

1 **Circulating multimeric immune complexes drive immunopathology in COVID-19**

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31 **Conflict of interest**

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

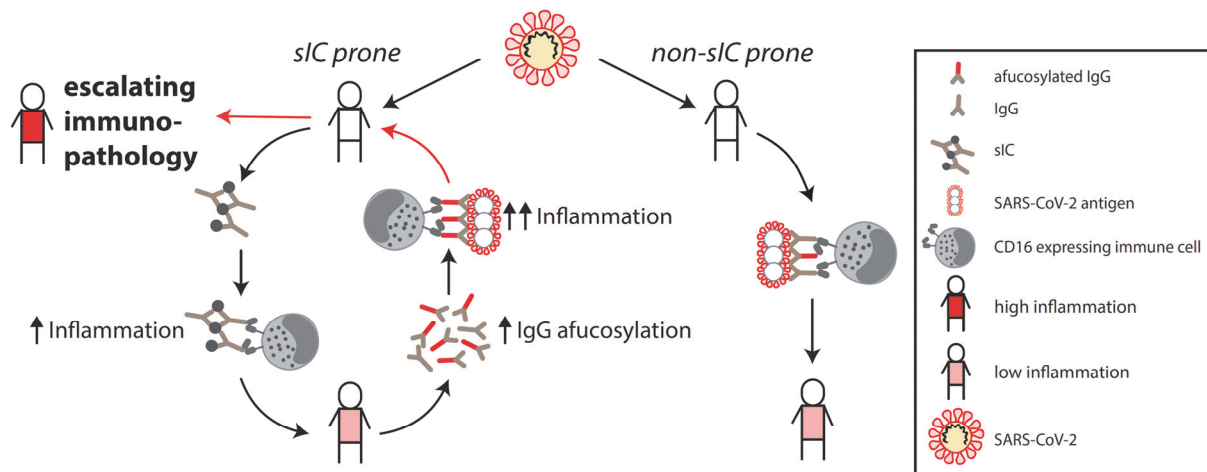
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49 **Graphical abstract**

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53 **A vicious cycle of immunopathology in COVID-19 patients is driven by soluble multimeric**
54 **immune complexes (sICs).** SARS-CoV-2 infection triggers sIC formation in prone
55 individuals. Activation of Fc γ RIII/CD16 expressing immune cells by sICs precedes a humoral
56 response to SARS-CoV2 infection. sICs and infection add to IgG afucosylation, further
57 enhancing Fc γ RIII/CD16 activation by opsonized targets. High inflammation induces further
58 sIC mediated immune cell activation ultimately leading to an escalating immunopathology.

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60

61 **Abstract**

62 A dysregulated immune response with high levels of SARS-CoV-2 specific IgG antibodies
63 characterizes patients with severe or critical COVID-19. Although a robust IgG response is
64 traditionally considered to be protective, excessive triggering of activating Fc-gamma-receptors
65 (Fc γ Rs) could be detrimental and cause immunopathology. Here, we document that patients
66 who develop soluble circulating IgG immune complexes (sICs) during infection are subject to
67 enhanced immunopathology driven by Fc γ R activation. Utilizing cell-based reporter systems
68 we provide evidence that sICs are predominantly formed prior to a specific humoral response
69 against SARS-CoV-2. sIC formation, together with increased afucosylation of SARS-CoV-2
70 specific IgG eventually leads to an enhanced CD16 (Fc γ RIII) activation of immune cells
71 reaching activation levels comparable active systemic lupus erythematosus (SLE) disease. Our
72 data suggest a vicious cycle of escalating immunopathology driven by an early formation of
73 sICs in predisposed patients. These findings reconcile the seemingly paradoxical findings of
74 high antiviral IgG responses and systemic immune dysregulation in severe COVID-19.

75

76 **Keywords**

77 Soluble immune complexes (sICs), Fc γ receptors, COVID-19, inflammation,
78 immunopathology

79

80 **Clinical implications**

81 The identification of sICs as drivers of an escalating immunopathology in predisposed patients
82 opens new avenues regarding intervention strategies to alleviate critical COVID-19
83 progression.

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87 Introduction

88

89 Since the emergence of SARS-CoV-2 in late December 2019 (1), more than 199 million
90 laboratory confirmed infections (as of August 3rd, 2021) have been reported, with cases
91 continuously rising (2). Accordingly, rapid insights into the disease manifestations and
92 pathogenesis have been globally obtained. A hallmark of the coronavirus disease 2019
93 (COVID-19) is a respiratory infection which can progress to an acute respiratory distress
94 syndrome (ARDS). Next to asymptomatic infections, COVID-19 symptoms differ widely
95 according to the disease process and may comprise fever, coughing, pneumonia, dyspnea,
96 hypoxia and lymphopenia (3). While fever and coughing are common symptoms, pneumonia,
97 hypoxia, dyspnea, certain organ manifestations and lymphopenia indicate critical or fatal
98 infections (3-6). Pronounced dyspnea can eventually progress to ARDS, a severe complication
99 frequently observed in critically ill patients (7, 8). Although overall disease severity and in
100 particular breathing difficulties are related to viral load (9), age (4, 10-13) and underlying
101 medical conditions (4, 11, 12), the delayed kinetics of respiratory failure strongly suggest an
102 essential role of the host immune response (3, 11). Typically, aggravation occurs between 9-11
103 days after symptom onset (12) and correlates with high levels of SARS-CoV-2 specific IgG
104 antibodies and systemic effects of pro-inflammatory cytokines such as IL-6 and TNF α (3, 14-
105 16). This cytokine release, primarily the result of macrophage and T helper (T_H) cell activation
106 (17), includes pattern recognition receptor (PRR) signaling in the context of innate immunity
107 but can also occur by Fc γ receptor (Fc γ R) activation (18). Triggered by immune complexes
108 (antibody-antigen complex), the cytokine release following Fc γ R activation represents a potent
109 defense mechanism against invading pathogens. A prototypical activating Fc γ R in this regard
110 is Fc γ RIII (CD16) expressed by NK cells (19, 20), monocyte-derived macrophages (CD16A)
111 (21) or neutrophils (CD16B, 98% sequence identical ectodomains). Specifically, CD16 is able
112 to sense circulating soluble immune complexes (sICs) as they are formed in certain autoimmune
113 diseases such as systemic lupus erythematosus (SLE) (22-25) and viral infections (26).
114 Overstimulation of activating Fc γ Rs in these cases is associated with disease severity (26-28)
115 and thus an Fc γ R-driven overshooting inflammatory response (18) might be an explanation for
116 the pronounced immunopathology observed during severe courses of COVID-19 (29).
117 Consistently, hyper-inflammation in SARS-CoV-1 and MERS infected patients has been
118 previously proposed as a possible pathogenic factor (30) and could be demonstrated in mice
119 and macaques infected with SARS-CoV-1 (31, 32). Furthermore, N297-dependent glycan-
120 modifications such as afucosylation within the constant region of IgG antibodies are known to
121 enhance Fc γ R binding, in turn promoting inflammation. It has been shown that enhanced
122 Fc γ RIII activation by low-fucosylated anti-SARS-CoV-2-S IgG leads to excessive alveolar
123 macrophage activation, driving severe COVID-19 disease progression (33). Further, it has been
124 proposed that uncleared antigen-antibody immune complexes (ICs) might be involved in the
125 pathogenesis of severe disease leading to systemic complement activation and tissue damage,
126 neutrophil activation, cytokine storm, systemic vasculitis, microvascular thrombosis and organ
127 failure (34-39). However, comprehensive evidence that circulating sICs impact disease
128 progression is still missing. Here, we aimed to further delineate the contribution of IgG-
129 mediated effector functions regarding COVID-19 severity in patient cohorts with varying
130 disease progressions. This revealed a marked correlation between CD16 activation by patient
131 IgG and severity of disease. Additionally, we identified circulating CD16-reactive sICs to be
132 abundantly present in the serum of patients with critical and severe disease, but not in the serum
133 of patients with a mild disease. sIC levels were comparable to those found in SLE patients with
134 active disease. As sIC formation preceded a SARS-CoV-2 specific humoral response in most
135 cases, we conclude that a so far undisclosed predisposing condition divides patients into sIC-
136 prone and non-sIC-prone individuals with patients developing sICs in response to an infectious
137 trigger also developing enhanced disease. Our data suggest a vicious cycle leading to an

138 escalating immunopathology driven by the early formation of sICs. Our findings enable new
139 avenues of intervention against COVID-19 and highly warrant further investigation into the
140 origin and composition of sICs predisposing to COVID-19 disease.

141
142

143 **Materials and Methods**

144

145 **Subjects and specimens**

146 Between March 2020 and April 2020, 41 patients with SARS-CoV-2 infection confirmed by
147 real-time PCR were hospitalized in the University Medical Center, Freiburg. Serum samples
148 were collected during hospitalization for routine laboratory testing. Clinical data were obtained
149 from electronic medical records. A total of 27 patients necessitating invasive mechanical
150 ventilation were included in the critical group. Fourteen patients requiring O₂ supplementation
151 were included in the severe group. Additionally, serum samples from 29 mild COVID-19 cases
152 and 30 healthy donor (HD) plasma samples were used as controls in this study. For the SLE
153 patient control cohort, sera were obtained from the Immunologic, Rheumatologic Biobank (IR-
154 B) of the Department of Rheumatology and Clinical Immunology.

155

156 **Cell culture**

157 African green monkey kidney Vero E6 cells (ATCC CRL-1586) were cultured at 37°C in
158 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal calf
159 serum (FCS, Biochrom), sodium pyruvate (1x, Gibco) and 100 U/ml penicillin-Streptomycin
160 (Gibco). BW5147 mouse thymoma cells (BW, obtained from ATCC: TIB-47) were stably
161 transduced with human FcγR as previously described (40, 41). Cells were maintained at 3x10⁵
162 to 9x10⁵ cells/ml in Roswell Park Memorial Institute medium (RPMI GlutaMAX, Gibco)
163 supplemented with 10% (vol/vol) FCS, sodium pyruvate (1x, Gibco), 100 U/ml penicillin-
164 Streptomycin (Gibco) β-mercaptoethanol (0.1 mM, Gibco). Cells were cultured at 37°C, 5%
165 CO₂. All cell lines were routinely tested for mycoplasma.

166

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

168 Serum IgG antibody titers targeting S1- and N-SARS-CoV-2 proteins were measured using
169 commercial enzyme-linked immunosorbent assay (ELISA). Anti-S1- SARS-CoV-2 IgG was
170 measured by the anti-SARS-CoV-2 ELISA (IgG) Euroimmune Kit (Euroimmune, Lübeck,
171 Germany) according to manufacturer's protocol. Results, expressed as arbitrary units (AU),
172 were evaluated semi-quantitatively by calculation of the ratio of the extinction of the control or
173 patient sample over the extinction of the calibrator. This ratio is interpreted as follows: < 0.8
174 negative; ≥ 0.8 to <1.0 borderline; ≥ 1.1 positive. Anti-N SARS-CoV-2 IgG was detected using
175 the recomWell SARS-CoV-2 IgG Kit (Mikrogen Diagnostik GmbH, Neuried, Germany)
176 according to manufacturer's protocol. The corresponding antibody activity expressed in AU/ml
177 is calculated using the formula (absorbance of sample / absorbance of cut-off) × 20. Results are
178 interpreted as follow: < 20 negative; ≥ 20 to < 24 borderline; > 24 positive. IgG against the
179 SARS-CoV-2 Spike Glycoprotein Receptor Binding Domain (RBD) were detected using
180 SARS-CoV-2 IgG ELISA Reagent Set, kindly provided by InVivo (InVivo Biotech Services
181 GmbH, Hennigsdorf, Germany) according to manufacturer's protocol.

