed by IgG
376 glycan profiles and sIC formation (Figure 5) and plays a major role in disease progression and
377 severity. Indeed, there is evidence in this direction from a clinical perspective provided by a
378 recent study that finds the administration of intravenous immunoglobulin (IVIg) to alleviate
379 COVID-19 disease[52]. Although no direct proof, this heavily implies that the saturation of
380 FcγRs mitigates immunopathology. Therefore, our findings provide an explanation for the
381 sustained immunopathology following SARS-CoV-2 infection as well as for the efficacy of
382 IVIg treatment in severe to critical COVID-19 disease.
383
384
385 **Material and Methods**
386
387 **Subjects and specimens**
388 Between March 2020 and April 2020, 41 patients with SARS-CoV-2 infection confirmed by
389 real-time PCR were hospitalized in the University Medical Center, Freiburg. Serum samples
390 were collected during hospitalization for routine laboratory testing. Clinical data were
391 obtained from electronic medical records. A total of 27 patients necessitating invasive
392 mechanical ventilation were included in the critical group. Fourteen patients requiring O_2
393 supplementation were included in the severe group. Additionally, serum samples from 29
394 mild COVID-19 cases and 30 healthy donor (HD) plasma samples were used as controls in
395 this study.

396

397 **Cell culture**

398 African green monkey kidney Vero E6 cells (ATCC CRL-1586) were cultured at 37°C in
399 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal calf
400 serum (FCS, Biochrom), sodium pyruvate (1x, Gibco) and 100 U/ml penicillin-Streptomycin
401 (Gibco). BW5147 mouse thymoma cells (BW, obtained from ATCC: TIB-47) were stably
402 transduced with human FcγR as previously described[53]. Cells were maintained at $3x10^5$ to
403 $9x10^5$ cells/ml in Roswell Park Memorial Institute medium (RPMI GlutaMAX, Gibco)
404 supplemented with 10% (vol/vol) FCS, sodium pyruvate (1x, Gibco), 100 U/ml penicillin-
405 Streptomycin (Gibco) β-mercaptoethanol (0.1 mM, Gibco). Cells were cultured at 37°C, 5%
406 CO_2. All cell lines were routinely tested for mycoplasma.

407

408 **Monitoring of antibody response to SARS-CoV-2 by ELISA**

409 Serum IgG antibody titers targeting S1- and N-SARS-CoV-2 proteins were measured using
410 commercial enzyme-linked immunosorbent assay (ELISA). Anti-S1- SARS-CoV-2 IgG was
411 measured by the anti-SARS-CoV-2 ELISA (IgG) Euroimmune Kit (Euroimmune, Lübeck,
412 Germany) according to manufacturer's protocol. Results, expressed as arbitrary units (AU),
413 were evaluated semi-quantitatively by calculation of the ratio of the extinction of the control
414 or patient sample over the extinction of the calibrator. This ratio is interpreted as follows: <
415 0.8 negative; ≥ 0.8 to <1.0 borderline; ≥ 1.1 positive. Anti-N SARS-CoV-2 IgG was detected
416 using the recomWell SARS-CoV-2 IgG Kit (Mikrogen Diagnostik GmbH, Neuried,
417 Germany) according to manufacturer's protocol. The corresponding antibody activity
418 expressed in AU/ml is calculated using the formula (absorbance of sample / absorbance of
419 cut-off) × 20. Result are interpreted as follow: < 20 negative; ≥ 20 to < 24 borderline; > 24
420 positive. IgG against the SARS-CoV-2 Spike Glycoprotein Receptor Binding Domain (RBD)
421 were detected using SARS-CoV-2 IgG ELISA Reagent Set, kindly provided by InVivo
422 (InVivo Biotech Services GmbH, Hennigsdorf, Germany) according to manufacturer's
423 protocol.

424

425 **Fcγ receptor activation assay**

426 FcγRIIIA (CD16A, 158V) activation was measured by a cell-based assay as previously
427 described[28]. For detection of anti-S and anti-RBD-specific FcγR activation we utilized SARS-
428 CoV-2-S- and RBD-coated plates (kindly provided by InVivo Biotech Services GmbH,
429 Hennigsdorf, Germany). The recombinant (S)-protein was produced under serum-free

430 conditions in mammalian cells and contains amino acid residues 1 to 1213 of the SARS-CoV-
431 2 Wuhan-Hu-1-isolate (GenBank annotation QHD43416.1). The furin cleavage site was
432 mutated, two mutations for protein stabilization were included, and the C-terminal domain
433 was replaced by a T4 trimerization sequence and a C-terminal hexa-His-Tag[54] The
434 recombinant RBD-protein represented amino acids 319 to 541 of the (S)-protein mentioned
435 before. Both recombinant proteins were purified using immobilized metal exchange
436 chromatography (IMAC) and preparative SEC under standard conditions in a regulated
437 environment. Microtiter plates were coated using 0.2 µg recombinant (S)-protein or RBD-
438 protein per well. N-specific FcγR activation was determined using plates coated with SARS-
439 CoV-2-N (Mikrogen Diagnostik GmbH, Neuried, Germany). Respective plates were
440 subsequently incubated with serial dilutions of SARS-CoV-2 positive sera or control sera in
441 RPMI supplemented with 10% (vol/vol) FCS for 30 min at 37°C. All wells were thoroughly
442 washed before co-cultivation with BW5147 reporter cells for 16 h at 37°C, 5% CO_2. Cross-
443 link activation of reporter cells was performed by direct coating of target antibody to ELISA
444 plate (Nunc Maxisorp; 96 well, flat transparent), followed by a blocking step and incubation
445 with 2×10^5 reporter cells per well. For all activation assays, mouse IL-2 secretion was
446 quantified by anti-IL-2 ELISA, as described earlier. FcγRIIIA (CD16A) activation by sICs
447 was measured by a recently developed cell-based assay[17]. Briefly, $2x10^5$ BW5147-CD16
448 reporter cells were incubated with SARS-CoV-2 sera in a total volume of 200 µl for 16 h at
449 37°C, 5% CO_2. Incubation was performed in a 96-well ELISA plate (Nunc Maxisorp) pre-
450 treated with PBS containing10% FCS for 1 h at 4°C to avoid direct binding of serum IgG to
451 the plate. Reporter cell mIL-2 secretion was quantified via ELISA as described previously[28].
452
453 **Purification of SARS-CoV2-S and –N specific antibodies from serum**
454 SARS-CoV-2-specific antibodies were purified using SARS-CoV-2 spike protein (S)-coated
455 plates (kindly provided by InVivo BioTech Services) and - nucleocapsid (N) - coated plates
456 recomWell SARS-CoV-2 IgG (Mikrogen Diagnostik GmbH, Neuried, Germany). Patient sera
457 were diluted 1:5 in 100 µl (two wells per serum sample) and incubated for one hour at 37°C
458 with the S- and N-precoated plates. After washing using PBS-T (0.05% Tween 20) 100 mM
459 formic acid (30 µl/well) was added and incubated for 5 min on an orbital shaker at room
460 temperature (RT) to elute bound IgG. Following pH neutralization using TRIS buffer (1 M),
461 the eluates were either directly processed or stored at 4°C.
462
463 **Quantitation of antigen-specific IgG amount**

464 In order to determine the relative S1- and N-SARS-CoV-2 specific IgG antibody
465 concentration of the generated eluates, S1- and N-ELISA were performed by the anti-SARS-
466 CoV-2 ELISA (IgG) Euroimmune Kit (Euroimmune, Lübeck, Germany) and anti-N SARS-
467 CoV-2 IgG ELISA (recomWell SARS-CoV-2 IgG Kit (Mikrogen Diagnostik GmbH,
468 Neuried, Germany) as aformentioned.
469
470 **Analysis of antigen-specific IgG-Fcγ fucosylation**
471 Fucosylation levels of S- and N-specific IgG were measured using a lectin-based ELISA
472 assay. Briefly, 96-well Maxisorb plates (Nunc®) were coated with 50µl/well anti-human IgG-
473 Fab fragment (MyBiosource, MBS674607) at a concentration of 2 µg/ml, diluted in PBS for
474 one hour at 37°C. After three washing steps with PBS-T (0.05% Tween20) unspecific binding
475 sites were blocked adding 300 µl/well Carbo-free™ blocking solution (VectorLab, Inc., SP-
476 5040, LOT: ZF0415) for one hour at room temperature. After three further washing steps,
477 eluted antibodies were 2-fold serially diluted with PBS in a total volume of 30 µl/well and
478 incubated for one hour at 37°C and 5% CO_2. After washing (3x) using PBS-T, 50 µl/well of 4
479 µg/ml biotinylated Aleuria Aurantia lectin (AAL, lectin, VectorLab, B-1395) diluted in lectin
480 buffer (10 mM HEPES, 0.1 mM $CaCl_2$, 0.15 M NaCl, 0.1% Tween20) was added and
481 incubated for 45 min at room temperature (RT). Following another three washing steps using
482 PBS-T, Streptavidin-Peroxidase Polymer (Sigma, S 2438), at 1 µg/ml final concentration
483 diluted in LowCross-HRP®-buffer (Candor, Order #.: 200 500) was added and incubated for
484 one hour at RT. After washing five times with PBS-T, 50 µl/well of 1-Step™ Ultra TMB-
485 ELISA Substrate Solution (ThermoFisher, 34028) was applied and the enzyme-substrate
486 reaction was stopped after six minutes using 50 µl/well sulphuric acid (1 M H_2SO_4).
487 Quantification of absorbance, OD_{450nm}, was performed using a Tecan M2000.
488
489 **PEG Precipitation**
490 Sera pools, consisting of eight different sera per pool, were diluted with varying amounts of
491 PEG8000, in order to reach a final PEG8000 concentration of 1, 2, 3.5, 5 and 7.5%
492 respectively. Mixtures were vortexed and incubated overnight at 4°C. For supernatant
493 analysis, precipitates were sedimented via centrifugation at 13.000 rpm for 30 minutes at 4°C.
494 For Mass Spectrometry analysis, PEG8000-precipitated sICs were shortly run into 10%
495 polyacrylamide gels. After over-night fixation (40% ethanol, 10% acetic acid, 50% water) and
496 washing (3x), complete lanes were excised.
497

