

1 **Original article:**

2

3 **Neutrophil and monocyte dysfunctional effector response towards bacterial challenge in**
4 **critically-ill COVID-19 patients**

5

6 Srikanth Mairpady Shambat^{**a}; Alejandro Gómez-Mejía^{*a}; Tiziano A. Schweizer^{*a}; Markus Huemer^a;
7 Chun-Chi Chang^a; Claudio Acevedo^a; Judith Bergada Pijuan^a, Clement Vulin^a, Nataliya
8 Miroshnikova^a; Daniel A. Hofmänner^b; Pedro D. Wendel Garcia^b, Matthias P. Hilty^b; Philipp Bühler
9 Karl^b; Reto A. Schüpbach^b; Silvio D. Brugger^a; Annelies S. Zinkernagel^a;

10

11 a, Department of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich,
12 University of Zurich, Switzerland

13 b, Institute of Intensive Care, University Hospital of Zurich, University of Zurich, Switzerland

14

15 *, These authors contributed equally

16

17

18 Correspondence: Annelies S. Zinkernagel

19 Email: Annelies.Zinkernagel@usz.ch

20

21

22 **Key words: COVID-19, Neutrophils, Monocytes, secondary bacterial infections,**
23 **hypercytokinemia**

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42 **Abstract**

43 COVID-19 displays diverse disease severities and symptoms. Elevated inflammation mediated by
44 hypercytokinemia induces a detrimental dysregulation of immune cells. However, there is limited
45 understanding of how SARS-CoV-2 pathogenesis impedes innate immune signaling and function
46 against secondary bacterial infections. We assessed the influence of COVID-19 hypercytokinemia
47 on the functional responses of neutrophils and monocytes upon bacterial challenges from acute and
48 corresponding recovery COVID-19 ICU patients. We show that severe hypercytokinemia in COVID-
49 19 patients correlated with bacterial superinfections. Neutrophils and monocytes from acute COVID-
50 19 patients showed severely impaired microbicidal capacity, reflected by abrogated ROS and MPO
51 production as well as reduced NETs upon bacterial challenges. We observed a distinct pattern of
52 cell surface receptor expression on both neutrophils and monocytes leading to a suppressive
53 autocrine and paracrine signaling during bacterial challenges. Our data provide insights into the
54 innate immune status of COVID-19 patients mediated by their hypercytokinemia and its transient
55 effect on immune dysregulation upon subsequent bacterial infections

56

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

80 **Introduction**

81 While most patients with Coronavirus-disease-2019 (COVID-19) exhibit only mild to moderate
82 symptoms, approximately 10% to 15% of patients progress to a severe disease. This severe course
83 of COVID-19 may require intensive care unit (ICU) support (Wu and McGoogan, 2020) and is
84 characterized by acute respiratory distress syndrome (ARDS) as well as cardiovascular,
85 gastrointestinal and neurological dysfunctions (Guan et al., 2020; Shi et al., 2020; The, 2012; Wu et
86 al., 2020; Zhou et al., 2020).

87 Despite limited data, bacterial superinfections in COVID-19 pneumonia (Hughes et al., 2020;
88 Lansbury et al., 2020), contribute to mortality (Chen et al., 2020; He et al., 2020; Zhou et al., 2020).

89 In our recent prospective single centre cohort study, we showed that 42.2% of the ICU COVID-ARDS
90 patients had bacterial superinfections (Buehler et al., 2020). These were associated with reduced
91 ventilator-free survival and significantly increased ICU length of stay (LOS) (Buehler et al., 2020).

92 Beyond ARDS, COVID-19 patients have been reported to show a complex immune dysregulation,
93 characterized by misdirected host responses and altered levels of inflammatory mediators (Chen et
94 al., 2020; Giamarellos-Bourboulis et al., 2020b; Wang et al., 2020; Wen et al., 2020). Severe COVID-
95 19 is characterized by lymphopenia, neutrophilia and myeloid cell-dysregulation (Kuri-Cervantes et
96 al., 2020; Tan et al., 2020; Wang et al., 2020; Wen et al., 2020; Wu et al., 2020) as well as high
97 plasma cytokine levels (Arunachalam et al., 2020; Lucas et al., 2020). These high cytokine levels
98 have been suggested to result in functional paralysis of the immune cells, causing respiratory and
99 multiple organ failure (Giamarellos-Bourboulis et al., 2020b). However, there is a limited
100 understanding of how SARS-CoV-2 pathogenesis impedes innate immune signaling and function
101 against secondary bacterial infections. Similarly, the role of neutrophils and monocytes and their
102 ability to respond to bacterial infection during COVID-19 remains to be elucidated. Here, we sought
103 to investigate the functional response of neutrophils and monocytes derived from critically-ill COVID-
104 19 patients during their acute illness and their subsequent recovery (rec)-phase towards bacterial
105 challenge as well as the signaling mediators underlying this response.