182

183 **Fcγ receptor activation assay**

184 FcγRIIIA (CD16A, 158V) activation was measured by a cell-based assay as previously
185 described (42). For detection of anti-S and anti-RBD-specific FcγR activation we utilized
186 SARS-CoV-2-S- and RBD-coated plates (kindly provided by InVivo Biotech Services GmbH,
187 Hennigsdorf, Germany). The recombinant (S)-protein was produced under serum-free
188 conditions in mammalian cells and contains amino acid residues 1 to 1213 of the SARS-CoV-

189 2 Wuhan-Hu-1-isolate (GenBank annotation QHD43416.1). The furin cleavage site was
190 mutated, two mutations for protein stabilization were included, and the C-terminal domain was
191 replaced by a T4 trimerization sequence and a C-terminal hexa-His-Tag (43). The recombinant
192 RBD-protein represented amino acids 319 to 541 of the (S)-protein mentioned before. Both
193 recombinant proteins were purified using immobilized metal exchange chromatography
194 (IMAC) and preparative SEC under standard conditions in a regulated environment. Microtiter
195 plates were coated using 0.2 µg recombinant (S)-protein or RBD-protein per well. N-specific
196 FcγR activation was determined using plates coated with SARS-CoV-2-N (Mikrogen
197 Diagnostik GmbH, Neuried, Germany). Respective plates were subsequently incubated with
198 serial dilutions of SARS-CoV-2 positive sera or control sera in RPMI supplemented with 10%
199 (vol/vol) FCS for 30 min at 37°C. All wells were thoroughly washed before co-cultivation with
200 BW5147 reporter cells for 16 h at 37°C, 5% CO₂. Cross-link activation of reporter cells was
201 performed by direct coating of target antibody to ELISA plate (Nunc Maxisorp; 96 well, flat
202 transparent), followed by a blocking step and incubation with 2 × 10⁵ reporter cells per well.
203 For all activation assays, mouse IL-2 secretion was quantified by anti-IL-2 ELISA, as described
204 earlier. FcγRIIIA (CD16A) activation by multimeric sICs was measured by a recently
205 developed cell-based assay (25, 44). Briefly, 2x10⁵ BW5147-CD16 reporter cells were
206 incubated with SARS-CoV-2 sera in a total volume of 200 µl for 16 h at 37°C, 5% CO₂.
207 Incubation was performed in a 96-well ELISA plate (Nunc Maxisorp) pre-treated with PBS
208 containing 10% FCS for 1 h at 4°C to avoid direct binding of serum IgG to the plate. Reporter
209 cell mIL-2 secretion was quantified via ELISA as described previously (42).

210

211 **Purification of SARS-CoV2-S and -N specific antibodies from serum**

212 SARS-CoV-2-specific antibodies were purified using SARS-CoV-2 spike protein (S)-coated
213 plates (kindly provided by InVivo BioTech Services) and - nucleocapsid (N) - coated plates
214 recomWell SARS-CoV-2 IgG (Mikrogen Diagnostik GmbH, Neuried, Germany). Patient sera
215 were diluted 1:5 in 100 µl (two wells per serum sample) and incubated for one hour at 37°C
216 with the S- and N-precoated plates. After washing using PBS-T (0.05% Tween 20) 100 mM
217 formic acid (30 µl/well) was added and incubated for 5 min on an orbital shaker at room
218 temperature (RT) to elute bound IgG. Following pH neutralization using TRIS buffer (1 M),
219 the eluates were either directly processed or stored at 4°C.

220

221 **Quantitation of antigen-specific IgG amount**

222 In order to determine the relative S1- and N-SARS-CoV-2 specific IgG antibody concentration
223 of the generated eluates, S1- and N-ELISA were performed by the anti-SARS-CoV-2 ELISA
224 (IgG) Euroimmune Kit (Euroimmune, Lübeck, Germany) and anti-N SARS-CoV-2 IgG ELISA
225 (recomWell SARS-CoV-2 IgG Kit (Mikrogen Diagnostik GmbH, Neuried, Germany) as
226 aforementioned.

227

228 **Analysis of antigen-specific IgG-Fc fucosylation**

229 Fucosylation levels of S- and N-specific IgG were measured using a lectin-based ELISA assay.
230 Briefly, 96-well Maxisorb plates (Nunc®) were coated with 50µl/well anti-human IgG-Fab
231 fragment (MyBiosource, MBS674607) at a concentration of 2 µg/ml, diluted in PBS for one
232 hour at 37°C. After three washing steps with PBS-T (0.05% Tween20) unspecific binding sites
233 were blocked adding 300 µl/well Carbo-free™ blocking solution (VectorLab, Inc., SP-5040,
234 LOT: ZF0415) for one hour at room temperature. After three further washing steps, eluted
235 antibodies were serially diluted (2-fold) with PBS in a total volume of 30 µl/well and incubated
236 for one hour at 37°C and 5% CO₂. After washing (3x) using PBS-T, 50 µl/well of 4 µg/ml
237 biotinylated Aleuria Aurantia lectin (AAL, lectin, VectorLab, B-1395) diluted in lectin buffer
238 (10 mM HEPES, 0.1 mM CaCl₂, 0.15 M NaCl, 0.1% Tween20) was added and incubated for
239 45 min at room temperature (RT). Following another three washing steps using PBS-T,

240 Streptavidin-Peroxidase Polymer (Sigma, S 2438), at 1 µg/ml final concentration diluted in
241 LowCross-HRP®-buffer (Candor, Order #: 200 500) was added and incubated for one hour at
242 RT. After washing five times with PBS-T, 50 µl/well of 1-Step™ Ultra TMB-ELISA Substrate
243 Solution (ThermoFisher, 34028) was applied and the enzyme-substrate reaction was stopped
244 after six minutes using 50 µl/well sulphuric acid (1 M H₂SO₄). Quantification of absorbance,
245 OD_{450nm}, was performed using a Tecan M2000. Relative fucosylation for each generated pool-
246 eluate was calculated by normalizing OD_{450nm} (fucosylation) to its respective relative antigen-
247 specific IgG amount.

248

249 **PEG Precipitation**

250 Sera pools, consisting of eight different sera per pool, were diluted with varying amounts of
251 PEG8000, in order to reach a final PEG8000 concentration of 1, 2, 3.5, 5 and 7.5% respectively.
252 Mixtures were vortexed and incubated overnight at 4°C. For supernatant analysis, precipitates
253 were sedimented via centrifugation at 13.000 rpm for 30 minutes at 4°C. For Mass Spectrometry
254 analysis, PEG8000-precipitated sICs were shortly run into 10% polyacrylamide gels. After
255 over-night fixation (40% ethanol, 10% acetic acid, 50% water) and washing (3x), complete
256 lanes were excised.

257

258 **Benzonase treatment of sera**

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

266

267 **Immune precipitation**

268 For mass spectrometry analysis of SARS-CoV-2-S specific precipitates, individual sera
269 containing CD16-reactive soluble immune complexes were subjected to immune precipitation
270 (IP) using Pierce MS-compatible magnetic IP kit (ThermoFisher Scientific, Darmstadt,
271 Germany) according to manufacturer's protocol. Briefly 250 µl serum was incubated overnight
272 at 4°C with 5 µg of biotinylated anti-RBD-specific TRES-1-224.2.19 mouse monoclonal
273 antibody or TRES-II-480 (isotype control) (kind gift of H.M. Jäck, Erlangen) before addition
274 of streptavidin magnetic beads. Beads were subsequently collected via centrifugation and
275 elution buffer was added to detach putative precipitated antigen. The elution was dried in a
276 speed vacuum concentrator and shortly run into 10% polyacrylamide gels. After over-night
277 fixation (40% ethanol, 10% acetic acid, 50% water) and washing (3x), complete lanes were
278 excised. Antibody biotinylation was performed using a Pierce antibody biotinylation Kit for IP
279 (ThermoFisher Scientific, Darmstadt, Germany) according to manufacturer's protocol.

280

281 **Mass Spectrometry**

282 Proteins were in-gel digested with sequencing grade modified trypsin (Promega GmbH,
283 Walldorf, Germany) similar to the procedure described by Pandey et al. (45). Vacuum-dried
284 peptides were dissolved in 0.5% trifluoroacetic acid, loaded onto a trap column (C18
285 PepMap100, 5 µm particles, Thermo Fisher Scientific GmbH, Dreieich, Germany) with 0.05%
286 trifluoroacetic acid (4 min, 10 µL/min) and separated on a C18 reversed phase column
287 (SilicaTip™ emitter, 75 µm i.d., 8 µm tip, New Objective, Inc, Littleton, USA, manually packed
288 23 cm with ReproSil-Pur ODS-3, 3 µm particles, Dr. A. Maisch HPLC GmbH, Ammerbuch-
289 Entringen, Germany; flow rate: 300 nL/min). For sample injection and multi-step gradient
290 formation (eluent "A": 0.5% acetic acid in water; eluent "B": 0.5% acetic acid in 80%

291 acetonitrile / 20% water; gradient length / acquisition time: 100 min or 175 min) an UltiMate
292 3000 RSLCnano system (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used.
293 Eluting peptides were electrosprayed at 2.3 kV via a Nanospray Flex ion source into a Q
294 Exactive HF-X hybrid quadrupole-orbitrap mass spectrometer (both Thermo Fisher Scientific
295 GmbH, Dreieich, Germany) and analyzed by data-dependent acquisition with HCD (higher
296 energy collisional dissociation) fragmentation of doubly, triply and quadruply charged ions
297 (loop count and dynamic exclusion dependent on the gradient length). Peak lists were generated
298 with ProteoWizard msConvert (<http://proteowizard.sourceforge.net/>; version 3.0.11098), linear
299 shift mass recalibrated (after a preliminary database search) using software developed in-house
300 and searched against a database containing the SARS-CoV-2 UniProtKB reference proteome
301 (proteome ID: UP000464024), all human UniProtKB/Swiss-Prot entries, and optionally (to
302 reduce the number of incorrectly assigned matches) selected bacterial proteins (finally the
303 *Pseudomonas fluorescens* (strain SBW25) reference proteome; proteome ID: UP000002332)
304 with Mascot 2.6.2 (Matrix Science Ltd, London, UK; peptide mass tolerance: ± 5 ppm;
305 fragment mass tolerance: ± 20 mmu; one missed trypsin cleavage and common variable
306 modifications allowed).

307

308 **Neutralization assay**

309 Serum neutralization capacity was analyzed as previously described (46). Briefly, VeroE6 cells
310 were seeded in 12-well plates at a density of 2.8×10^5 cells/well 24 h prior to infection. Serum
311 samples were diluted at ratios of 1:16, 1:32 and 1:64 in 50 μ L PBS total volume. Negative
312 controls (PBS without serum) were included for each serum. Diluted sera and negative controls
313 were subsequently mixed with 90 plaque forming units (PFU) of authentic SARS-CoV-2 (B.1)
314 in 50 μ l PBS (1600 PFU/mL) resulting in final sera dilution ratios of 1:32, 1:64, and 1:128.
315 Following incubation at RT for 1 h, 400 μ L PBS was added to each sample and the mixture
316 was subsequently used to infect VeroE6 cells. After 1.5 h of incubation at RT, inoculum was
317 removed and the cells were overlaid with 0.6% Oxoid-agar in DMEM, 20 mM HEPES (pH
318 7.4), 0.1% NaHCO_3 , 1% BSA and 0.01% DEAE-Dextran. Cells were fixed 48 h post-infection
319 (4% formaldehyde for 30 minutes). Upon removal of the agar overlay, plaque neutralization
320 was visualized using 1% crystal violet. PFU were counted manually. Plaques counted for
321 serum-treated wells were compared to the average number of plaques in the untreated negative
322 controls, which were set to 100%.

323

324 **Ethics**

325 The protocol of this study conforms to the ethical guidelines of the 1975 Declaration of Helsinki
326 and was approved by the institutional ethical committee of the University of Freiburg (EK
327 153/20). Written informed consent was obtained from participants and the study was conducted
328 according to federal guidelines, local ethics committee regulations (Albert-Ludwigs-
329 Universität, Freiburg, Germany: No. F-2020-09-03-160428 and no. 322/20; No 507/16 and
330 624/14 for the SLE patients).

331

332 **Statistical analyses**

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

341

342 **Data and materials availability**

343 All data associated with this study are present in the paper or Supplementary Materials.

344

345

346 **Results**

347

348 **Patients and clinical information.**

349 We retrospectively analyzed serial serum samples collected for routine diagnostic testing from
350 41 patients hospitalized at our tertiary care center between March and June 2020 with SARS-
351 CoV-2 infection confirmed by real-time PCR. Based on the clinical course, we categorized
352 patients as either severely diseased (hospitalized with COVID-19 related pneumonia) versus
353 critically diseased (COVID-19 related pneumonia and eventually in need of invasive
354 mechanical ventilation). In total, 27 patients with critical and 14 with severe courses of disease
355 were grouped into separate cohorts (Table 1). Most patients were older than 60 years with an
356 overall mean age of 68 years (63 years and 76 years in the critically and severely diseased
357 patients respectively). The majority of patients in both groups had comorbidities of different
358 origin with cardiovascular diseases including hypertension representing the most frequent
359 pathology (35/41, 85%). Similar to previous reports, high Interleukin 6 (IL-6) and C-reactive
360 protein (CRP) levels were associated with severity of disease ($\bar{\text{O}}$ IL-6: 1452.1 pg/ml in the
361 critical group vs 46.1 pg/ml in the severe group and $\bar{\text{O}}$ CRP: 162.2 mg/l vs 65.3 mg/l, $\bar{\text{O}}$ 13-25
362 days post symptom onset respectively). Similarly, procalcitonin, a biomarker of microbial
363 coinfection, was significantly higher in critically diseased patients ($\bar{\text{O}}$ value 9.9 ng/ml vs 0.17
364 ng/ml). Bacterial superinfection represented a further complication in 39% of the patients and
365 was only slightly more frequent in patients with critical disease (11/27, 41% vs 5/14, 33%).
366 More than half of the patients (59%) were treated with hydroxychloroquine/Lopinavir and
367 Ritonavir (Kaletra®), (18/27, 67% in the critical group vs 6/14, 43% in the severe group).
368 Notably, at the time of serum acquisition, only one patient received steroid treatment, which
369 was given due to underlying chronic obstructive pulmonary disease. Finally, mortality rate was
370 37% (10/27) in critically and 7% (1/14) in severely diseased patients.