498 **Benzonase treatment of sera**

499 Serum from six individual patients containing CD16-reactive soluble immune complexes,
500 were treated with 250 units (U) of Benzonase Nuclease (Sigma-Aldrich Chemie Gmbh,
501 Munich Germany) for 1 h at 4°C. After treatment, sera were titrated in complete BW5147
502 culture medium and tested for CD16 reactivity. Non-treated sera served as control. To verify
503 Benzonase activity in the presence of human serum, 3 µg of pIRES-eGFP plasmid DNA
504 (Addgene) were digested with 250 U of Benzonase. Successful nucleic acid digestion was
505 visualized using a 1% agarose gel stained with Midori Green.

506

507 **Immune precipitation**

508 For mass spectrometry analysis of SARS-CoV2-S specific precipitates, individual sera
509 containing CD16-reactive soluble immune complexes were subjected to immune precipitation
510 (IP) using Pierce MS-compatible magnetic IP kit (ThermoFisher Scientific, Darmstadt,
511 Germany) according to manufacturer's protocol. Briefly 250 µl serum was incubated
512 overnight at 4°C with 5 µg of biotinylated anti-RBD-specific TRES-1-224.2.19 mouse
513 monoclonal antibody or TRES-II-480 (isotype control) (kind gift of H.M. Jäck, Erlangen)
514 before addition of streptavidin magnetic beads. Beads were subsequently collected via
515 centrifugation and elution buffer was added to detach putative precipitated antigen. The
516 elution was dried in a speed vacuum concentrator and shortly run into 10% polyacrylamide
517 gels. After over-night fixation (40% ethanol, 10% acetic acid, 50% water) and washing (3x),
518 complete lanes were excised. Antibody biotinylation was performed using a Pierce antibody
519 biotinylation Kit for IP (ThermoFisher Scientific, Darmstadt, Germany) according to
520 manufacturer's protocol.

521

522 **Mass Spectrometry**

523 Proteins were in-gel digested with sequencing grade modified trypsin (Promega GmbH,
524 Walldorf, Germany) similar to the procedure described by Pandey et al.[55]. Vacuum-dried
525 peptides were dissolved in 0.5% trifluoroacetic acid, loaded onto a trap column (C18
526 PepMap100, 5 µm particles, Thermo Fisher Scientific GmbH, Dreieich, Germany) with
527 0.05% trifluoroacetic acid (4 min, 10 µL/min) and separated on a C18 reversed phase column
528 (SilicaTip™ emitter, 75 µm i.d., 8 µm tip, New Objective, Inc, Littleton, USA, manually
529 packed 23 cm with ReproSil-Pur ODS-3, 3 µm particles, Dr. A. Maisch HPLC GmbH,
530 Ammerbuch-Entringen, Germany; flow rate: 300 nL/min). For sample injection and multi-
531 step gradient formation (eluent "A": 0.5% acetic acid in water; eluent "B": 0.5% acetic acid in

532 80% acetonitrile / 20% water; gradient length / acquisition time: 100 min or 175 min) an
533 UltiMate 3000 RSLCnano system (Thermo Fisher Scientific GmbH, Dreieich, Germany) was
534 used. Eluting peptides were electrosprayed at 2.3 kV via a Nanospray Flex ion source into a Q
535 Exactive HF-X hybrid quadrupole-orbitrap mass spectrometer (both Thermo Fisher Scientific
536 GmbH, Dreieich, Germany) and analyzed by data-dependent acquisition with HCD (higher
537 energy collisional dissociation) fragmentation of doubly, triply and quadruply charged ions
538 (loop count and dynamic exclusion dependent on the gradient length). Peak lists were
539 generated with ProteoWizard msConvert (http://proteowizard.sourceforge.net/; version
540 3.0.11098), linear shift mass recalibrated (after a preliminary database search) using in-house
541 developed software and searched against a database containing the SARS-CoV-2 UniProtKB
542 reference proteome (proteome ID: UP000464024), all human UniProtKB/Swiss-Prot entries,
543 and optionally (to reduce the number of incorrectly assigned matches) selected bacterial
544 proteins (finally the Pseudomonas fluorescens (strain SBW25) reference proteome;
545 proteome ID: UP000002332) with Mascot 2.6.2 (Matrix Science Ltd, London, UK; peptide
546 mass tolerance: ± 5 ppm; fragment mass tolerance: ± 20 mmu; one missed trypsin cleavage
547 and common variable modifications allowed).
548
549 **Neutralization assay**
550 Serum neutralization capacity was analyzed as previously described[56]. Briefly, VeroE6 cells
551 were seeded in 12-well plates at a density of $2.8x10^5$ cells/well 24 h prior to infection. Serum
552 samples were diluted at ratios of 1:16, 1:32 and 1:64 in 50 µL PBS total volume. Negative
553 controls (PBS without serum) were included for each serum. Diluted sera and negative
554 controls were subsequently mixed with 90 plaque forming units (PFU) of authentic SARS-
555 CoV-2 (B.1) in 50 µl PBS (1600 PFU/mL) resulting in final sera dilution ratios of 1:32, 1:64,
556 and 1:128. Following incubation at RT for 1 h, 400 µL PBS was added to each sample and the
557 mixture was subsequently used to infect VeroE6 cells. After 1.5 h of incubation at RT,
558 inoculum was removed and the cells were overlaid with 0.6% Oxoid-agar in DMEM, 20 mM
559 HEPES (pH 7.4), 0.1% $NaHCO_3$, 1% BSA and 0.01% DEAE-Dextran. Cells were fixed 48h
560 post-infection (4% formaldehyde for 30 minutes). Upon removal of the agar overlay, plaque
561 neutralization was visualized using 1% crystal violet. PFU were counted manually. Plaques
562 counted for serum-treated wells were compared to the average number of plaques in the
563 untreated negative controls, which were set to 100%.
564
565 **Statistical analyses**

Statistical analyses were performed using linear statistical models. i.e. the two-group comparisons were made based on the t-statistic of the estimated effects. Differences over more than two groups were tested by Analysis of Variance (ANOVA) and multiple testing for subsequent two-group comparisons was then considered by performing Games-Howell post-hoc tests. For the time course data, patient differences were treated as random effects in a linear mixed effects model with time and clinical course (severe vs. critical) as fixed main and interaction effects. All analyses were performed at the $\log_2$ scale. Assumptions about variance heterogeneity and normal distribution were checked by visual inspection of diagnostic plots.

Ethics

The protocol of this study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional ethical committee of the University of Freiburg (EK 153/20). Written informed consent was obtained from participants and the study was conducted according to federal guidelines, local ethics committee regulations (Albert-Ludwigs-Universität, Freiburg, Germany: No. F-2020-09-03-160428 and no. 322/20)

582 **Figure Legends**

583

584 **Figure 1**

585 **IgG responses against different SARS-CoV-2 proteins across severe and critical clinical**
586 **course of disease.**

587 IgG antibody levels were analyzed in longitudinal serum samples from hospitalized SARS-
588 CoV-2 infected individuals. 27 patients were categorized as critically diseased when in need
589 of invasive mechanical ventilation (red symbols) compared to 14 severely diseased patients
590 who did not require invasive ventilation (blue symbols). (A) IgG response against SARS-
591 CoV-2 S1 –protein and (B) SARS-CoV-2 N-protein as determined by commercial ELISA
592 assays. Dotted lines represent cut-off values for commercial S1- and N- specific ELISA
593 assays. Each dot represents the mean value obtained by the analysis of all samples which were
594 available at the indicated time points following symptom onset. Solid black lines indicate the
595 median. (C) Serum neutralization capacity against SARS-CoV-2 measured by a plaque
596 reduction assay. Sera were considered neutralizing upon 50% plaque reduction (dotted line) at
597 a 1:64 dilution. Solid black lines indicate the median. Significant differences were tested
598 using a linear mixed effects model (***, $p<0.001$; *, $p<0.05$).