106 **Results**

107 **Extensive COVID-19-mediated hypercytokinemia correlates with subsequent bacterial** 108 **superinfections**

109 We first assessed the plasma levels of cytokines involved in neutrophil and monocyte functional
110 responses in our prospective cohort of critically-ill COVID-19 ICU patients (acute, n=27), including
111 the same patients in their recovery phase (rec, n=21), as well as healthy donors (n=16) (Table S1
112 and S2). As shown previously (Blanco-Melo et al., 2020; Huang et al., 2020; Lucas et al., 2020) we
113 observed that the cytokines affecting neutrophil function granulocyte colony-stimulating factor (G-
114 CSF), interleukin (IL)-8, IL-4, macrophage inflammatory protein (MIP-1 α , MIP-2 α , MIP-1 β) and
115 stromal cell-derived factor 1 alpha (SDF-1 α) had significantly increased levels in acute-patients. In
116 addition, we showed that these levels decreased upon recovery and were similar to values measured
117 in healthy donors (Fig. S1). For monocyte effectors, we found the most significant changes in the
118 levels of fractalkine (CX₃CL1), interferon gamma-induced protein 10 (IP10) and monocyte
119 chemotactic protein-1 (MCP-1) (Fig. S1).

120 In a next step, we sought to investigate whether the cytokine levels varied in COVID-19 patients who
121 developed secondary bacterial infections as compared to patients who did not. Principal component
122 analysis (PCA) showed that cytokines measured in COVID-19 patients clustered apart from healthy
123 donors (Fig. 1A). Both, acute- (Fig. 1A, right top panel) and rec-phase COVID-19 patients (Fig. 1A,
124 right bottom panel), who developed a secondary bacterial infection displayed higher degrees of
125 hypercytokinemia with increased separation on the density curve as compared to those without (Fig.
126 1A). This was confirmed by calculating the normalised cytokine values (sum of Z-scores) in the
127 plasma. Patients who developed a secondary bacterial infection showed significantly elevated
128 cumulative cytokine levels in both acute- and rec-phase (Fig. 1B). Additionally, we found a distinct
129 clustering of specific cytokines among critically-ill COVID-19 patients who developed bacterial
130 superinfections versus patients without any bacterial superinfections (Fig. S2A-B). Furthermore,
131 integrative correlation mapping of clinical parameters taken within 24 hours from sampling revealed
132 that cytokine levels correlated with myoglobin levels and bacterial superinfection status, which in
133 turn, correlated with ICU LOS and ventilation days (Fig. 1C) (Buehler et al., 2020). Overall, extensive
134 COVID-19 hypercytokinemia correlated with the development of bacterial superinfections.

135

136 **Reduced elimination of intracellular bacteria by neutrophils and monocytes in acute-phase** 137 **COVID-19 patients**

138 These above described clinical findings indicated increased susceptibility towards bacterial
139 superinfection in critically-ill COVID-19 patients associated with alterations in plasma cytokine levels.
140 Aiming to further dissect these findings, we assessed neutrophil and monocyte function upon
141 bacterial challenge *ex vivo*. Neutrophils and monocytes derived from critically-ill COVID-19 patients
142 or healthy donors were incubated with either autologous or heterologous plasma prior to bacterial
143 challenge with *Streptococcus pneumoniae* (SP) or *Staphylococcus aureus* (SA) (Fig. 1D-1G and

144 S2C-F). Neutrophils from acute patients internalized significantly less SP. Stimulation with healthy
145 donor plasma partially restored the internalization ability (Fig. S2C). We did not observe significant
146 differences in the phagocytosis ability of monocytes challenged with SP (Fig. S2D). No plasma-
147 mediated effect on phagocytosis ability was observed when either neutrophils or monocytes were
148 challenged with SA (Fig. S2E-F).

149 Our data show that acute COVID-19 neutrophils and monocytes had impaired bactericidal function
150 with a significant reduction in their ability to clear bacteria as compared to the same cells stimulated
151 with healthy plasma (Fig. 1D-G). Similarly, stimulation of healthy neutrophils and monocytes with
152 acute plasma showed significantly impaired clearance of intracellular bacteria (Fig. 1D-G).
153 Neutrophils from rec-phase patients did not show any impairment in their ability to eliminate
154 intracellular bacteria compared to healthy cells (Fig. 1D and F). In contrast, monocytes from rec-
155 phase patients still displayed reduced bacterial killing capacity (Fig. 1E and G). Neutrophils and
156 monocytes derived from COVID-19 patients who developed subsequent bacterial superinfections
157 showed a tendency towards decreased intracellular killing capacity compared to COVID-19 patients
158 without (Fig. 1D-G). Further confirmation was achieved by stimulating healthy monocytes with acute
159 plasma, which showed significantly impaired ability to clear intracellular bacteria as compared to
160 monocytes stimulated with rec-phase- patients' or healthy plasma (Fig. S2G-H). These data
161 suggested that hypercytokinemia during COVID-19 impairs neutrophils' and monocytes' ability to
162 eradicate intracellular bacteria.