371

372

373 **Table 1: Clinical characteristics of the hospitalized SARS-CoV-2 patients.**
 374 Patients were categorized as either severely (hospitalized, requiring O₂ supplementation, n=14)
 375 or critically diseased (hospitalized and in need of invasive mechanical ventilation, n=27).
 376 Diagnostic markers are depicted as mean and SD (in brackets) of all analyzed laboratory
 377 parameters obtained 13-25 days post symptom onset. Percentage [%] is indicated.
 378

	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

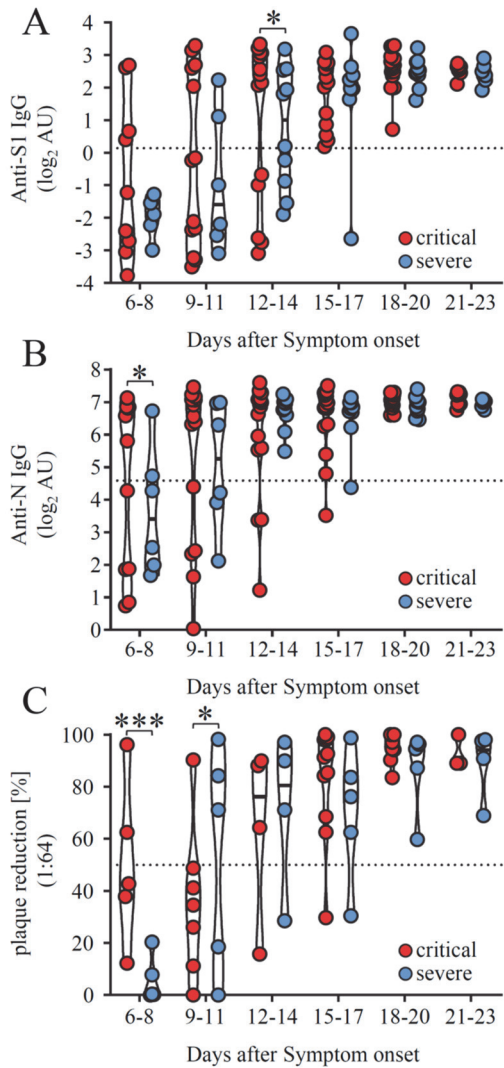
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Kinetics of IgG antibody responses following symptom onset across severe and critical courses of disease.

It has been observed that elevated SARS-CoV-2 antibody titers are associated with disease severity (15) and speculated to play a role not only in the clearance but also in the pathogenesis of SARS-CoV-2 infection (47). We initially analyzed the levels and kinetics of SARS-CoV-2 specific IgG in serial serum samples from patients hospitalized with critical (n=27) or severe (n=14) illness, a setting we also used in the following experiments. A total of 125 (critically diseased) and 79 (severely diseased) serum samples, obtained from the aforementioned patients at different time points within 6-25 days following symptom onset were analyzed by commercially available S1- and N- specific ELISA-based assays. Assay specificity was confirmed analyzing healthy donor (HD) serum samples (n=30) as negative control (Figure 1-figure supplement 1 A, B). Most patients developed detectable SARS-CoV-2 specific IgG responses within 9-14 days after symptom onset. SARS-CoV-2 specific IgG gradually increased over time in both severely and critically diseased patients reaching a plateau at 18-20

396 days after symptom onset (Figure 1 A, B). Varying antibody response kinetics were observed
397 for each individual patient (Figure 1-figure supplement 2 A-D) with anti-N IgG titers rising
398 significantly earlier than anti-S1 IgG (12.5 ± 3.3 days vs 10.6 ± 3.8 ; $p = 0.0091$). A trend
399 towards earlier seroconversion for anti-S1 IgG could be observed in critically diseased patients
400 (mean time of seroconversion 11.4 ± 3.0 days in critically diseased patients vs 12.9 ± 3.8 days
401 for severely diseased patients; $p = 0.24$), whereas time of seroconversion for anti-N IgG was
402 similar in both groups (10.1 ± 3.2 and 10.4 ± 4.2 days for critically and severely diseased
403 patients, respectively; $p = 0.83$). S1- and N-specific IgG levels at plateau did not significantly
404 differ between the two groups. No significant difference between deceased and discharged
405 patients was measured 13-25 days after symptom onset (Figure 1- figure supplement 1 C, D,
406 E). Next, we evaluated and compared the neutralizing capacity of SARS-CoV-2 antibodies in
407 either critically versus severely diseased patients in a plaque-reduction assay (Figure 1 C). All
408 patients mounted a robust neutralizing antibody response ($91\% \pm 10.5\%$ neutralization at a
409 1:64 serum dilution), with peaking titers at 18-20 days following symptom onset. Of note, two
410 critically diseased patients developed a neutralizing response already at 6-8 days after symptom
411 onset. In summary, we observed only minor differences in cohort wide kinetics of S1- or N-
412 specific IgG levels between patients hospitalized with severe or critical clinical courses
413 indicating that antibody levels per se did not correlate with severity of disease in our study.
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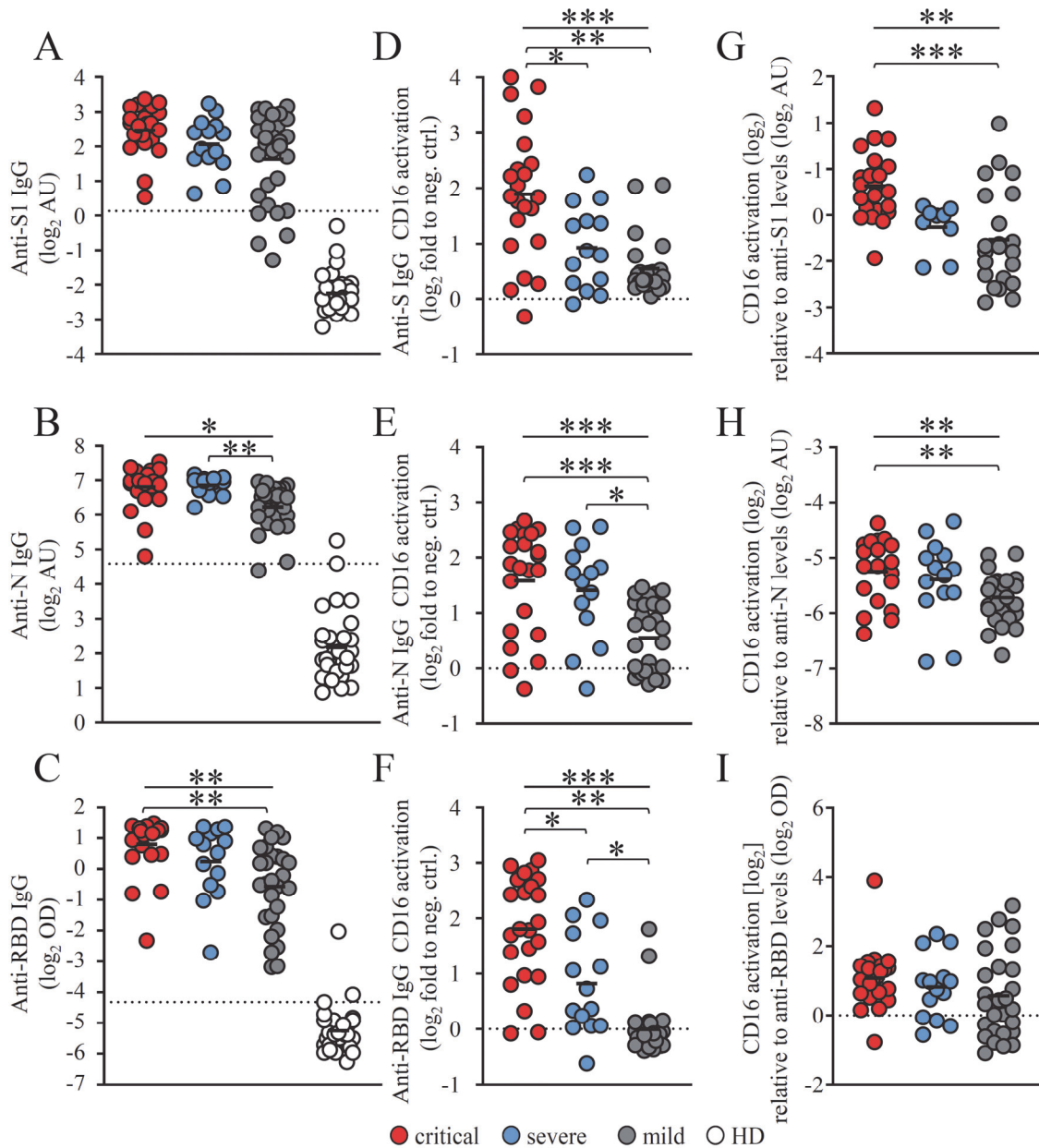
418 **Figure 1. IgG responses against different SARS-CoV-2 proteins across severe and**
419 **critical clinical course of disease.**

420 IgG antibody levels were analyzed in longitudinal serum samples from hospitalized SARS-
421 CoV-2 infected individuals. 27 patients were categorized as critically diseased when in need of
422 invasive mechanical ventilation (red symbols) compared to 14 severely diseased patients who
423 did not require invasive ventilation (blue symbols). (A) IgG response against SARS-CoV-2 S1
424 -protein and (B) SARS-CoV-2 N-protein as determined by commercial ELISA assays. Dotted
425 lines represent cut-off values for commercial S1- and N- specific ELISA assays. Each dot
426 represents the mean value obtained by the analysis of all samples which were available at the
427 indicated time points following symptom onset. Solid black lines indicate the median. (C)
428 Serum neutralization capacity against SARS-CoV-2 measured by a plaque reduction assay. Sera
429 were considered neutralizing upon 50% plaque reduction (dotted line) at a 1:64 dilution. Solid
430 black lines indicate the median. Significant differences were tested using a linear mixed effects
431 model (***, $p < 0.001$; *, $p < 0.05$).
432

433 **Patients with severe COVID 19 show enhanced FcγRIII/CD16 activation by S-specific IgG**
434 **antibodies.**

435 FcγRIII (CD16) activation initiates multiple protective effector functions such as antibody-
436 dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells as well as antibody-
437 dependent cytokine and chemokine secretion by NK cells and macrophages (18, 48). However,
438 excessive FcγR stimulation can have severe adverse effects such as elevated cytokine release
439 as observed in systemic autoimmune diseases or viral infections (18). Therefore, we
440 hypothesized that an exaggerated FcγR mediated activation triggered by SARS-CoV-2 specific
441 IgG might contribute to the exacerbation of COVID-19 in severely compared to critically
442 diseased patients. To address this, we analyzed the ability of SARS-CoV-2 specific antibodies
443 to activate CD16 (158V) using a previously validated cell-based reporter system (40-42, 49,
444 50) (Figure 2- figure supplement 1A). Considering the typically late time point of health
445 deterioration, we performed an analysis of CD16 activation triggered by SARS-CoV-2 specific
446 IgG with serum samples obtained 13-25 days following symptom onset (Figure 2). Sera were
447 analyzed at a 1:500 dilution to stay within the dynamic range of detection (Figure 2- figure
448 supplement 2). Depending on the availability of sample material 2-8 samples/patient/time-point
449 were included in this analysis. If available in sufficient quantity, sera were reanalyzed.
450 Reproducibility was tested using available serum surplus (Figure 2- figure supplement 3). Sera
451 from 28 patients with mild SARS-CoV-2 infection and 30 healthy blood donors were included
452 for reference. Semi-quantitative assessment of IgG titers using antigen-specific ELISA revealed
453 comparable levels between critically and severely diseased patient cohorts (Figure 2 A, B, C).
454 In contrast, S- ($p=0.0147$) and RBD-specific ($p=0.0120$) but not N-specific IgG-mediated CD16
455 activation was significantly increased in critically compared to severely diseased patients
456 (Figure 2 D-F). Furthermore, normalizing CD16 activation to antigen-specific IgG titers,
457 revealed significantly stronger CD16 activation by S- ($p=0.0033$) and N-specific ($p=0.006$) IgG
458 compared to mildly diseased patients (Figure 2 G-I). Intriguingly, we observed a heterogeneous
459 CD16 activation pattern characterized by either high or low CD16-activating sera irrespective
460 of the clinical manifestation (Figure 2 D-F). Overall, a significant positive correlation could be
461 determined between anti-SARS-CoV-2 antigen IgG titers and CD16 activation (Figure 2-figure
462 supplement 4). Our data document a sustained CD16 activation by SARS-CoV-2 specific
463 antibodies particularly in patients suffering from critical COVID-19 disease. Based on these
464 results we confirmed the notion that elevated FcγRIII/CD16 activation by S- and or RBD-
465 specific IgG might contribute to disease severity of COVID-19.