599

600 **Figure 1-figure supplement 1**

601 **SARS-CoV-2 specific IgG levels in seronegative patients and according to disease**
602 **outcome.**

603 A) S1- and B) N-specific IgG levels in 30 healthy donors. Solid black lines indicate the
604 median. C) Cumulative S1-, D) N- and E) RBD-specific IgG levels measured 13-25 days after
605 symptom onset in deceased (black symbols) and not deceased COVID-19 patients (blue
606 symbols). Solid black lines indicate the median.

607

608 **Figure 1-figure supplement 2**

609 **Longitudinal changes in anti- SARS-CoV-2 IgG titers in severely and critically diseased**
610 **patients.**

611 Serial serum samples were collected from hospitalized COVID-19 patients and used for
612 SARS-CoV-2–specific IgG measurement. IgG responses against SARS-CoV-2 S1- and N-
613 protein in (A, C) critically (red symbols) and (B, D) severely (blue symbols) diseased patients.
614 Dotted lines represent cut-off values for commercial S1- and N- specific ELISA assays. Each
615 symbol represents the mean value of all samples which were available for each patient at the

616 indicated time range after symptom onset. There are no significant t-tests (i.e. $p>0.05$ for all
617 comparisons).
618
619 **Figure 2**
620 **CD16 activation by SARS-CoV-2 - specific IgG is enhanced in critically diseased**
621 **patients**.
622 FcγRIII activation by SARS-CoV-2-specific IgG on BW5147 reporter cells in serum samples
623 obtained 13-25 days following symptom onset from 23 critically (red symbols) and 14
624 severely (blue symbols) diseased patients. Between 2 to 8 samples/patient were analyzed
625 depending on the availability of sample material. Sera from 29 non-hospitalized patients with
626 mild SARS-CoV-2 infection (grey symbols) and 30 healthy donors (open circles) served as
627 reference. Each symbol represents the mean value of all available samples per patient. (A, B,
628 C) ELISA levels for S1- N- and RBD-specific IgG. Dotted lines represent cut-off values for
629 commercial S1-, N- and RBD - specific ELISA assays. Solid black lines indicate the mean.
630 (D, E, F) FcγRIII activation by S-, N- and RBD-specific IgG expressed as $\log_2$ fold change
631 relative to negative control. Solid black lines indicate the mean. (G, H, I) FcγRIII activation,
632 expressed as $\log_2$ values relative to SARS-CoV-2-spcific IgG titers. Solid black lines indicate
633 the mean. Significant differences over all three groups were tested by ANOVA and pairwise
634 group comparison was made by Games-Howell post-hoc tests (***, $p<0.001$; **, $p<0.01$; *,
635 $p<0.05$).
636
637 **Figure 2-figure supplement 1**
638 **Cell-based reporter assay measuring CD16 activation in response to immobilized IgG**
639 **and sICs**. BW5147 reporter cells expressing chimeric human FcγRIII secrete IL-2 in response
640 to FcγR activation by A) clustered viral specific IgG binding solid-phase antigen or B) soluble
641 ICs. Solubility of sICs is achieved by pre-blocking an ELISA plate with PBS supplemented
642 with 10% FCS as previously described[17].
643
644 **Figure 2-figure supplement 2**
645 **Dose dependent CD16 activation by SARS-CoV-2 specific IgG.**
646 CD16 activation by A) S- , B) N- and C) RBD-specific IgG in 9 representatively selected
647 serum samples and one SARS-CoV-2 negative serum (dark blue bars). Sera were serially
648 diluted at 1:20, 1:100, 1:500 and 1:2500. FcγRIII activation initiates IL-2 secretion by
649 reporter cells, which is subsequently measured via ELISA (OD 450 nm). Based on this

650 empirical pretesting all sera were thereafter tested at 1:100 and 1:500 dilutions to reach an
651 optimal dynamic range of response. The OD values obtained by the 1:500 dilutions were used
652 for subsequent data analysis.
653
654 **Figure 2-figure supplement 3**
655 **Reproducibility of CD16 activation measurements by SARS-CoV-2 specific IgG.**
656 Selected sera which were available in sufficient amount from patients with critical (red
657 symbols) or severe (blue symbols) SARS-CoV-2 infection were tested in two independent
658 experiments to show reproducibility and consistency of results. CD16 activation by S-, N- and
659 RBD specific IgG is shown. Statistical tests using a Kolmogorov-Smirnov test indicate no
660 significant differences.
661
662 **Figure 2-figure supplement 4**
663 **Correlation of CD16 activation by virus specific IgG and ELISA levels.**
664 Pearson's correlation coefficient was used to assess the relationship between virus–specific
665 IgG levels and their capability to trigger CD16 activation on BW5147 reporter cells in 22
666 paired samples from patients with critical disease (red symbols), 14 paired samples from
667 patients with severe disease (blue symbols) and 28 samples from patients with mild disease
668 (grey symbols). Each dot represents the mean value obtained by the analysis of all samples
669 available at the indicated time points. (A-C) anti-S IgG, (D-F) anti-N IgG and anti-RBD-IgG
670 (E-I).
671
672 **Figure 3**
673 **Anti SARS-CoV-2 IgG Fc core fucosylation in critical and severe COVID-19 cases.**
674 IgG-Fc core fucosylation levels of SARS-CoV-2 –specific IgG in critically (red bars) and
675 severely (blue bars) diseased COVID-19 patients. Analysis was carried out on a pool of 5
676 different sera. Measured OD values for fucosylation of the generated eluates were normalized
677 to their respective IgG titers determined by antigen-specific S1 and N ELISA. A) S-IgG-Fc-
678 fucosylation and B) N-IgG-Fc-fucosylation in critically and severely diseased patients
679 characterized by either high (red) or low (patterned) CD16-activation levels in the FcγR
680 activation reporter assay. The mean and standard deviation (SD) of at least three independent
681 experiments is depicted. Statistical tests using a two-factorial linear model indicate three
682 significant differences between the low and high categories (***, $p<0.001$; **, $p<0.01$; *,
683 $p<0.05$; ns = not significant).

684 **Figure 4**

685 **Severe COVID-19 disease coincides with high CD16 activation by sICs.**

686 Serial serum samples obtained 13-25 days after onset of symptoms were analyzed in a cell-
687 based reporter assay which is sensitive to sIC amount and size[16]. FcγR activation is shown as
688 $\log_2$ fold change relative to negative control. Each symbol represents the mean value obtained
689 by the analysis of all samples available in the indicated time range for each individual patient.
690 A) Analysis of CD16 activation by sICs in SARS-CoV-2-infected patients compared to
691 healthy blood donors B) Levels of IC-mediated CD16 activation across severe, critical and
692 mild clinical courses of COVID-19 disease, in healthy donors (HD) and in non-COVID-19-
693 patients who developed acute respiratory distress syndrome (ARDS). Solid black lines
694 indicate the mean. Two-group comparisons with the linear model indicate significant
695 differences between critical cases and all other groups, as well as between severe cases and all
696 other groups (***, $p<0.001$; **, $p<0.01$). No significant differences ($p>0.05$) have been found
697 for the comparisons mild vs. healthy and for HD vs. ARDS. C) Kinetics of IC-mediated CD16
698 activation in critically and severely diseased patients. Solid black lines indicate the median.
699 The mixed effects model indicates two time points with significant differences (**, $p<0.01$; *,
700 $p<0.05$).

701

702 **Figure 4-figure supplement 1**

703 **CD16 activation by sICs in non-COVID-19 patients with ARDS.**

704 Serum samples from 47 patients with ARDS in response to infections of different etiology
705 were analyzed in a cell-based reporter assay which is sensitive to sIC amount and size[16]. FcγR
706 activation is shown as $\log_2$ fold change relative to negative control. Each symbol represents
707 one sample from one patient. CMV: Cytomegalovirus reactivation under immunosuppression;
708 HIV: HIV infection; TBC: Mycobacterium tuberculosis infection; Influenza: influenza virus
709 infection; TX: solid organ transplantation. Solid black lines indicate the median.

710

711 **Figure 4-figure supplement 2**

712 **PEG precipitation eliminates sIC-mediated CD16 activation.**

713 Pools of 8 sera were incubated with equal volumes of PEG8000 to reach the indicated final
714 PEG concentrations. A) CD16 activation after PEG-precipitation in the pool supernatant,
715 showing either high (sICs+) or no (sICs-) CD16 activation. Sera from healthy donors (HD)
716 were included as a negative control. Activation levels are expressed as IL-2 levels (OD 450
717 nm) released by reporter cells. The mean and SD of two independent experiments is depicted.