163

164 **Impaired neutrophil and monocyte effector response against bacterial challenges in acute-** 165 **phase COVID-19 patients**

166 To assess the factors involved in the reduced intracellular killing capacity of acute phase neutrophils,
167 we analyzed key neutrophil effector responses. Neutrophils from acute phase patients stimulated
168 with autologous plasma produced significantly lower levels of reactive oxygen species (ROS) upon
169 bacterial challenge compared to stimulation with healthy plasma (Fig. 2A SA and SP). The same
170 effect was observed when healthy neutrophils were stimulated with acute patients' plasma prior to
171 bacterial challenge (Fig. 2A). Conversely, neutrophils from rec-patients stimulated with autologous
172 plasma displayed the same ROS production levels as neutrophils derived from healthy donors (Fig.
173 2B SA and SP).

174 Additionally, stimulation of neutrophils from acute patients and healthy donors with acute patients'
175 plasma resulted in significantly lower levels of myeloperoxidase (MPO) compared to stimulation with
176 healthy plasma upon bacterial challenge (Fig. 2C, left). In line with the normalized ROS levels during
177 recovery, neutrophils from rec-phase stimulated with plasma from rec-phase patients exhibited MPO
178 levels comparable to cells stimulated with healthy plasma, after bacterial challenge (Fig. 2C, right).
179 Since increased rates of dysregulated cell death of various cell types during COVID-19 has been
180 described in literature (Varga et al., 2020; Xu et al., 2020), we investigated whether neutrophils from
181 critically-ill COVID-19 patients showed increased sensitivity towards cell death during bacterial

182 infection. Neutrophils stimulated with acute- COVID-19 plasma, irrespective of their origin, showed
183 increased cell death upon bacterial challenge (Fig. 2D, left). In contrast, neutrophils stimulated with
184 plasma from rec patients or healthy donors prior to bacterial challenge remained viable (Fig. 2D,
185 right).

186 Recently, it has been proposed that neutrophil extracellular traps (NETs) contribute to the formation
187 of microthrombi in COVID-19 ARDS and that sera from COVID-19 patients triggered NETs release
188 in healthy neutrophils (Middleton et al., 2020; Zuo et al., 2020). Since NETs formation is a strategy
189 to eliminate extracellular pathogens (Brinkmann et al., 2004), we tested the hypothesis that bacterial
190 challenge-mediated cell death of neutrophils isolated from acute patients is due to increased NETs
191 release. Neutrophils from acute patients exhibited a higher amount of spontaneous extracellular
192 DNA-release (Fig. 2E, top) and elevated levels of MPO-DNA (Fig. 2E, bottom) than neutrophils from
193 recovery patients or healthy donors. However, bacterial challenge resulted in significantly lower
194 release of NETs and MPO-DNA from neutrophils derived from acute patients as compared to
195 neutrophils from rec-phase or healthy donors (Fig. 2F-H). The inability to release NETs upon
196 bacterial challenge was confirmed by fluorescence microscopy (Fig. 2I and J, Fig. S3B).

197 Analysis of monocyte subsets revealed significantly lower proportions of classical (CD14+ CD16-)
198 monocytes during acute COVID-19. Similarly, non-classical (CD14dim CD16+) monocytes
199 proportions were reduced during both acute and rec-phase COVID-19 compared to healthy donors
200 (Fig. S4A). We observed the same plasma-mediated decrease of ROS levels upon bacterial
201 challenge in the acute-phase in classical monocytes, whereas no differences in nitric oxide
202 production were found (Fig. S4B-F). Non-classical monocytes exhibited no difference in ROS
203 production, irrespective of disease status (Fig. S4G-H). Together, these data suggest that neutrophils
204 from acute- COVID-19 patients are in a state of exhaustion causing inability to produce ROS, MPO
205 and to trigger NETs release upon secondary bacterial challenge, whereas classical monocytes were
206 skewed towards a significantly impaired ROS, but not nitric oxide, production.