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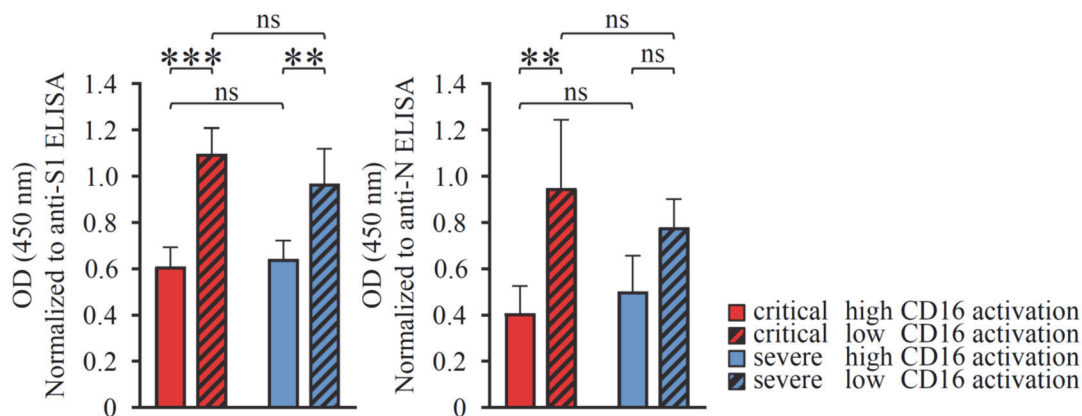
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469 **Figure 2. CD16 activation by SARS-CoV-2 - specific IgG is enhanced in critically diseased**
470 **patients.**

471 FcγRIII activation by SARS-CoV-2-specific IgG on BW5147 reporter cells in serum samples
472 obtained 13-25 days following symptom onset from 23 critically (red symbols) and 14 severely
473 (blue symbols) diseased patients. Between 2 to 8 samples/patient were analyzed depending on
474 the availability of sample material. Sera from 29 non-hospitalized patients with mild SARS-
475 CoV-2 infection (grey symbols) and 30 healthy donors (open circles) served as reference. Each
476 symbol represents the mean value of all available samples per patient. (A, B, C) ELISA levels
477 for S1- N- and RBD-specific IgG. Dotted lines represent cut-off values for commercial S1-, N-
478 and RBD - specific ELISA assays. Solid black lines indicate the mean. (D, E, F) FcγRIII
479 activation by S-, N- and RBD-specific IgG expressed as log₂ fold change relative to negative
480 control. Solid black lines indicate the mean. (G, H, I) FcγRIII activation, expressed as log₂
481 values relative to SARS-CoV-2-specific IgG titers. Solid black lines indicate the mean.
482 Significant differences over all three groups were tested by ANOVA and pairwise group
483 comparison was made by Games-Howell post-hoc tests (***, p<0.001; **, p<0.01; *, p<0.05).
484

485 **Enhanced Fc γ -afucosylation of S-specific IgG in critically and severely diseased patients**
486 **results in increased Fc γ RIII/CD16 activation.**

487 Based on the findings described above we speculated that differences in Fc γ mediated effector
488 functions might contribute to disease severity of COVID-19. We compared CD16 high- versus
489 CD16 low-activating patient sera regarding their SARS-CoV-2 specific IgG core fucosylation.
490 Inspired by previous findings (51-53) we focused on determining IgG core fucosylation of S-
491 and N- specific SARS-CoV2 IgG. To determine IgG core fucosylation we used a lectin-based
492 ELISA preceded by antigen-specific antibody purification from immobilized SARS-CoV-2-
493 antigen. Analysis of anti-S and anti-N IgG core fucosylation was performed on serum pools
494 containing five sera of either critically or severely diseased patients obtained 13-25 days post
495 symptom onset. Given the aforementioned heterogeneity in CD16-activation, we analyzed
496 pools of 5 sera of either critically or severely diseased patients characterized by either high or
497 low CD16-activation. To stay within the dynamic detection range, relative fucosylation was
498 analyzed at a dilution of 1:4 (Figure 3). When analyzing serum pools from critically and
499 severely diseased patients we determined a significantly lower level of core fucosylation among
500 the high CD16 activators (Figure 3, plain-colored bars) compared to the low CD16 activators
501 (Figure 3, shaded bars). This applied for both the S- and N-specific antibodies. These results
502 are in line with previously published findings regarding the effect of Fc γ -afucosylation on
503 Fc γ RIII/CD16 effector functions (51, 54) and recapitulate similar findings in the context of
504 COVID-19 (52, 53). However, we did not observe significant differences between critically
505 and severely diseased patients.
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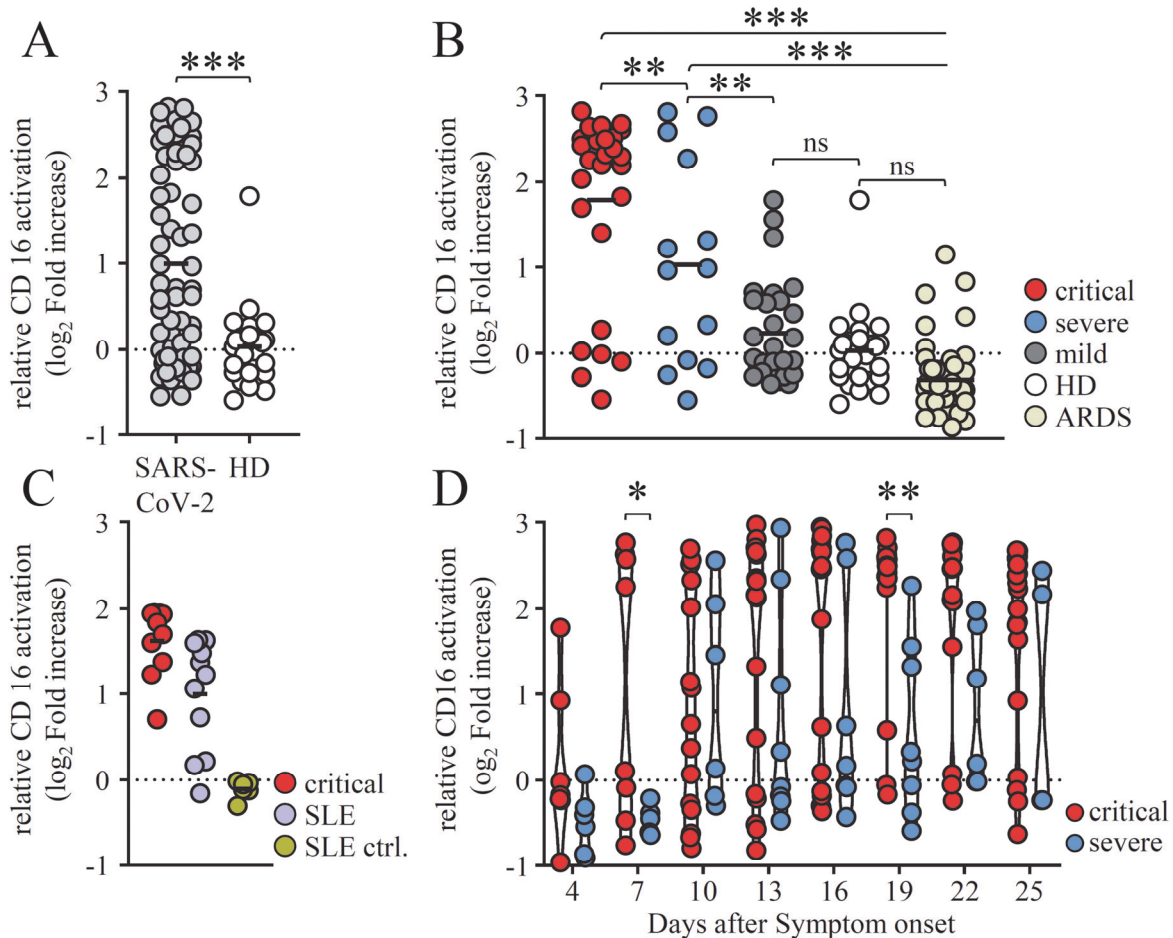
509 **Figure 3. Anti SARS-CoV-2 IgG Fc core fucosylation in critical and severe COVID-19**
510 **cases.**

511 IgG-Fc core fucosylation levels of SARS-CoV-2 –specific IgG in critically (red bars) and
512 severely (blue bars) diseased COVID-19 patients. Analysis was carried out on a pool of 5
513 different sera. Measured OD values for fucosylation of the generated eluates were normalized
514 to their respective IgG titers determined by antigen-specific S1 and N ELISA. A) S-IgG-Fc-
515 fucosylation and B) N-IgG-Fc-fucosylation in critically and severely diseased patients
516 characterized by either high (red) or low (patterned) CD16-activation levels in the Fc γ R
517 activation reporter assay. The mean and standard deviation (SD) of at least three independent
518 experiments is depicted. Statistical tests using a two-factorial linear model indicate three
519 significant differences between the low and high categories (***, $p < 0.001$; **, $p < 0.01$; *,
520 $p < 0.05$; ns = not significant).
521

522 **COVID-19 disease severity correlates with an increase in FcγRIII/CD16-reactive soluble**
523 **IgG complexes.**

524 Next to afucosylation, it has been proposed that uncleared antigen-antibody immune complexes
525 (ICs) might be involved in the pathogenesis of severe COVID-19.(34-36, 38). However, the
526 actual presence of circulating, multimeric soluble ICs (sICs) in critically or severely diseased
527 patients has not been shown yet. As extensive FcγR activation by sICs might contribute to the
528 severe systemic inflammatory state occurring in some COVID-19 patients with prolonged
529 disease, we surmised that sICs might be a putative explanation for the marked differences in
530 IL-6, PCT and CRP levels between critically and severely diseased patients (Table 1). We thus
531 set out to characterize our patient cohort regarding the presence of sICs in serum samples taken
532 at various time points during disease and after hospitalization. To this end, we deployed a novel
533 cell-based reporter assay developed to quantify CD16 (158V) activation by IgG-containing
534 sICs, measuring their bioactivity (25, 44). This assay does not react to monomeric IgG or small
535 dimeric complexes in solution, but specifically identifies multimeric sICs and has been
536 successfully used to detect sICs in patients with systemic lupus erythematosus (SLE). In SLE,
537 sICs are major driver of inflammation (24). This assay showed, as judged by conventional
538 biomarkers, that sIC bioactivity correlates with SLE disease severity. Moreover, the assay is
539 sensitive to sICs size with larger complexes leading to stronger receptor activation compared
540 to small complexes (25). Analysis of serum samples, obtained 13-25 days after symptom onset,
541 revealed the presence of highly CD16-reactive sICs in SARS-CoV-2 infected patients
542 compared to healthy individuals (Figure 4A). Next, we compared sIC-mediated CD16
543 activation between COVID-19 patients of varying disease severity. While all COVID-19
544 patient groups tested positive for reactive sICs compared to healthy control (HD) sera, we found
545 that critically diseased patients show a striking increase in reactive sICs compared to patients
546 with severe or mild disease (Figure 4B). We then compared sIC bioactivity between sera from
547 critically diseased COVID-19 patients and sera from SLE patients with active disease (Figure
548 4C). We conclude that sICs formed in COVID-19 are comparable to sICs formed during active
549 SLE regarding their potential to drive inflammation. Only 6 out of 27 patients with critical
550 disease (22%) showed no sIC-mediated CD16 activation. As we did not detect highly reactive
551 sICs in the serum of 47 patients with acute respiratory distress syndrome (ARDS; mean age
552 57.5 years) in response to infections of different etiology including CMV reactivation,
553 HIV/AIDS, influenza or pulmonary TBC infection, we conclude that the formation of reactive
554 sICs is associated with severe SARS-CoV-2 disease (Figure 4-figure supplement 1).
555 Remarkably, longitudinal analysis of reactive sICs in the serum of critically or severely diseased
556 patients revealed high CD16 activation levels in 4 critically diseased patients already 6 to 8
557 days after symptom onset (Figure 4D). Of note, 2 of 4 patients with an early increase of
558 circulating reactive sIC eventually died. sIC-mediated CD16 activation persisted in 14 of 19
559 critically diseased patients at high levels until day 26 after symptom onset. sIC-mediated CD16
560 activation in severely diseased patients was slightly delayed compared to critically diseased
561 patients and was first detected in 4 patients 9-11 days after symptom onset (Figure 4D). Only 4
562 of 14 patients with severe disease showed detectable sIC-mediated CD16 activation. To verify
563 that sICs represent the CD16-reactive component in the serum of COVID-19 patients, we
564 analyzed serum-mediated CD16 activation before and after PEG8000-precipitation. This
565 treatment was previously shown to selectively precipitate large IgG complexes from solution
566 (25, 55). For this analysis, pools of 8 sera, showing either high (IC+) or no (IC-) CD16
567 activation, were compared. Sera from healthy donors (HD) served as a negative control.
568 Compatible with the hypothesis of serum-derived sICs driving CD16 activation, no activation
569 was observed following incubation with 3.5% PEG8000 (Figure 4-figure supplement 2 A). To
570 ensure that the treatment did not precipitate monomeric IgG, we tested the depleted sera for
571 remaining S1- and N-specific IgG. As depicted S1- and N- specific IgG could still be detected
572 at unchanged high levels in samples treated with 3.5% PEG8000 (Figure 4-figure supplement