718 B) Anti SARS-CoV-2 IgG levels against S1 (left panel) or N (right panel) IgG following PEG
719 precipitation. The mean and SD of two independent experiments (sIC+/sIC-) is depicted.
720
721 **Figure 4-figure supplement 3**
722 **Individual CD16 activation by sICs and anti-S1 ELISA IgG kinetics post symptoms**
723 **onset.**
724 Individual sera for either critically or severely diseased patients were analyzed in order to
725 detect anti S1-IgG antibodies (top panel) via Euroimmune ELISA [AU] and CD16 activation
726 by soluble immune complexes (lower panel, relative CD16 activation depicted as fold
727 increase to the negative control) over time (1-40 days post symptom onset). White panel: not
728 tested.
729
730 **Figure 4-figure supplement 4**
731 **Benzonase treatment of sIC-reactive sera does not abolish CD16 activation.**
732 Left panel: sIC-mediated CD16 reactivity expressed as $\log_2$ fold increase to the negative
733 control, in serum of six individual patients before and after treatment with 250 Units of
734 Benzonase Nuclease. Right panel: As positive control, 3 µg plasmid DNA was digested. M:
735 1kb DNA ladder, Lane 1: benzonase digestion in the presence of human serum, lane 2:
736 plasmid DNA w/o benzonase in the presence of human serum, lane 3: benzonase digestion in
737 medium only and lane 4: plasmid DNA w/o benzonase in medium only.
738
739
740 **Figure 5**
741 **Summary of antibody features from SARS-CoV-2-infected patients with critical and**
742 **severe disease.**
743 Relative multivariate antibody features illustrated as radar chart in critically (red) or severely
744 (blue) diseased COVID-19 patients normalized to the corresponding features of patients with
745 mild infection (grey). Each spoke represents one of the following variables: ELISA (S1-IgG,
746 N-IgG,) and CD16 activation (S-IgG, N-IgG, sICs). Arithmetic mean values of $\log_2$ values
747 were calculated for each group (days 13-25 post symptom onset) respectively. The fold
748 change compared to mildly diseased patients is shown.
749
750
751

752 **Table 1**

753 **Clinical characteristics of the hospitalized SARS-CoV-2 patients.**

754 Patients were categorized as either severely (hospitalized, requiring O_2 supplementation,
755 n=14) or critically diseased (hospitalized and in need of invasive mechanical ventilation,
756 n=27). Diagnostic markers are depicted as mean and SD (in brackets) of all analyzed
757 laboratory parameters obtained 13-25 days post symptom onset. Percentage [%] is indicated.

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Performed the experiments: J.A., N.G., U.S., S.G., K. C., W.B.

Analyzed the data: J.A., S.G., P.K., V.F., K.C., A.M-P., W.B., C.K.

Contributed/reagent/sample material: A.B.G., D.H., T.W., N.G.M.

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Review and editing: H.H., M.S., K.C.

Conceptualization: V.F., H.H.

Competing interests: The authors declare the following competing financial interest(s): InVivo BioTech Services is a biotechnology company producing antibodies and proteins, including SARS-CoV-2 antigens.

Data and materials availability: All data associated with this study are present in the paper or Supplementary Materials.

937 **Figures and Tables**

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939 **Table 1**

	All patients n: 41	%	critical n: 27	%	severe n: 14	%
Age [years]	Ø 68 (31-90)	-	Ø 63 (39-79)	-	Ø 76 (31-90)	-
Female	8	19.5	5	18.5	3	21.4
Male	33	80.5	22	81.5	11	78.6
Comorbidities						
Hypertension	21	51.2	12	44.4	9	64.3
Cardiovascular disease	14	34.1	5	18.5	9	64.3
Pulmonary disease	6	14.6	2	7.4	4	28.6
Chronic kidney disease	6	14.6	1	3.7	5	35.7
Diabetes	10	24.4	6	22.2	4	28.6
Malignancy	8	19.5	4	14.8	4	28.6
none	6	14.6	6	22.2	0	0
Diagnostic markers						
Interleukin-6 [pg/ml] Ø	1012.8	-	1452.1 (3774.6)	-	46.1 (26.8)	-
Procalcitonin [ng/ml] Ø	7	-	9.9 (21.9)	-	0.17 (0.11)	-
C- reactive protein [mg/l] Ø	128.1	-	162.2 (75.8)	-	65.3 (47.1)	-
Complications						
Bacterial superinfection	16	39	11	40.7	5	35.7
Treatment						
Hydroxychloroquine, Ritonavir+ Lopinavir (Kaletra®)	24	58.5	18	66.7	6	42.9
Fatal outcome						
Total	11	26.8	10	37	1	7.1

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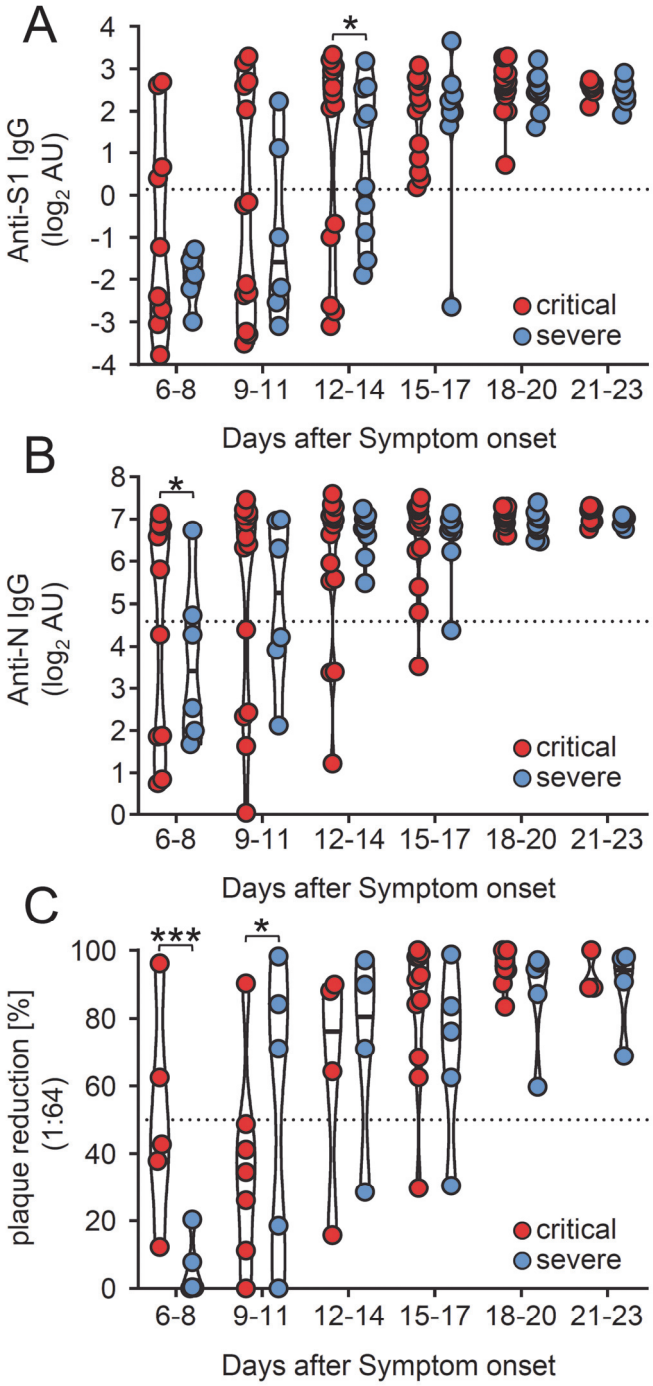
942 **Figure 1**



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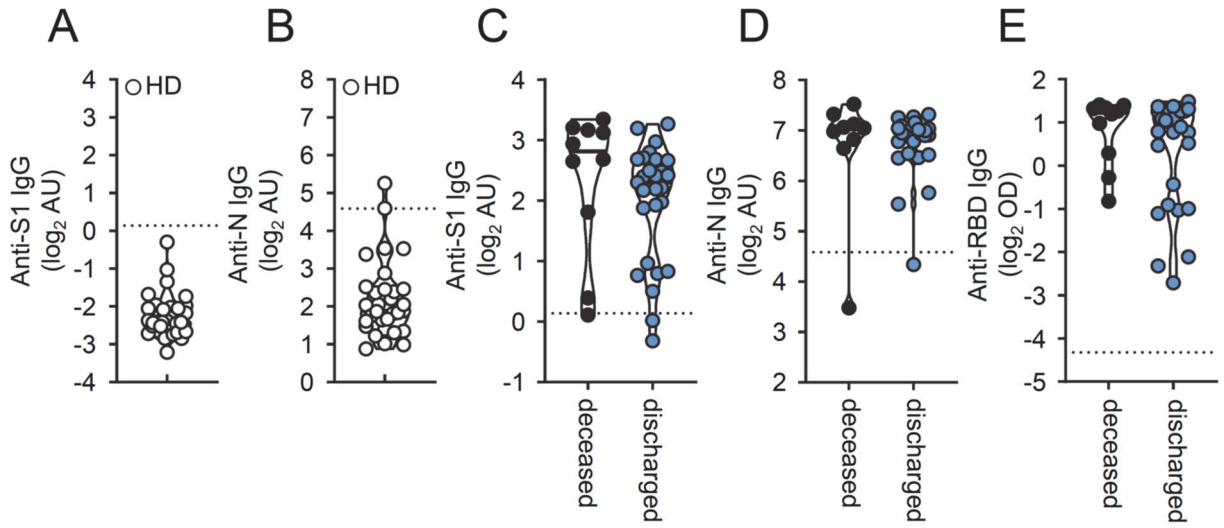
946 **Figure 1 – figure supplement 1**