207

208 **Neutrophils cell surface receptor alterations in acute COVID-19 patients contribute to an** 209 **dysfunctional phenotype**

210 Given the observed impaired neutrophil effector response to bacterial challenges in acute COVID-
211 19 patients, we investigated potentially pivotal signaling mechanisms and receptor phenotypes of
212 neutrophils. Neutrophils from acute patients showed a significant decrease in the expression of the
213 receptors CXCR 1, 2, 3, CCR1 and CCR5 (Fig. 3A, B, E, and F) and of the maturation marker CD15
214 compared to neutrophils during recovery or from healthy donors (Fig. 3D). Additionally, we observed
215 higher levels of the activation marker CD66b and chemokine receptor CXCR4 in neutrophils from
216 acute patients, indicating the presence of immature or dysfunctional neutrophils in the blood. (Fig.
217 3C and S5D). Furthermore, upon bacterial challenge a similar pattern of cell surface receptor
218 phenotype with reduced expression of CXCR1, 2, 3, CCR1, 5 and CD15, with increased expression
219 of CXCR4 and CD66b was found in neutrophils from acute COVID-19 (Fig. 3A-F and S5C-F).

220 Overall, PCA analysis of acute-COVID-19 patients showed clear separation from rec-phase patients
221 and healthy donors, exhibiting a strong clustering for their receptor phenotype, whereas rec-phase
222 and healthy controls largely overlapped (Fig. 3G-I). Finally, we studied whether this distinct neutrophil
223 phenotype in COVID-19 patients contributed to an impaired cytokine production involved in the
224 autocrine-paracrine signaling upon bacterial challenge. We found that acute COVID-19 neutrophils
225 were characterized by reduced secretion of G-CSF, MIP-1 α , MIP-1 β , MCP-1, IL-2, SDF-1 α , IL-9, IL-
226 17A, IL-18, IL-20 and IL-23, but increased secretion of soluble PD1, IP-10 and MIP-2 α compared to
227 rec-phase and healthy neutrophils (Fig. 3J-K). Collectively, these data suggest that acute COVID-19
228 is marked by the presence of dysfunctional neutrophils, displaying reduced effector responses upon
229 secondary bacterial challenge. Together with plasma cytokine levels affecting neutrophil function
230 (Fig. S1) and the clinical observation that the patients in our prospective cohort presented with
231 neutrophilia, our data helps to explain the role of neutrophil dysfunction in increased risk of
232 secondary bacterial infections in critically-ill COVID-19 patients.

233

234 **Monocyte subpopulation alterations in COVID-19 contribute to impaired response against** 235 **bacterial challenges**

236 The myeloid compartment, especially monocytes, is particularly affected by COVID-19 (Schulte-
237 Schrepping et al., 2020). Classical monocytes from acute patients displayed more heterogeneity,
238 with higher expression of CD163, CX₃CR1 and low expression of HLA-DR compared to recovery
239 and healthy monocytes (Fig. 4A and C, S6A). Upon bacterial challenge, classical monocytes from
240 COVID-19 patients (acute and recovery) displayed high expression of CD163 and CD11b, but low
241 expression of the activation markers HLA-DR, CD86 and CD80 (Fig. 4A-D, and S6). Overall, PCA
242 analysis showed that the acute COVID-19 classical monocyte clustering pattern was strongly
243 associated with low expression of HLA-DR, CD86, CD80 and a high expression of CD163, CX3CR1
244 and CD11b (Fig. 4G-I).

245 Moreover, non-classical monocytes showed higher expression of CCR2, CD11b, CD163 and CD86
246 in acute-phase, while CD64 was increased in both acute- and rec-phase compared to healthy
247 controls, both in the presence and absence of bacterial challenge (Fig. 4E-F, and S6B). Similarly,
248 PCA analysis showed that the non-classical monocyte cluster in the acute-phase was characterized
249 by an increased expression of CCR2, CD163 CD120b, CD11b and low expression of HLA-DR (Fig.
250 S6C). Finally, COVID-19 monocytes showed a dampened cytokine response to challenge with
251 bacteria compared to healthy controls (Fig. 4J and K). Particularly, monocytes from patients with
252 acute COVID-19 showed reduced secretion of G-CSF, MIP-1 α , MIP-1 β , MCP-1, TNF- α and IL2 (Fig.
253 4J and K).

254 Taken together, these data suggest that dynamic changes of monocyte receptor and cytokine
255 secretion profile associated with acute- COVID-19- were involved in an aberrant antibacterial
256 response.

257

258 **Discussion**

259 We show that a higher degree of COVID-19 mediated hypercytokinemia in the plasma is positively
260 associated with bacterial superinfections in COVID-19 patients. Neutrophils and monocytes from
261 acute-phase COVID-19 patients exhibited impaired microbicidal capacity, reflected by abrogated
262 ROS and MPO production as well as NETs formation by neutrophils and impaired ROS production
263 in monocytes. This immunosuppressive phenotype was characterized by a high expression of CD15,
264 CXCR4 and low expression of CXCR1, CXCR2 and CD15 in neutrophils and low expression of HLA-
265 DR, CD86 and high expression of CD163 and CD11b in monocytes. Additionally, neutrophils and
266 monocytes from acute COVID-19 exhibited a blunted cytokine production capacity upon bacterial
267 challenge.