573 2 B). When resolving sIC-mediated CD16 activation over the complete time of hospitalization
574 for select patients from which samples at different time points were available, we observed that
575 sIC reactivity predominantly precedes anti-S1 IgG in ELISA as well as CD16 activation by
576 SARS-CoV-2-specific IgG (Fig. 4-figure supplement 3, Fig. 4-figure supplement 5). This
577 implies that sIC formation does not depend on the presence of SARS-CoV-2 antigens.
578 Accordingly, we were not able to identify any SARS-CoV-2-derived antigens in PEG8000-
579 precipitated sICs using tandem mass spectrometry (data not shown). To further exclude the
580 formation of multimeric sICs formed from circulating S1 antigen, we also specifically targeted
581 S1 for precipitation from patient serum using biotinylated S1-specific monoclonal antibodies.
582 However and in line with our previous approach, S1-specific precipitation using streptavidin-
583 sepharose beads and subsequent mass spectrometry analysis for any SARS-CoV-2-specific
584 antigens in sICs remained without result (data not shown). Recently, the role of neutrophil
585 mediated intravascular NETosis was reported to play a critical role in thrombosis formation and
586 subsequent organ damage observed in severe clinical forms of COVID-19 (56-58). Since this
587 process could mediate the formation of aggregated IgG as a form of sICs, we next tested
588 whether Benzonase® nuclease treatment of patient serum would dissolve reactive sICs. To this
589 end we tested sera from critically diseased patients or healthy individuals and compared CD16
590 reactivity before and after nuclease treatment (Figure 4-figure supplement 4). Nuclease activity
591 in diluted human serum was controlled using plasmid DNA for reference. This revealed that
592 nucleic acid was not involved in the formation of CD16-reactive sICs in critically diseased
593 patients. Finally, we tested pooled patient sera for autoantibodies against a panel of prototypical
594 autoantigens associated with autoimmune disease including anti-nuclear autoantibodies (ANA)
595 by indirect immunofluorescence, dsDNA autoantibodies by ELISA and autoantibodies against
596 the extractable nuclear antigens (nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1,
597 CENP B, PCNA, nucleosomes, histones, ribosomal P-protein, AMA-M2, DFS70) by dot blot
598 in case SARS-CoV-2 infection triggers autoantibody formation and possible sIC formation.
599 However, no significant autoantibody titers could be detected in any sera pool (data not shown).
600 Although we were not able to identify their origin, our data clearly indicates the presence of
601 circulating sICs in COVID-19 patients with an increase in CD16-reactive sICs corresponding
602 with severity of disease and reaching activation levels comparable to those observed in SLE
603 patients with active disease. Accordingly, we conclude that circulating sICs are a hitherto
604 unknown, yet contributing factor to COVID-19 disease severity and, regarding infectious
605 diseases, our findings represent an observation unique to severely diseased COVID-19 patients.
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Figure 4. Severe COVID-19 disease coincides with high CD16 activation by sICs.

Serial serum samples obtained 13-25 days after onset of symptoms were analyzed in a cell-based reporter assay which is sensitive to sIC amount and size (25, 44). FcγR activation is shown as log₂ fold change relative to negative control. Each symbol represents the mean value obtained by the analysis of all samples available in the indicated time range for each individual patient. A) Analysis of CD16 activation by sICs in SARS-CoV-2-infected patients compared to healthy blood donors B) Levels of IC-mediated CD16 activation across severe, critical and mild clinical courses of COVID-19 disease, in healthy donors (HD) and in non-COVID-19 patients who developed acute respiratory distress syndrome (ARDS). Solid black lines indicate the mean. Two-group comparisons with the linear model indicate significant differences between critical cases and all other groups, as well as between severe cases and all other groups (***, p<0.001; **, p<0.01). No significant differences (p>0.05) have been found for the comparisons mild vs. healthy and for HD vs. ARDS. C) Select sera from critically diseased patients were compared to sera from SLE patients with active disease regarding CD16 activation. Sera from healthy donors served as SLE-negative control. Solid black lines indicate the mean. D) Kinetics of IC-mediated CD16 activation in critically and severely diseased patients. Days after symptom onset are depicted as a range (+/- 1 day). Solid black lines indicate the median. The mixed effects model indicates two time points with significant differences (**, p<0.01; *, p<0.05).

629 Discussion

630

631 We collected and analyzed data from 41 COVID-19 patients hospitalized at the University
632 Hospital Freiburg. Patients were categorized by severity of disease into severely (n=14) and
633 critically diseased patients (n=27). Both groups were of comparable average age and had a
634 similar male-to-female ratio. For comparison we also analyzed 28 mildly diseased and 30
635 healthy individuals. As key findings we identify *de novo* produced afucosylated SARS-CoV-2
636 IgG and the presence of soluble circulating immune complexes (sICs) activating FcγRIII/CD16
637 as potential risk factors closely associated COVID-19 severity.

638

639 **Circulating sICs contribute to COVID-19 disease severity.**

640 Using an adapted reporter cell activation assay optimized to detect sICs (25, 44) we provide
641 first evidence of circulating sICs in the serum of COVID-19 patients and experimentally
642 confirm previous hypotheses suggesting immune complexes as potential drivers of disease
643 progression in COVID-19 (34-36, 38). In fundamental contrast to opsonized antigens
644 decorating virus-infected cells in tissues, sICs become distributed systemically. Thus
645 constitutive activation of CD16⁺ monocytes, granulocytes and NK cells could readily explain
646 systemic responses which potentiate local inflammation in virus-infected tissues intensifying
647 organ damage and dysfunction. Although the origin of the circulating immune complexes and
648 the nature of the bound antigens still remains elusive, we clearly show that the presence of IgG-
649 containing sICs during SARS-CoV-2 infection is directly responsible and sufficient for the
650 observed FcγRIII/CD16 activation by patient serum. Recent work has shown that viral antigens
651 can be detected in the serum of patients (59, 60). However, as we find sIC reactivity to
652 predominantly precede SARS-CoV-2-S specific IgG responses, we conclude that circulating S
653 or shed S1-antigens are not involved in sIC formation. Since sICs are commonly associated
654 with immunopathology in autoimmunity (23, 24, 61) and several studies have described that a
655 variety of specific auto-antibodies can be detected in certain critically ill COVID-19 patients
656 (62-64), we could not identify a distinct culprit antigen linked to sIC formation when searching
657 for prototypical autoantibodies. However, as sIC formation is strongly reminiscent to SLE and
658 sICs initiate a common terminal pathway of inflammation we classified patients as sIC-prone
659 or non-sIC-prone (graphical abstract). Of note, besides sIC formation a range of additional
660 phenotypical abnormalities shared between B cell populations in autoimmune disorders
661 exemplified by active SLE and severe COVID-19 have been observed. This includes the
662 pronounced engagement of extrafollicular B cell responses, associated with the activation of
663 effector B cells lacking naïve (IgD) and memory markers (CD27) as well as class-switched
664 antibody secreting cells (62).

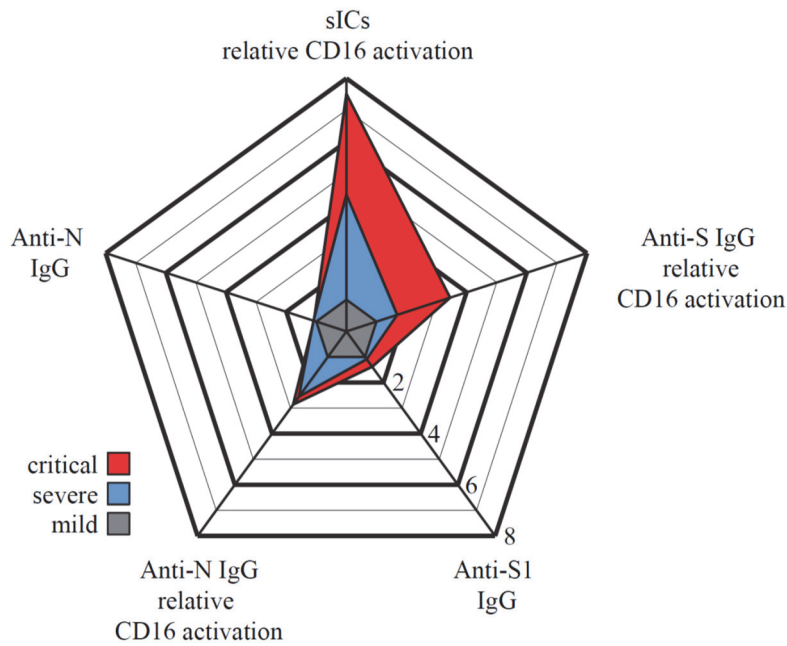
665 We suggest a hidden predisposition in sIC-prone patients resulting in a strong early
666 inflammatory response to SARS-CoV-2 infection possibly being a trigger for further sIC
667 formation and the generation of afucosylated SARS-CoV-2 IgG. Very recent work reports that
668 an acute SARS-CoV-2 infection triggers the *de novo* IgG production against multiple
669 autoantigens. In this study, 60-80% of all hospitalized COVID-19 patients exhibited anti-
670 cytokine IgG (ACA) (65). The authors show that ACA levels and specificity change over time
671 during hospitalization, suggesting ACA induction in response to viral infection and
672 inflammation. Further, it has been shown that pre-existing neutralizing anti-type I interferon
673 antibodies, which can be found in about 10% of patients with severe COVID-19 pneumonia,
674 are related to the highest risk of developing life-threatening COVID-19 disease (66). Therefore,
675 the *de novo* induction of anti-cytokine auto-antibodies in a large proportion of hospitalized
676 COVID-19 patients as described by Chang et al. (65), might indeed represent a source of
677 circulating sICs in COVID-19. In such a scenario, immune responses are deviated first by an
678 immunodepletion of critical cytokines and second through the formation of pathological sICs
679 which trigger immunological damage. We show that critically diseased patients exhibit

680 significantly higher levels of reactive sICs compared to less severely diseased patients. Notably,
681 CD16 activation levels in patients with critical disease were comparable to those measured in
682 SLE patients, where circulating sICs have long been shown to crucially contribute to tissue
683 damage and disease manifestations (67, 68). In addition, sIC responses can be found
684 significantly earlier in critically diseased patients, which was associated with a fatal disease
685 outcome. We also find that patients show a wide range of sIC reactivity. According to the
686 Heidelberger-Kendall precipitation curve (69), sIC size is critically dependent on the
687 antigen:antibody stoichiometry. As the used Fc γ R activation assay is highly sensitive to sIC
688 size (25), it is likely that this also plays a role when detecting this bioactivity in COVID-19
689 patient serum. Therefore, we propose that in addition to the presence of sICs, the size of sICs
690 plays a role in CD16 driven COVID-19 immunopathology. Based on these findings, together
691 with the higher levels of afucosylation, we conclude that CD16 activation in COVID-19 disease
692 is governed by sIC formation and IgG glycan profiles (Figure 5). It can be hypothesized that
693 the formation of sICs in predisposed patients initiates a vicious circle of Fc γ R-mediated
694 inflammation leading to an increase of IgG afucosylation, followed by enhanced Fc γ R
695 activation by SARS-CoV-2-specific IgG, further contributing to inflammation and,
696 conceivably, to *de-novo* sIC formation. Indeed, there is evidence in this direction from a clinical
697 perspective provided by a recent study that finds the administration of intravenous
698 immunoglobulin (IVIg) to alleviate COVID-19 disease (70). Although no direct proof, this
699 heavily implies that the saturation of Fc γ Rs mitigates immunopathology as previously reported
700 for autoimmune diseases (71). Therefore, our findings provide an explanation for the sustained
701 immunopathology following SARS-CoV-2 infection observed in some patients as well as for
702 the efficacy of IVIg treatment in severe to critical COVID-19 disease. Finally, when we
703 analyzed sera from COVID-19 patients obtained during the following waves of the pandemic,
704 we again found comparable levels of reactive sICs in the serum of critically diseased patients
705 (data not shown) implying that sIC formation in COVID-19 is conserved across different
706 SARS-CoV-2 strains. It will be important to investigate whether such sICs may persist in
707 reconvalescent patients and might be an explanation for immune alterations including auto-
708 antibodies observed in patients with persistent long COVID-19 symptoms (72).