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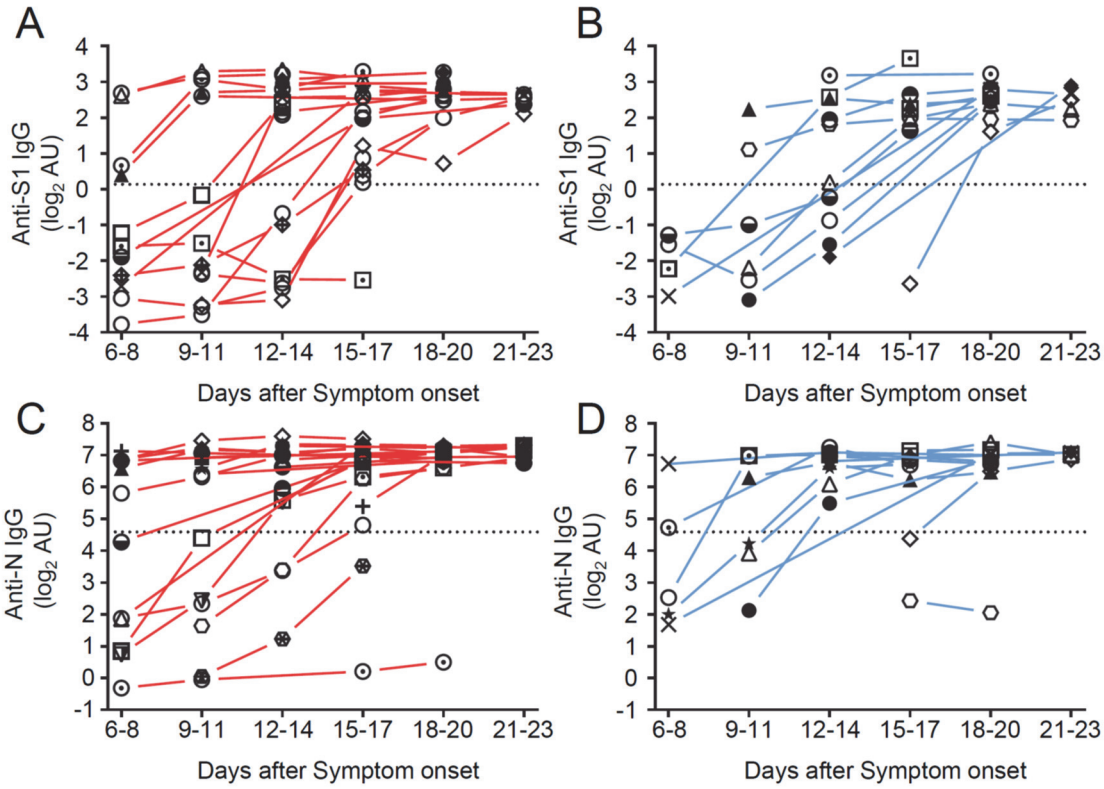

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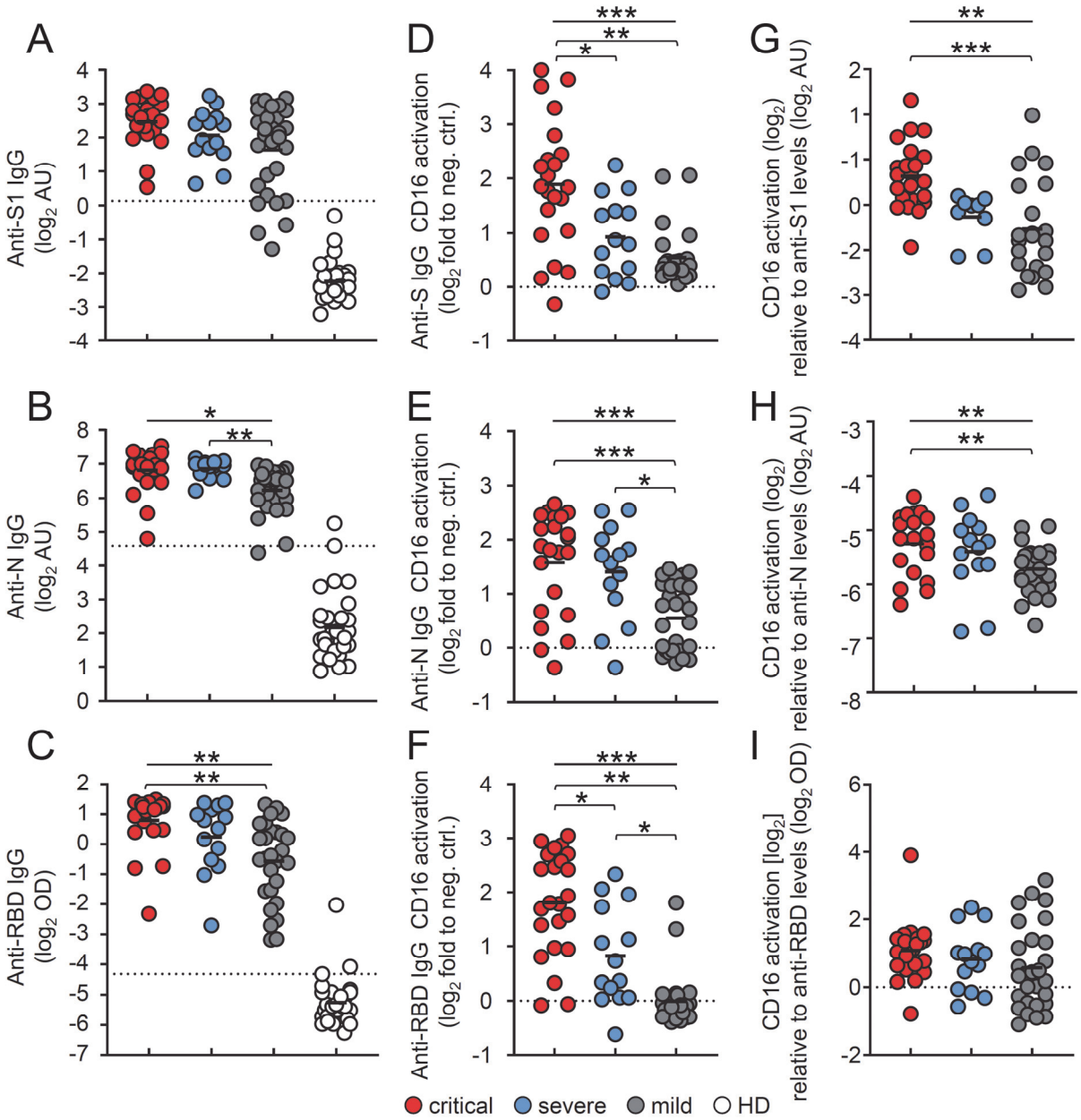