268 Studies have shown that severe COVID-19 is accompanied by hypercytokinemia with high levels of
269 pro-inflammatory cytokines such as IL-6 and IL-1 β as well as anti-inflammatory cytokines such as
270 IL-4 and IL-10 (Arunachalam et al., 2020; Coperchini et al., 2020; Lucas et al., 2020). Our initial
271 screening detected significantly higher levels of cytokines involved in recruitment and trafficking of
272 neutrophils such as IL-8, G-CSF and SDF-1 α , in accordance with previous reports showing that
273 acute COVID-19 patients have elevated neutrophil counts (Chevrier et al., 2020; Morrissey et al.,
274 2020; Schulte-Schrepping et al., 2020; Silvin et al., 2020; Wu et al., 2020). We also report a rise in
275 CX₃CL1, IP10 and MIP-1 β levels, indicating increased recruitment of monocytes (Chevrier et al.,
276 2020; Lucas et al., 2020). However, the concomitant presence of high levels of IL-4 and IL-10, with
277 broad anti-inflammatory functions, might cause functional impairment of neutrophils and monocytes
278 towards bacterial challenge (Woytschak et al., 2016). We found that higher degree of
279 hypercytokinemia in the plasma correlated with the occurrence of bacterial superinfections in
280 COVID-19 patients (Buehler et al., 2020). Specifically, TNF- α , IFN- γ , G-CSF, MIP-1 α , IL-10 and
281 CX₃CL1 were elevated in patients developing bacterial superinfection. However, further studies
282 using larger cohorts specifically looking at the correlation between elevated levels of certain
283 cytokines and the risk for developing bacterial superinfections are required to elaborate on these
284 observations.

285 We hypothesized that elevated levels of inflammatory mediators in the plasma might lead to impaired
286 functional responses to bacterial challenge. Indeed, neutrophils and monocytes derived from acute
287 COVID-19 showed a decreased capacity to kill intracellular bacteria. The capacity to clear
288 internalized bacteria could be restored when COVID-19 derived cells were stimulated with healthy
289 plasma. Additionally, monocytes but not neutrophils, from rec-patients also showed impaired ability
290 to clear intracellular bacteria. We were able to link this inefficiency in clearing intracellular bacteria
291 in acute COVID-19 by a significant decrease in their ability to produce ROS and intracellular MPO
292 (in neutrophils).

293 This altered functionality is consistent with a recent study showing reduced oxidative burst in
294 response to *E. coli* in severe COVID-19 patients (Schulte-Schrepping et al., 2020). Since neutrophils
295 and monocytes engage in a complex crosstalk with other immune cells to elicit efficient effector-

296 response, we were keen on identifying possible autocrine-paracrine signaling mechanisms.
297 Neutrophils and monocytes from critically-ill COVID-19 patients were functionally impaired in their
298 capacity to produce cytokines important for activation and subsequent antimicrobial actions.
299 Significantly lower levels of G-CSF and IL-17 as well as IL-18 in neutrophils from acute patients
300 could be linked to decreased ROS (Castellani et al., 2019; Hu et al., 2017) and MPO (Leung et al.,
301 2001) production, respectively. This was consistent with a recent observation regarding the
302 diminished or inexistent expression of cytokine genes (IL6, TNF- α) by monocytes upon stimulations
303 with TLR ligands (Arunachalam et al., 2020). Several recent studies have proposed that NETs can
304 contribute to inflammation-associated lung damage and microthrombi in severe COVID-19 patients
305 (Middleton et al., 2020; Radermecker et al., 2020). The concentration of NETs components was
306 found to be augmented in plasma, tracheal aspirate and lung autopsy tissues from COVID-19
307 patients (Veras et al., 2020; Zuo et al., 2020). Notably, it was found that SARS-CoV-2 infection could
308 directly induce the release of NETs by healthy neutrophils (Veras et al., 2020). In line with these
309 findings, we observed that neutrophils from acute patients released higher levels of DNA upon
310 plasma stimulation. However, upon bacterial challenge the induction of NETs against SA was
311 significantly reduced in acute COVID-19. This phenomenon might be due to neutrophil exhaustion
312 and a subsequent inability to properly respond to bacterial challenges.

313 These findings were confirmed by the fact that neutrophils isolated from acute patients showed lower
314 expression of key receptors CXCR1, CXCR2 as well as CXCR3, important for sensing IL-8 as well
315 as G-CSF (Cummings et al., 1999; Swamydas et al., 2016). On the other hand, SDF-1 α receptor
316 CXCR4, involved in neutrophil trafficking from the bone marrow, was significantly higher, whereas
317 the maturation marker CD15 was significantly lower in acute COVID-19 cells. Emergence of
318 CXCR4+ cells has been linked as a neutrophil precursor marker (Evrard et al., 2018) and similarly it
319 has been suggested that these immature neutrophils are being released into the blood during severe
320 COVID-19 (Silvin et al., 2020). Also, presence of abnormal neutrophils in patients with severe
321 COVID-19 has been observed (Wilk et al., 2020). A recent single-cell transcriptomic study proposed
322 that premature neutrophils in severe COVID-19 might be programmed towards an anti-inflammatory
323 start or even exert suppressive functions (Schulte-Schrepping et al., 2020). Our data here in addition
324 underline the severity of the impairment of neutrophils' ability to functionally respond to bacterial
325 infection.