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Figure 5. Summary of antibody features from SARS-CoV-2-infected patients with critical and severe disease.

Relative multivariate antibody features illustrated as radar chart in critically (red) or severely (blue) diseased COVID-19 patients normalized to the corresponding features of patients with mild infection (grey). Each spoke represents one of the following variables: ELISA (S1-IgG, N-IgG,) and CD16 activation (S-IgG, N-IgG, multimeric sICs). Arithmetic mean values of \log_2 values were calculated for each group (days 13-25 post symptom onset) respectively. The fold change compared to mildly diseased patients is shown.

724

725 **Acknowledgements**

726 We thank Sophia Ruben and Torsten Schulz from InVivo Bio Tech Services for preparing
727 SARS-CoV-2 (S)- and RBD- coated plates and preparing the SARS-CoV-2 IgG ELISA
728 Reagent Set. We thank Hans-Martin Jäck for providing TRES-1-224.2.19 and TRES-II-480
729 monoclonal antibodies and Quinnlan David for critically reading the manuscript. We also thank
730 Andreas Schlosser for validation of our Mass Spectrometry data.

731

732 **Author contribution**

733 Conceived and designed the experiments: J.A., A.M-P., S.G., P.K., A.L, V.F., M.S., H.H.

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

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

736 Contributed/reagent/sample material: A.B.G., D.H., T.W., NG.M., RE.V.

737 Writing and original draft preparation: J.A., S.G., P.K., V.F.

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

739 Conceptualization: V.F., H.H.

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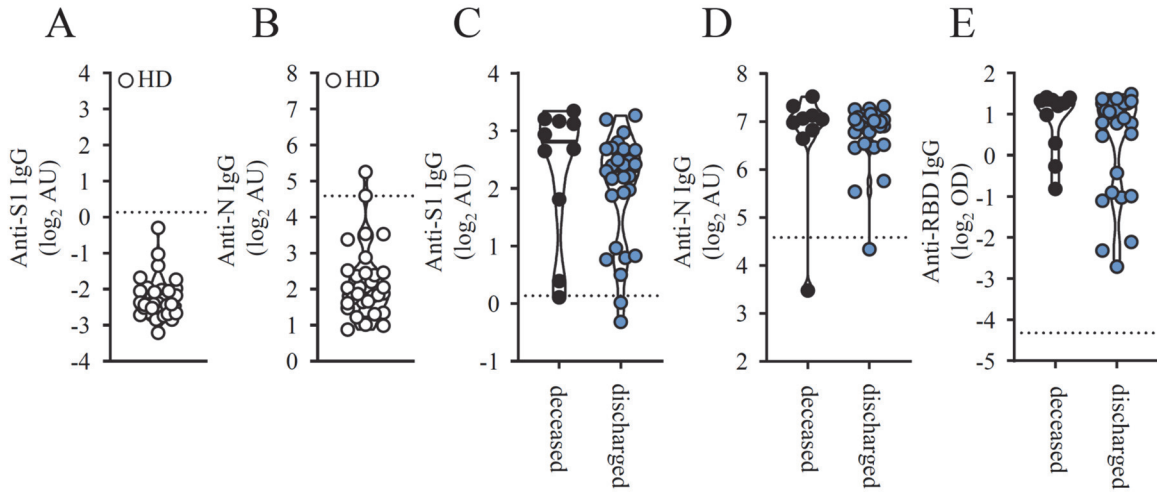
938 **Supplementary Figures**

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941 **Figure 1-figure supplement 1.**

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

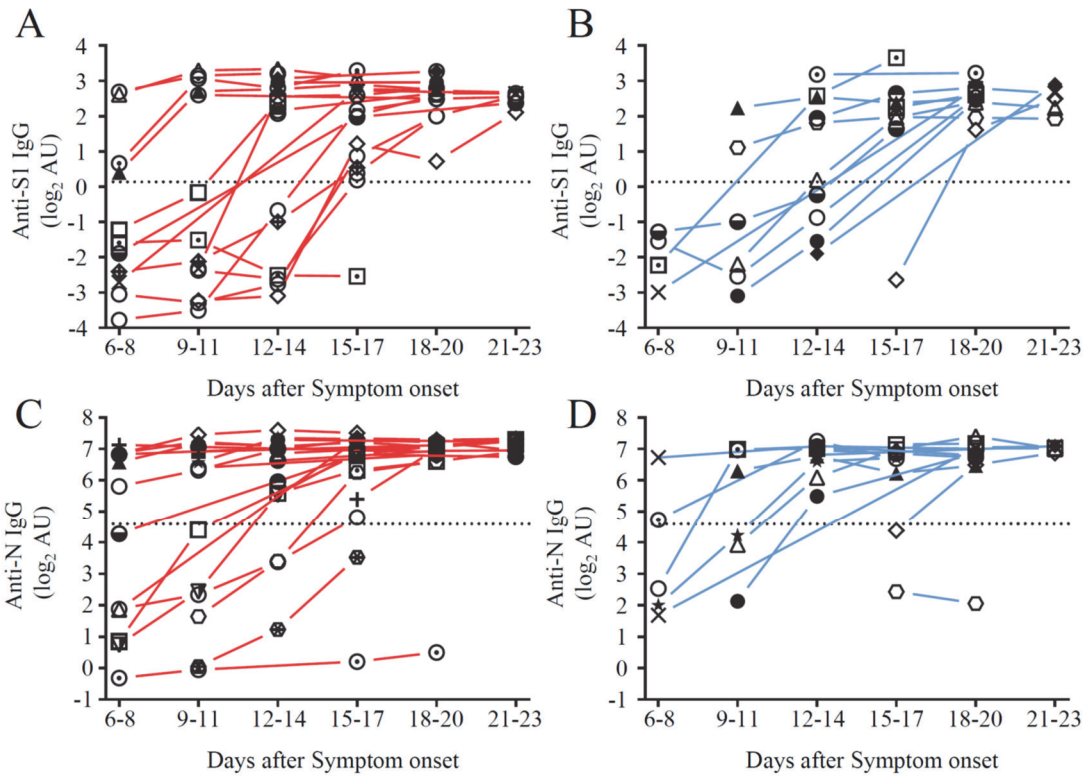
947 A) S1- and B) N-specific IgG levels in 30 healthy donors. Solid black lines indicate the median.
948 C) Cumulative S1-, D) N- and E) RBD-specific IgG levels measured 13-25 days after symptom
949 onset in deceased (black symbols) and not deceased COVID-19 patients (blue symbols). Each
950 symbol represents the mean value obtained by the analysis of all samples available in the
951 indicated time range for each individual patient. Solid black lines indicate the median.

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954 **Figure 1-figure supplement 2.**

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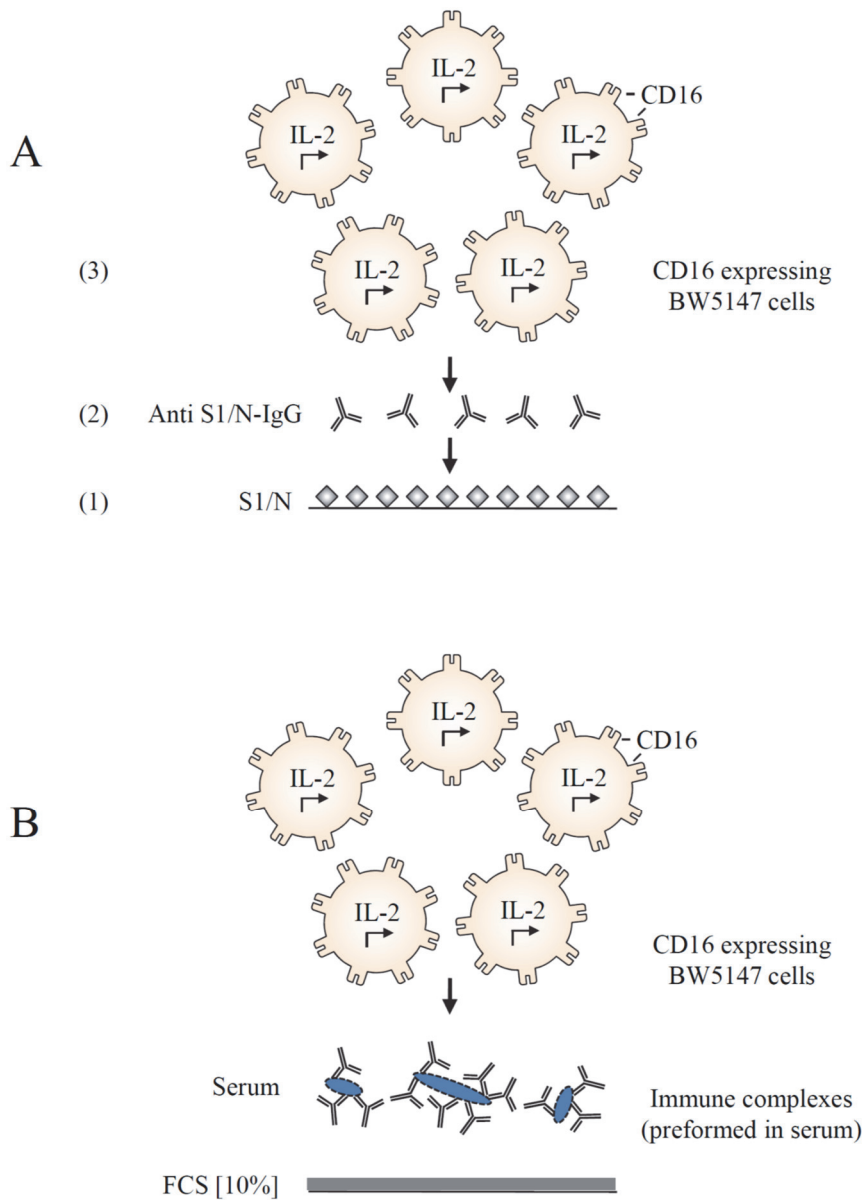
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958 **Longitudinal changes in anti- SARS-CoV-2 IgG titers in severely and critically diseased**
959 **patients.**