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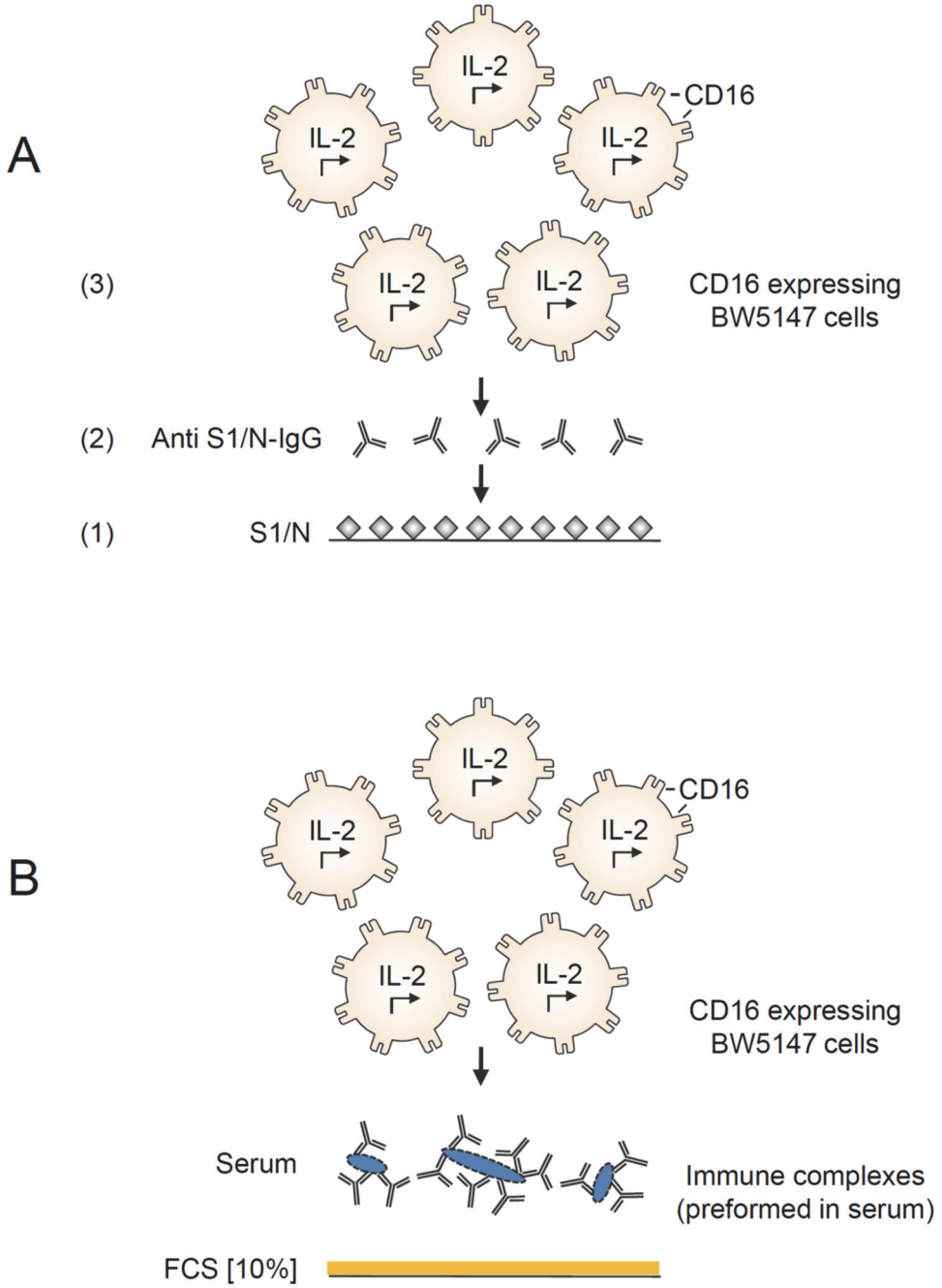

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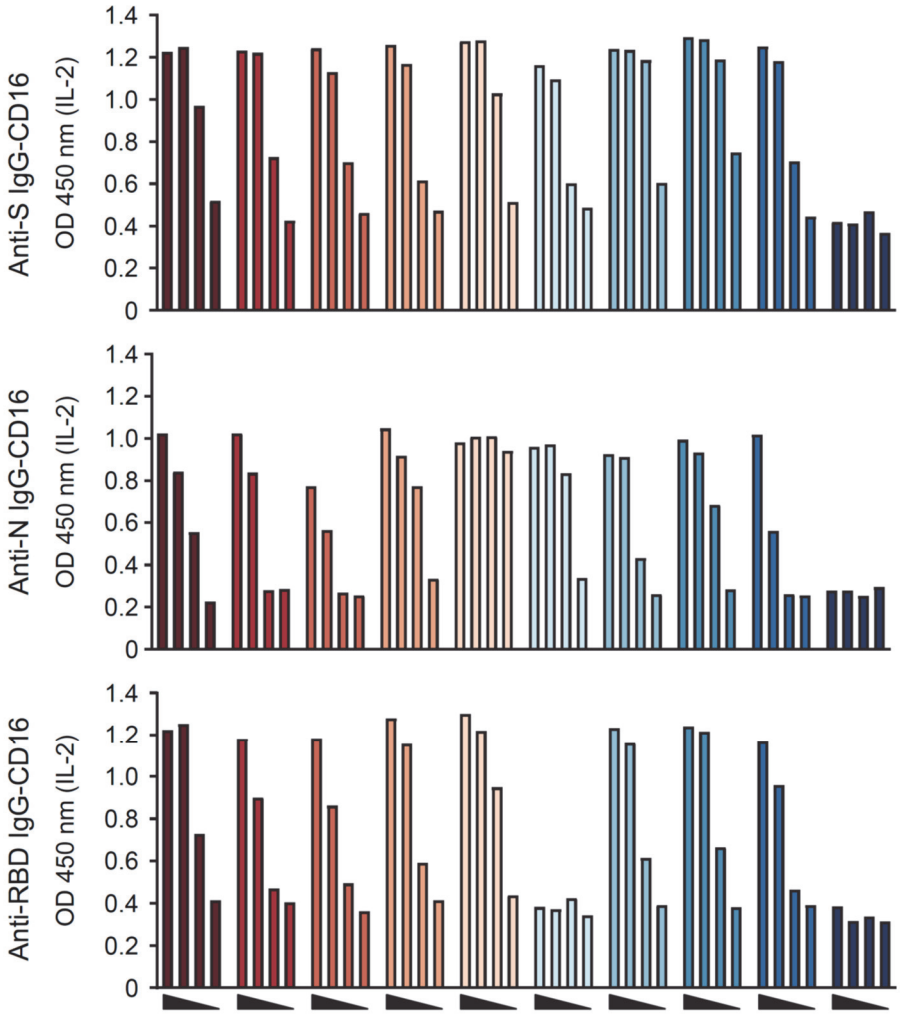

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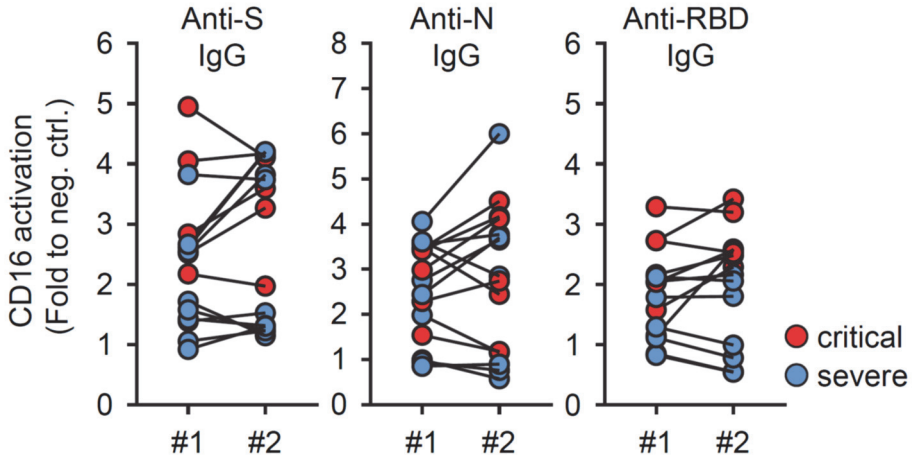

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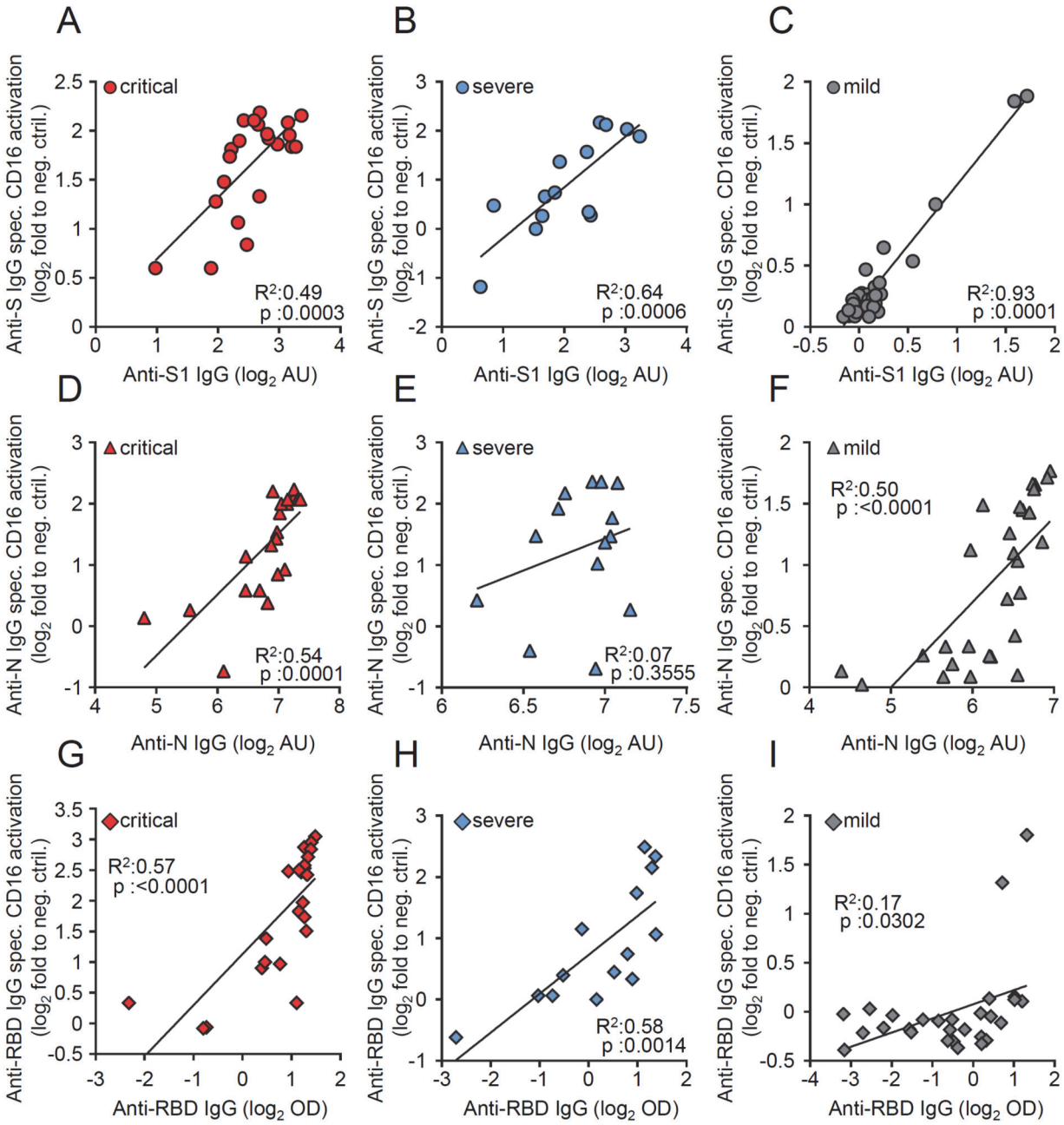