326 Acute patients showed a lower proportion of classical monocytes, crucial for anti-bacterial response,
327 compared to rec-phase patient and healthy donors. Additionally, monocytes were characterized by
328 lower numbers of non-classical monocytes that are important for maintaining vascular homeostasis
329 (Giamarellos-Bourboulis et al., 2020a; Hadjadj et al., 2020; Schulte-Schrepping et al., 2020; Silvin
330 et al., 2020; Thevarajan et al., 2020; Wilk et al., 2020). Similar to other studies, HLA-DR expression
331 on classical monocytes was also significantly reduced (Giamarellos-Bourboulis et al., 2020a;
332 Schulte-Schrepping et al., 2020; Silvin et al., 2020), which can be mediated by the IL-6
333 overproduction during severe COVID-19 (Giamarellos-Bourboulis et al., 2020a). Emergence of HLA-

334 DR_{low} monocytes during severe COVID-19 can be linked to a phenotype similar to myeloid derived
335 suppressor cells or dysfunctional monocytes. HLA-DR_{low}, CD163_{high} monocytes are usually
336 associated with anti-inflammatory tissue-homeostatic functions and are linked to an
337 immunosuppressive phenotype in sepsis (Fischer-Riepe et al.; MacParland et al., 2018; Veglia et
338 al., 2018; Venet et al., 2020). Thus, a defective or suppressed monocyte compartment can further
339 add to its inability to respond to bacterial infection.

340 In conclusion, we demonstrated that during acute COVID-19, patients presented with alterations in
341 neutrophil and monocyte effector cytokines, which severely affected their ability to respond to
342 bacterial challenges. These data corroborated the clinical disease course with increased bacterial
343 superinfections observed in critically-ill COVID-19 patients (Buehler et al., 2020). Our study further
344 emphasizes the importance of tailoring treatments, aiming to restore the antibacterial effector
345 functions of neutrophils and monocytes, thereby decreasing the risk of high lethality in COVID-19
346 due to secondary bacterial infections.

347

348

349

350

351

352

353

354

355

356

357

358

359

360

361

362

363

364

365

366

367

368

369

370 **Methods**

371 **Human subject details**

372 Patients recruited under the MicrobiotaCOVID prospective cohort study conducted at the Institute of
373 Intensive Care Medicine of the University Hospital Zurich (Zurich, Switzerland) registered at
374 clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT04410263). The study was approved by the local
375 ethics committee of the Canton of Zurich, Switzerland (Kantonale Ethikkommission Zurich BASEC
376 ID 2020 - 00646). Patients were considered to be in the acute phase on the first 5 days after initial
377 ICU admission while the recovery phase was defined as patients were discharged from the ICU or
378 were negative for COVID-19 and under a defined clinical score, in a non-critical state. A list of all
379 patient demographics and clinical scores is available in table S1 and S2.

380

381 **Bacterial strains**

382 *Staphylococcus aureus* (SA) strains JE2 (MRSA-USA300, NARSA) and Cowan I (MSSA, ATCC
383 12598) were grown in Tryptic Soy Broth (TSB) at 37°C and 220rpm for 16 hours. Stationary phase
384 cultures were diluted in fresh TSB and bacteria were grown to exponential phase for the infection.
385 For *Streptococcus pneumoniae* (SP), the 603 strain (serotype 6B) (Malley et al., 2001) was
386 passaged twice on blood agar plates (Columbia blood agar, Biomereux) and incubated at 37°C with
387 5% CO₂ for 14h. A liquid culture was prepared in Todd Hewitt Yeast broth (THY) with a starting
388 OD_{600nm} of 0.1 and grown at 37°C in a water bath until OD_{600nm} of 0.35 for the infection.

389

390 **Blood collection and plasma preparation**

391 COVID-19 patients and healthy donors' blood was sampled in EDTA tubes as per the protocol under
392 the Microbiota-COVID (ClinicalTrials.gov Identifier: NCT04410263), (BASEC ID 2020 - 00646) and
393 centrifuged at 3000 rpm for 10min for plasma collection. The collected plasma was centrifuged a
394 second time at same conditions to remove any additional debris and supernatants were collected
395 and aliquoted. Fresh plasma was immediately used to prepare a 10% plasma solution in RPMI 1640
396 (Gibco™) and used for *in vitro* experiments; the remaining plasma was utilized for cell stimulation as
397 well as cytokine quantification and remaining aliquots stored at -80°C until further use.