960 Serial serum samples were collected from hospitalized COVID-19 patients and used for SARS-
961 CoV-2-specific IgG measurement. IgG responses against SARS-CoV-2 S1- and N-protein in
962 (A, C) critically (red symbols) and (B, D) severely (blue symbols) diseased patients. Dotted
963 lines represent cut-off values for commercial S1- and N- specific ELISA assays. Each symbol
964 represents the mean value of all samples which were available for each patient at the indicated
965 time range after symptom onset. There are no significant t-tests (i.e. p>0.05 for all
966 comparisons).
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968 **Figure 2-figure supplement 1.**
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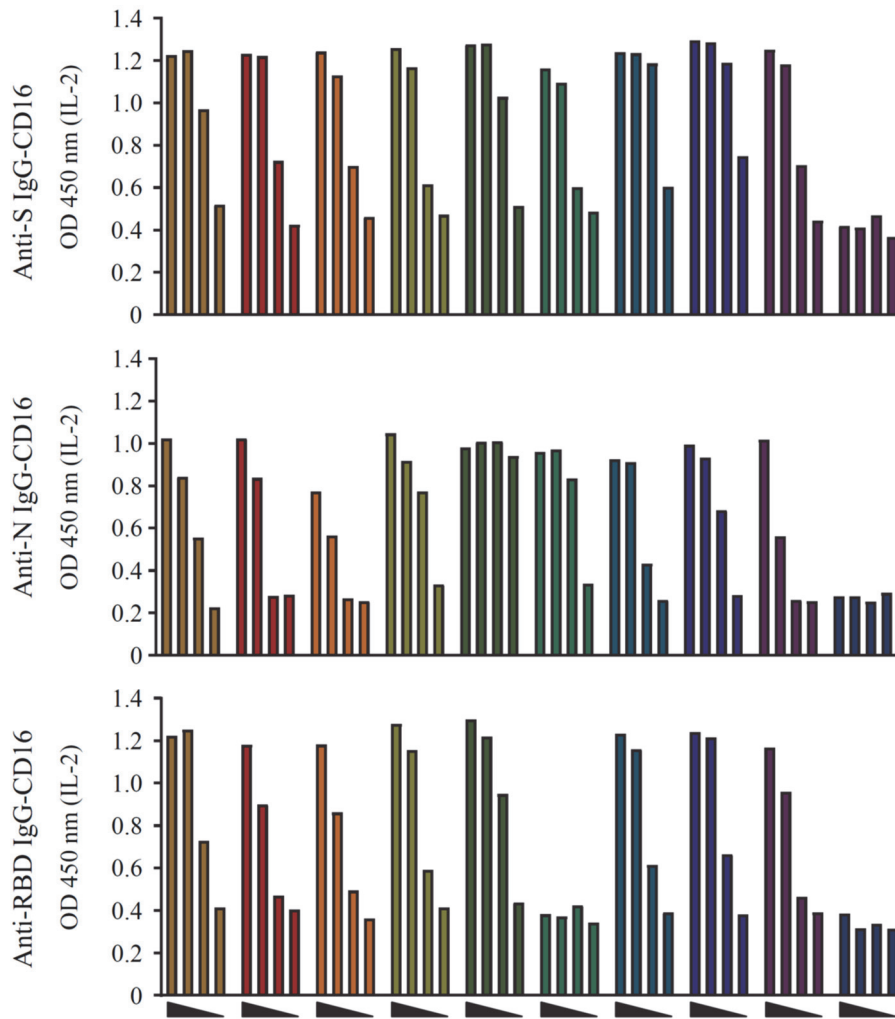


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Cell-based reporter assay measuring CD16 activation in response to immobilized IgG and sICs.

BW5147 reporter cells expressing chimeric human Fc γ RIII secrete IL-2 in response to Fc γ R activation by A) clustered viral specific IgG binding solid-phase antigen or B) soluble ICs. Solubility of sICs is achieved by pre-blocking an ELISA plate with PBS supplemented with 10% FCS as previously described (25, 44).

979 **Figure 2-figure supplement 2.**
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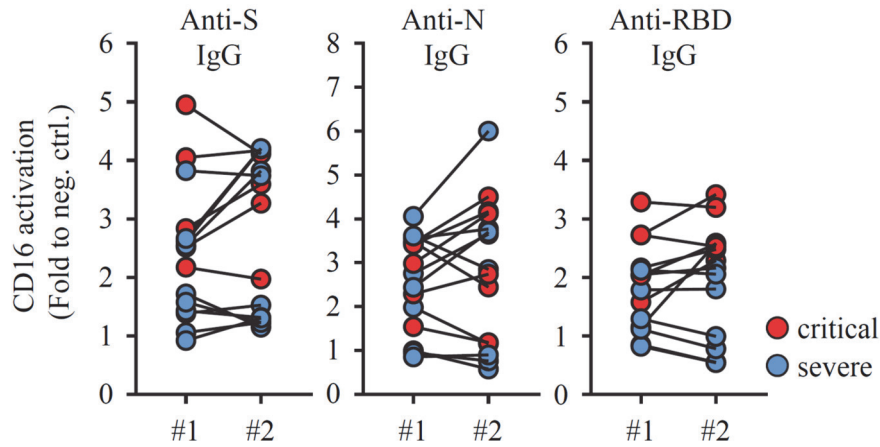
Dose dependent CD16 activation by SARS-CoV-2 specific IgG.

CD16 activation by A) S-, B) N- and C) RBD-specific IgG in 9 representatively selected serum samples and one SARS-CoV-2 negative serum (dark blue bars). Sera were serially diluted at 1:20, 1:100, 1:500 and 1:2500. FcγRIII activation initiates IL-2 secretion by reporter cells, which is subsequently measured via ELISA (OD 450 nm). Based on this empirical pretesting all sera were thereafter tested at 1:100 and 1:500 dilutions to reach an optimal dynamic range of response. The OD values obtained by the 1:500 dilutions were used for subsequent data analysis.

993 **Figure 2-figure supplement 3.**

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998 **Reproducibility of CD16 activation measurements by SARS-CoV-2 specific IgG.** Selected

999 sera which were available in sufficient amount from patients with critical (red symbols) or

1000 severe (blue symbols) SARS-CoV-2 infection were tested in two independent experiments to

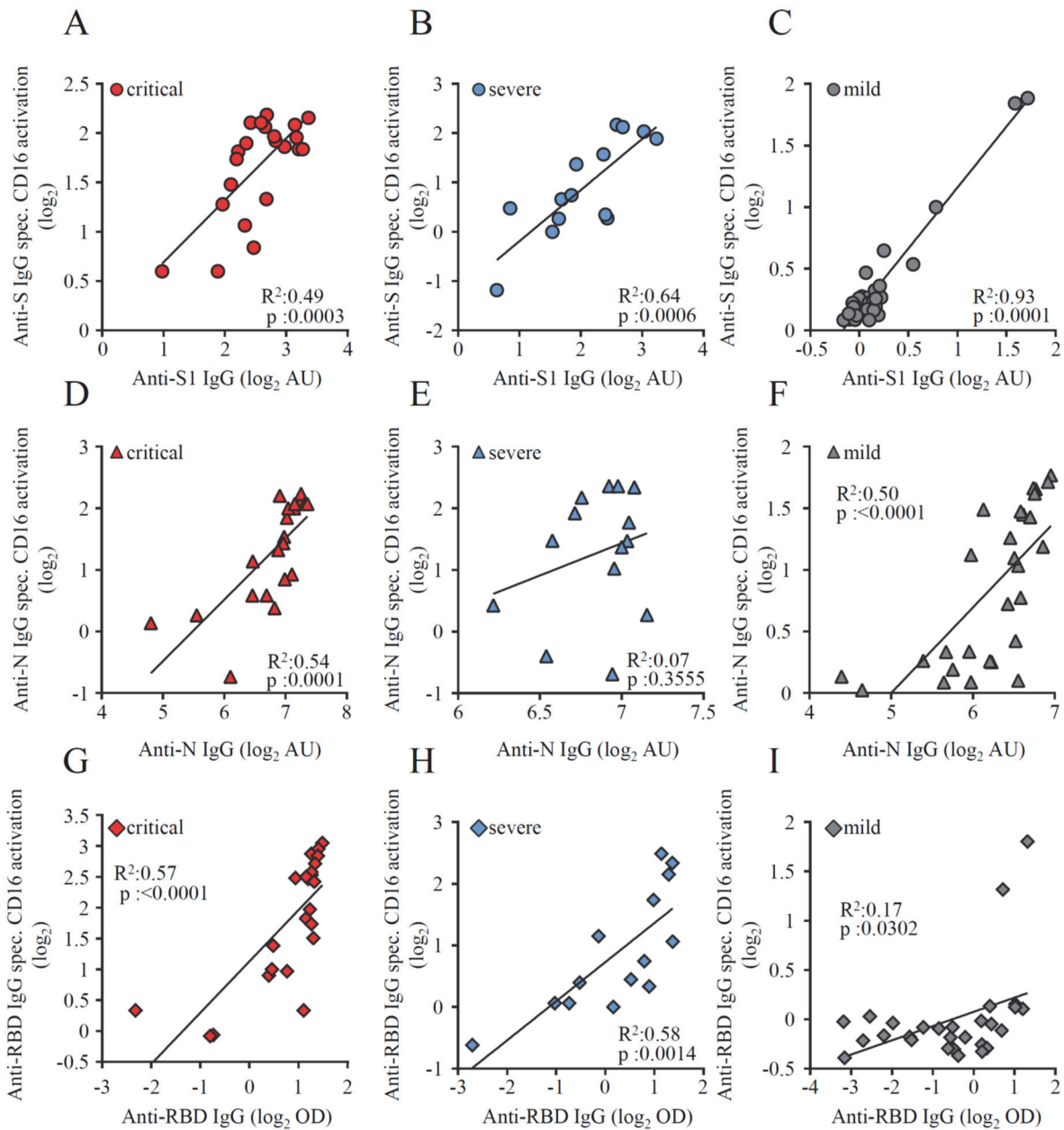
1001 show reproducibility and consistency of results. CD16 activation by S-, N- and RBD specific

1002 IgG is shown. Statistical tests using a Kolmogorov-Smirnov test indicate no significant

1003 differences.

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1005 **Figure 2-figure supplement 4.**
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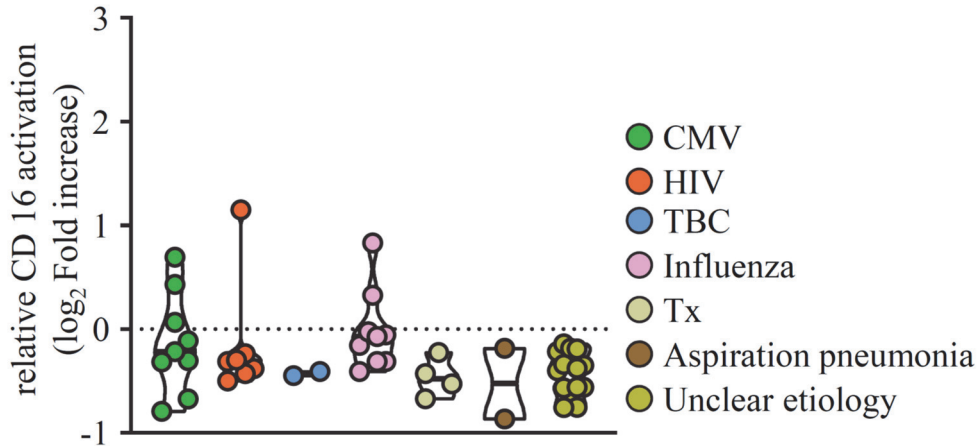


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Correlation of CD16 activation by virus specific IgG and ELISA levels.

Pearson’s correlation coefficient was used to assess the relationship between virus-specific IgG levels and their capability to trigger CD16 activation on BW5147 reporter cells in 22 paired samples from patients with critical disease (red symbols), 14 paired samples from patients with severe disease (blue symbols) and 28 samples from patients with mild disease (grey symbols). Each dot represents the mean value obtained by the analysis of all samples available at the indicated time points. (A-C) anti-S IgG, (D-F) anti-N IgG and anti-RBD-IgG (E-I).