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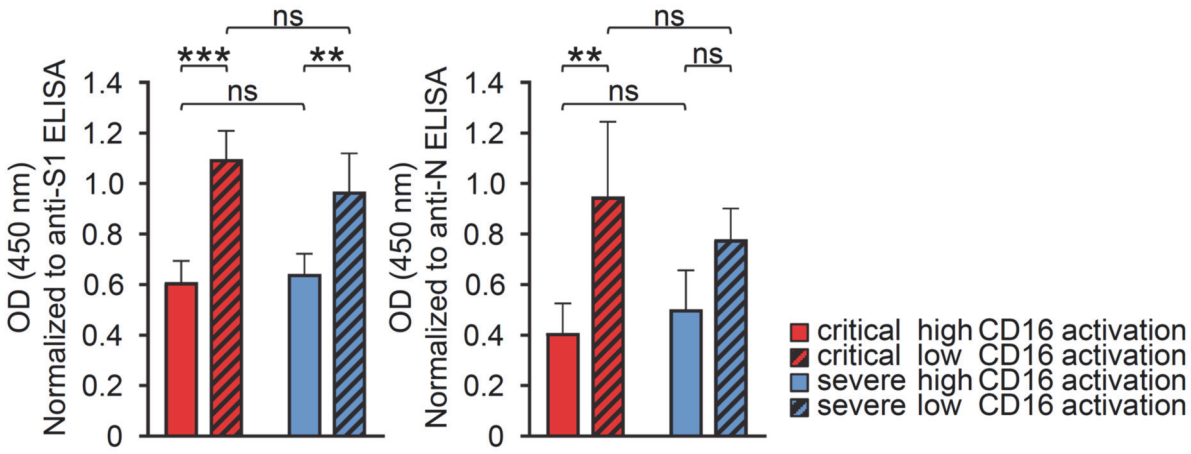

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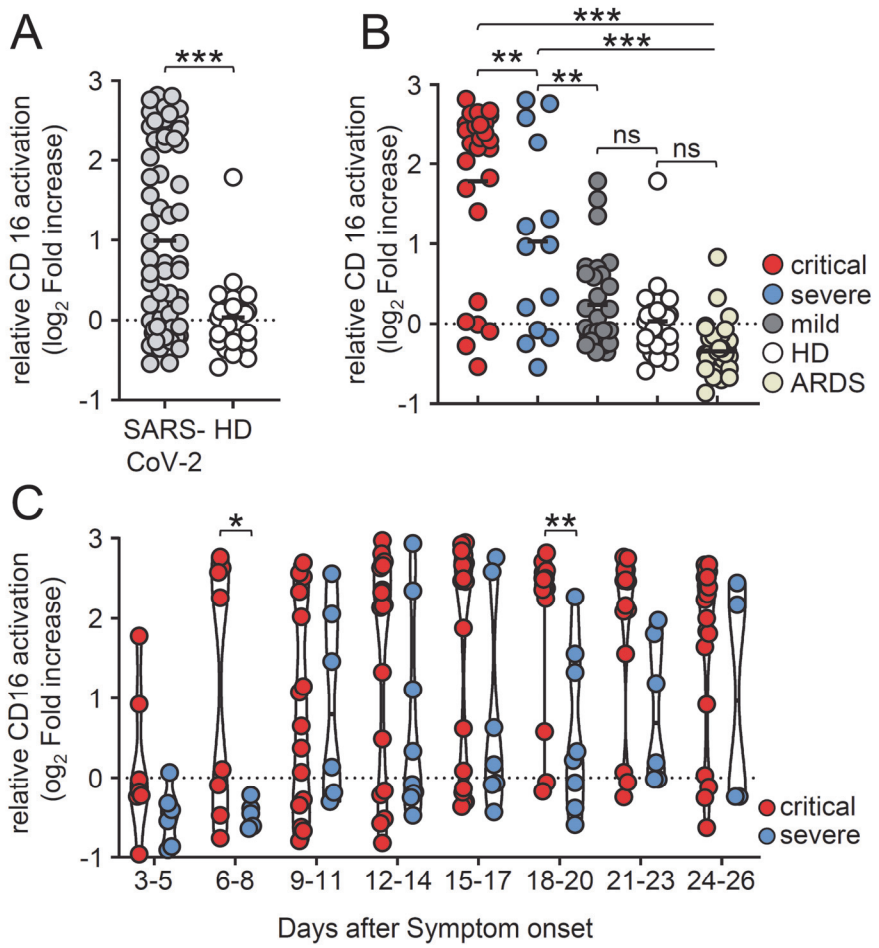

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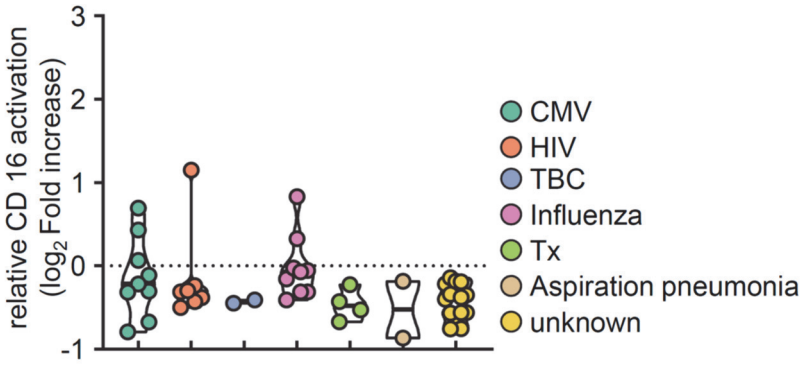

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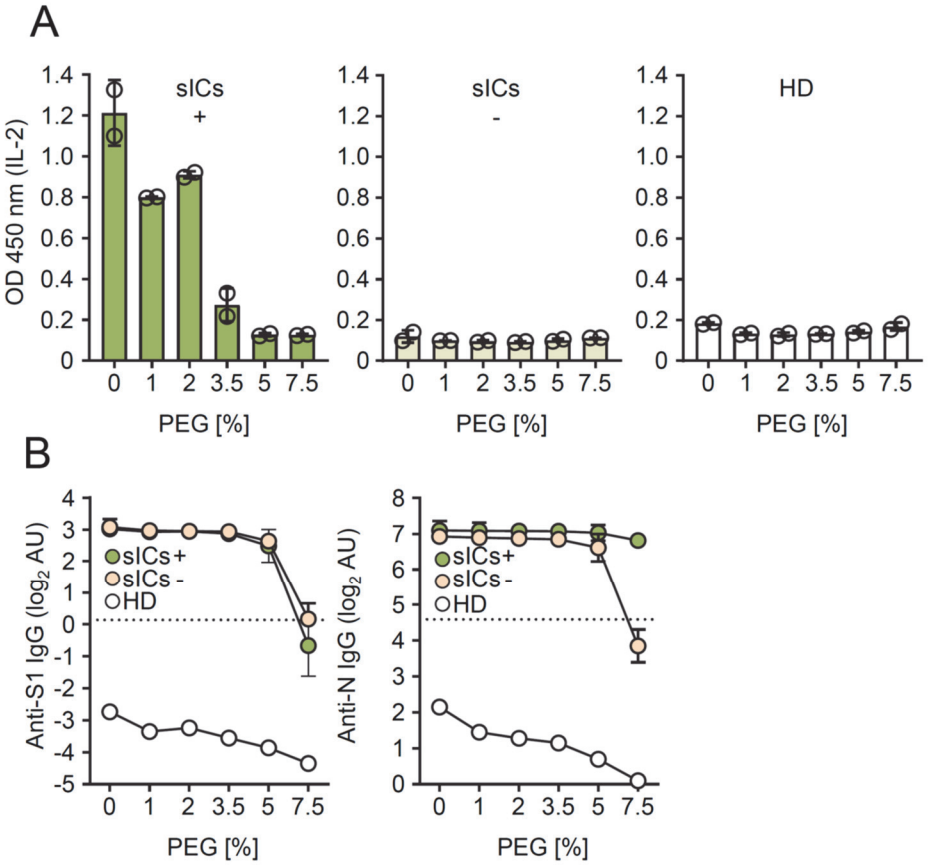