398

399 **Cytokine measurement by Luminex**

400 Cytokine levels in patients and healthy donors' plasma, as well as cell culture supernatants from *ex*
401 *vivo* experiments were assessed using the Luminex™ MAGPIX™ instrument (ThermoFisher).
402 Samples were thawed on ice and prepared according to the manufacturer's instructions using a
403 custom-made 33-plex human cytokine panel (Procartaplex ThermoFisher). In brief, Luminex™
404 magnetic beads were added to the 96-well plate placed on a magnetic holder and incubated for
405 2min. The plate was washed twice with assay buffer for 30sec. In parallel, provided standards and
406 plasma samples were diluted in assay buffer (cell culture media was used for cell culture
407 supernatants) and added to the plate. The plate was incubated for 2h at RT at 550rpm in a plate

408 orbital shaker. Next, the plate was washed twice with assay buffer and incubated for 30min at 550rpm
409 with detection antibodies. After two washing steps, the plate was incubated with Streptavidin-PE
410 solution for 30min at 550rpm. Finally, the plate was washed, reading buffer was added and incubated
411 for 10min at RT and 550rpm before running the plate. Data acquisition and analysis were performed
412 using the Xponent software (v. 4.3). Data were validated using the Procarta plex analyst software
413 (ThermoFisher).

414

415 **Principal Component and Integrated Correlation analysis**

416 PCA plots of the cytokine analysis from patient and healthy donor plasma as well as receptor analysis
417 from the ex vivo experiments were created using the 'PCA' and the 'fviz_pca_biplot' functions
418 available in 'FactoMineR' package in R. Correlation mapping was performed using the 'corrplot'
419 package in R. The color of the circles indicated positive (blue) and negative (red) correlations, color
420 intensity represented correlation strength as measured by the Pearson's correlation coefficient. The
421 correlation matrix was reordered manually to better visualize the variables of interest.

422

423 **Peripheral blood mononuclear cells (PBMCs) isolation**

424 Patients and healthy donor PBMCs were isolated from the cellular fraction of the blood after 1:2
425 dilution with DPBS using the Lymphoprep (Axis Shield) density gradient method. In brief, the diluted
426 blood was overlaid on Lymphoprep and centrifuged for 25min at 2000rpm with lowest acceleration
427 and break settings. Following the gradient separation, the PBMCs layer was transferred into a new
428 50ml conical tube and diluted with FACS buffer (2mM EDTA and 1% FBS). Cells were washed twice
429 with FACS buffer. Next, cells were resuspended in red blood cells (RBC) lysis buffer (ThermoFisher),
430 mixed gently and incubated for 10min at 37°C and 5% CO₂. The lysis reaction was stopped by adding
431 FACS buffer and the suspension was centrifuged. Cells were washed once and resuspended in
432 FACS buffer for counting using the Attune NxT flow cytometer (ThermoFisher).

433

434 **Monocytes enrichment from PBMCs**

435 Patient and healthy PBMCs were used for monocyte enrichment using the EasySep™ Human
436 Monocyte Enrichment Kit without CD16 Depletion (StemCell™) following the manufacturer's
437 instructions. In brief, PBMCs (<100 million) were transferred to a 5ml polystyrene tube, the human
438 monocyte enrichment cocktail was added and the sample was gently mixed and incubated for 10min
439 on ice. Following incubation, magnetic beads were added to the mixture and samples were mixed
440 and incubated for 10min on ice. Finally, the mixture was placed in a magnetic holder (StemCell™)
441 for 3min and the cells were decanted into a new tube. Monocytes were then washed, resuspended
442 in RPMI 1640 and counted using the Attune NxT flow-cytometer (ThermoFisher).

443

444

445

446 **Neutrophils isolation**

447 Neutrophils were isolated from the cellular fraction of the blood, after dilution with DPBS (Gibco™),
448 with the EasySep™ Human Neutrophil Isolation Kit (StemCell™) according to the manufacturer's
449 instruction. In brief, Neutrophil enrichment cocktail was added to the diluted blood and incubated for
450 15min at RT. Next, magnetic beads were added for another 15min, after which the tubes were placed
451 into a magnetic holder (StemCell™). PMNs were collected after 15min of cell separation. They were
452 centrifuged at 1500rpm for 6min (low acceleration and brakes) and subsequent red blood cells
453 (RBCs) lysis was performed with resuspension in H₂O followed by addition of DPBS. After a further
454 centrifugation step, neutrophils were resuspended in RPMI 1640 and counted using the Attune NxT
455 flow-cytometer (ThermoFisher).