1016 **Figure 4-figure supplement 1.**
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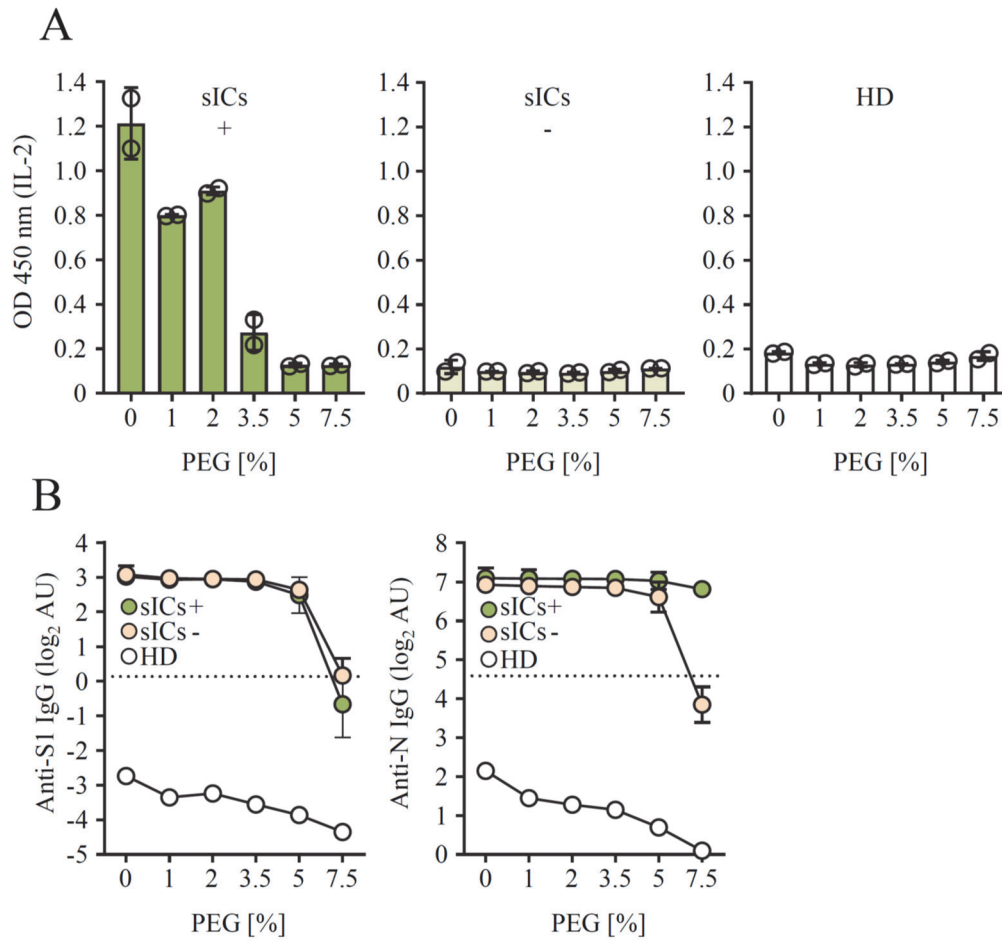


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1021 **CD16 activation by sICs in non-COVID-19 patients with ARDS.**

1022 Serum samples from 47 patients with ARDS in response to infections of different etiology were
1023 analyzed in a cell-based reporter assay which is sensitive to sIC amount and size (25, 44). FcγR
1024 activation is shown as log₂ fold change relative to negative control. Each symbol represents one
1025 sample from one patient. CMV: Cytomegalovirus reactivation under immunosuppression; HIV:
1026 HIV infection; TBC: Mycobacterium tuberculosis infection; Influenza: influenza virus
1027 infection; TX: solid organ transplantation. Solid black lines indicate the median.
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1029 **Figure 4-figure supplement 2.**
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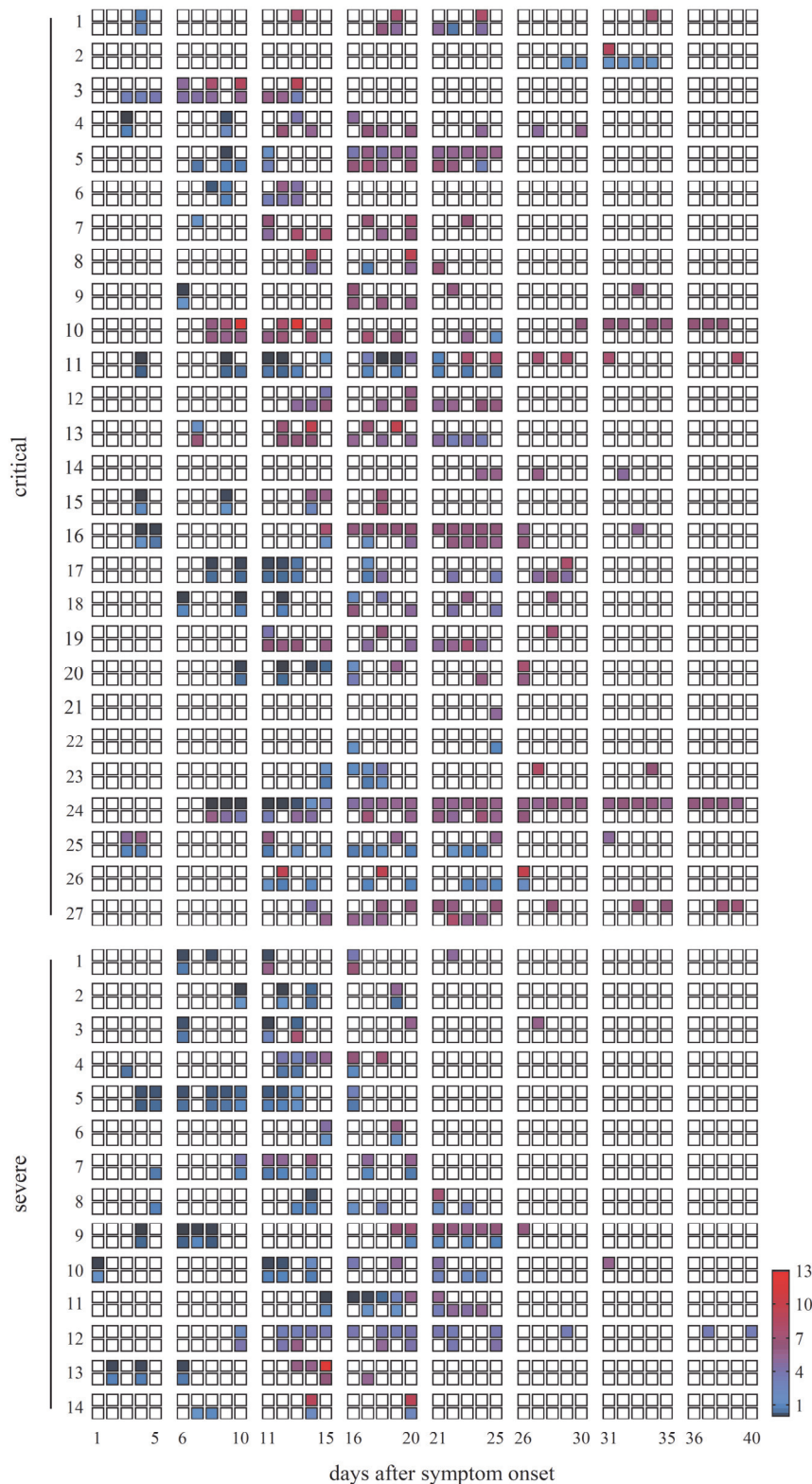


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PEG precipitation eliminates sIC-mediated CD16 activation.

Pools of 8 sera were incubated with equal volumes of PEG8000 to reach the indicated final PEG concentrations. A) CD16 activation after PEG-precipitation in the pool supernatant, showing either high (sICs+) or no (sICs-) CD16 activation. Sera from healthy donors (HD) were included as a negative control. Activation levels are expressed as IL-2 levels (OD 450 nm) released by reporter cells. The mean and SD of two independent experiments is depicted. B) Anti SARS-CoV-2 IgG levels against S1 (left panel) or N (right panel) IgG following PEG precipitation. The mean and SD of two independent experiments (sICs+/sICs-) is depicted.

1042 **Figure 4-figure supplement 3.**

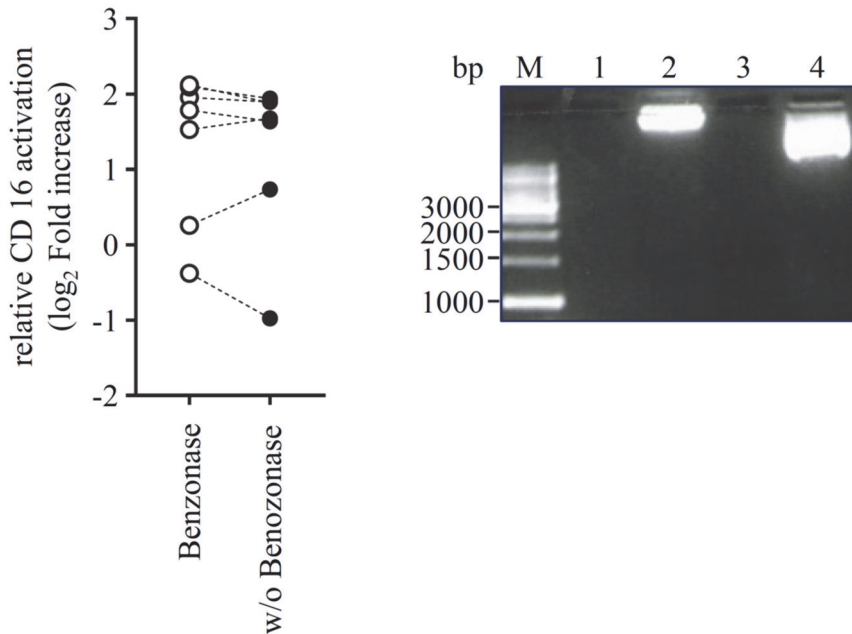


1043 **Individual CD16 activation by sICs and anti-S1 ELISA IgG kinetics post symptoms onset.**
1044 Individual sera from either critically (n = 27) or severely (n = 14) diseased patients were
1045 analyzed via ELISA [AU] for anti S1-IgG (upper row) and for CD16 activation by soluble
1046 immune complexes (lower row, relative CD16 activation depicted as fold increase to the
1047 negative control) over time (1-40 days post symptom onset). White squares: not tested.
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1050 **Figure 4-figure supplement 4.**

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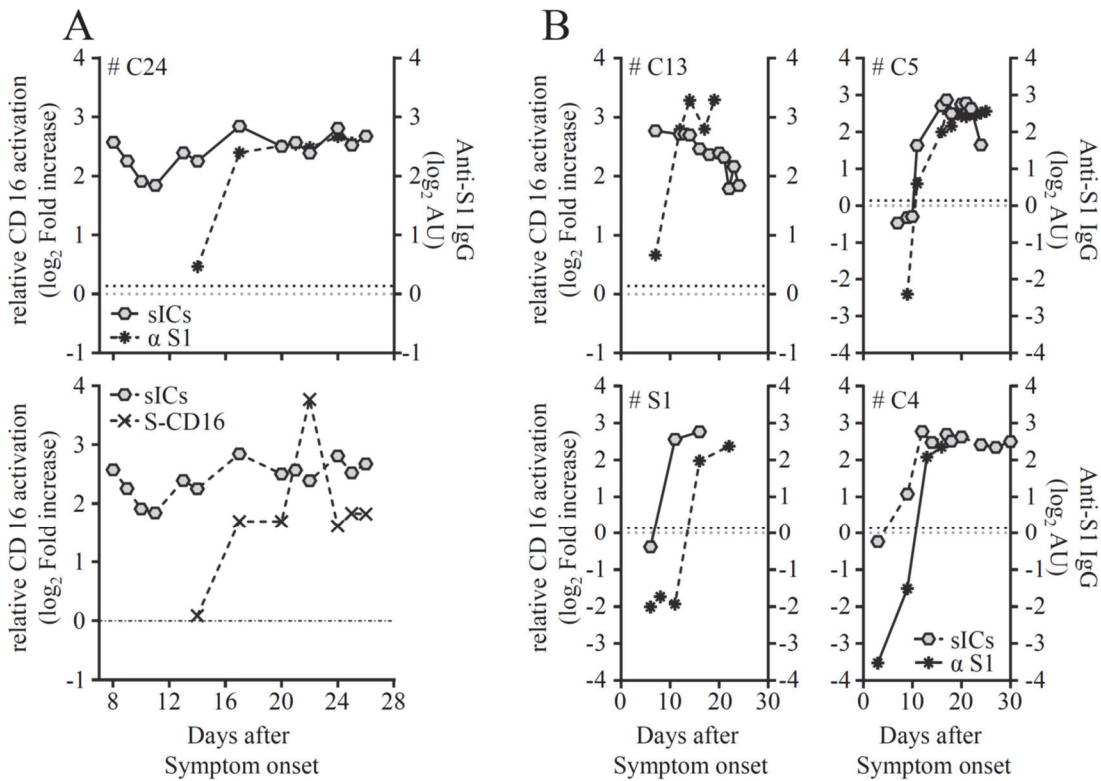
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1055 **Benzonase treatment of sIC-reactive sera does not abolish CD16 activation.**

1056 Left panel: sIC-mediated CD16 reactivity expressed as log₂ fold increase to the negative
1057 control, in serum of six individual patients before and after treatment with 250 Units of
1058 Benzonase Nuclease. Right panel: As positive control, 3 µg plasmid DNA was digested. M:
1059 1kb DNA ladder, Lane 1: benzonase digestion in the presence of human serum, lane 2: plasmid
1060 DNA w/o benzonase in the presence of human serum, lane 3: benzonase digestion in medium
1061 only and lane 4: plasmid DNA w/o benzonase in medium only.
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1063 **Figure 4-figure supplement 5.**
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sIC formation precedes SARS-CoV-2-IgG response.

Individual patients for which enough material was available were analyzed over time. A) CD16 activation by sICs- vs. S1-ELISA (top panel) and sIC –CD16 activation vs. anti-S- CD16 activation (bottom panel) in one critically ill patient, #C24. B) sIC-CD16 activation vs. S1-ELISA in four individual patients #C13, #C5, #S1 and #C4. Dashed line (black) represents commercial S1-ELISA-Cut-off level, whereas dashed line (grey) is set to 0. Individual longitudinal courses correspond to patients depicted in figure 4 supplement 3.