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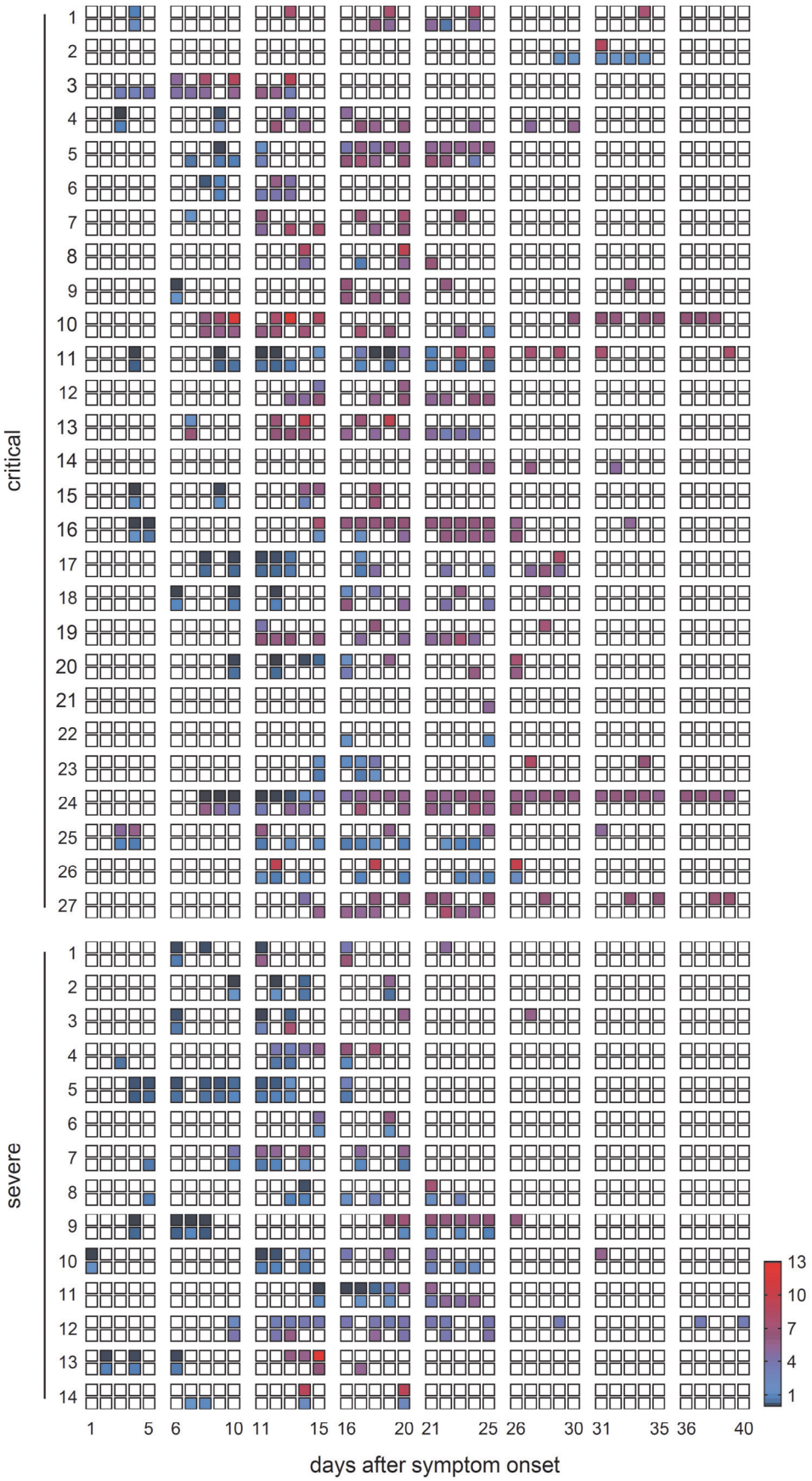

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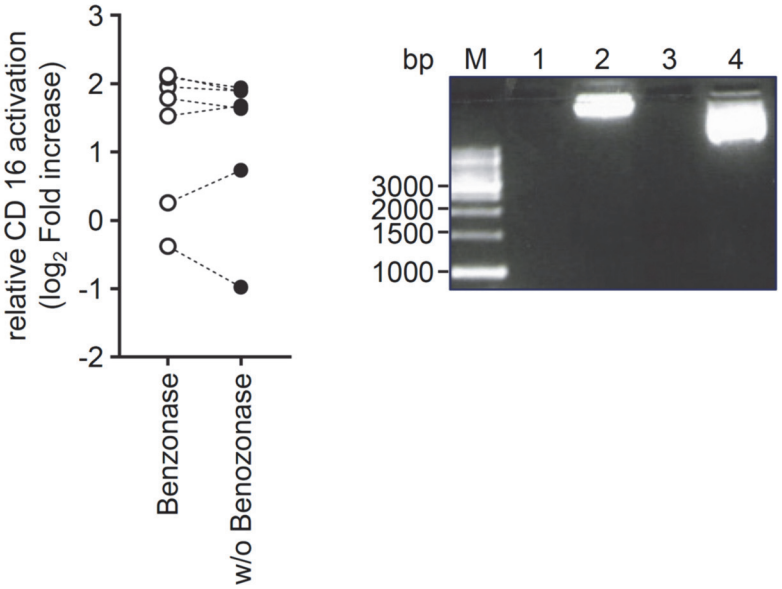

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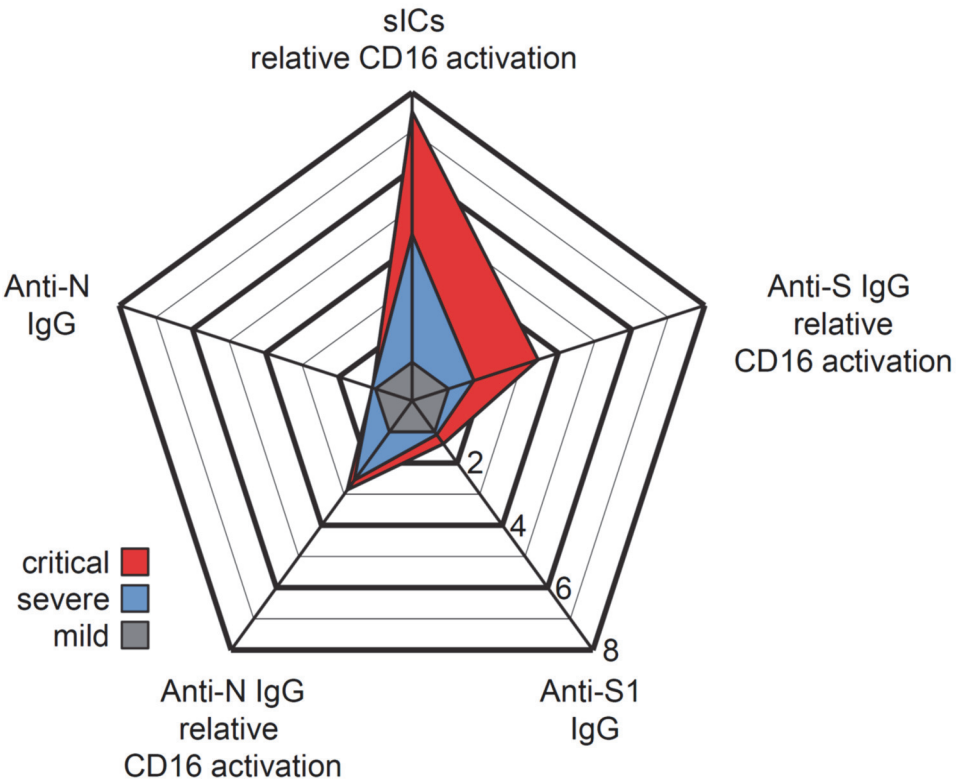
982 **Figure 5**



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