456

457 **Plasma stimulation**

458 Isolated neutrophils or monocytes, from both COVID-19 patients and healthy donors, were seeded
459 in conical 96-well V-bottom plates (for Flow cytometry assays, around 2×10^5 cells / well) or in 24-well
460 F-bottom plates (for phagocytosis and intracellular survival assays, approximately 2.5×10^5 to 3×10^5
461 cells/well) and stimulated with 10% autologous or heterologous (either COVID-19 or healthy donor
462 plasma) for 2.5h at 37°C + 5% CO₂.

463

464 **Bacterial challenge**

465 For phagocytosis and intracellular killing assays, bacteria were opsonized for 20min in RPMI 1640
466 supplemented with 2.5% of either patient or healthy plasma at a determined multiplicity of infection
467 (MOI) for neutrophils (50 for SP and 10 for JE2) and monocytes (50 for SP and Cowan I) infections
468 respectively.

469 To analyze intracellular survival of the bacteria in neutrophils, neutrophils were seeded into 24-well
470 plates (TPP) and infected with exponentially grown SA at a MOI of 10 or with exponentially grown
471 SP at a MOI of 50. After 40min, 1mg/ml flucloxacillin and 25µg/ml lysostaphin were added to kill all
472 extracellular SA or penicillin (10µg/ml) / streptomycin (10µg/ml) to kill extracellular SP. Infected cells
473 were harvested 30min and 4h after addition of antibiotics, washed twice with PBS, lysed with ddH₂O,
474 serially diluted and drop plated. Bacterial survival was analyzed and calculated relatively to the
475 invasion (30min time point).

476 To analyze intracellular survival of the bacteria within monocytes were seeded into 24-well plates
477 (TPP) and infected with exponentially grown SA or SP at a MOI of 50. After 40min, 1mg/ml
478 flucloxacillin and 25µg/ml lysostaphin were added to kill all extracellular SA or penicillin (10u/ml) /
479 streptomycin (10µg/ml) to kill extracellular SP. Infected cells were harvested 30min and 90min after
480 addition of antibiotics, washed twice with PBS, lysed with 0.02% of Triton X-100 in ddH₂O, serially
481 diluted and drop plated. Bacterial survival was analyses and calculated relatively to the invasion
482 (30min time point). End point bacterial free supernatant from both neutrophils and monocytes
483 bacterial infection experiments were utilized for cytokine measurement.

484 **Flow cytometry**

485 Staining of reactive oxygen species (ROS, for neutrophils and monocytes) and nitric oxide (NO,
486 formonocytes) was performed after 1 hour of bacterial challenge or plasma stimulation only by
487 incubation with 5 μ M CellROXTM green reagent and 5 μ M DAF-FMTM diacetate (ThermoFisher)
488 respectively, for 30min. After the incubation period, cells were washed with DPBS. Cells were stained
489 with either LIVE/DEADTM fixable Near-IR or Aqua stain (ThermoFisher) in DPBS for 25min at 4°C.
490 Next, cells were washed with FACS buffer and stained for surface antigens for 30min at 4°C.
491 Antibodies included anti-CD15 eFluor450 (clone: HI98), anti-CD181 FITC (8F1-1-4), anti-CD182
492 PerCP-eFluor710 (5E8-C7-F10), anti-CD183 PE-eFluor610 (CEW33D), anti-CD66b APC (G10F5),
493 anti-HLA-DR eFluor450 (LN3), anti-CD45 eFluor506 (HI30), anti-CD14 SB600 (61D3), anti-CD64
494 FITC (10.1), anti-CD163 PerCP-eFluor710 (GHI/61), anti-CD16 PE (CB16), anti-CD86 PE-Cyanine
495 5.5 (IT2.2), anti-CD206 PE-Cyanine 7 (19.2), anti-CD169 APC (7-239), anti-CD11b AF[®] 700
496 (VIM12), anti-CD3 APC-eFluor780 (UCHT1), anti-CD19 APC-eFluor780 (HIB19), anti-CD56 APC-
497 eFluor780 (CMSSB), anti-CD119 FITC (BB1E2) and anti-CX₃CR1 APC (2A9-1) from ThermoFisher,
498 anti-CD195 BV510 (J418F1), anti-CD184 BV605 (12G5), anti-CD191 PE-Cyanine7 (5F10B29), anti-
499 CD88 AF[®]700 (S5/1), anti-CD192 PerCP-Cyanine5.5 (K036C2), anti-CD80 PE-DazzleTM594 (2D10)
500 and anti-CD120b PE-Cyanine7 (3G7A02) from Biolegend. For intracellular MPO staining,
501 neutrophils were washed, fixed and permeabilized with the Cytofix/CytopermTM Fixation/
502 Permeabilization Solution Kit (BD) for 15min at 4°C and stained subsequently for another 30min with
503 anti-MPO eFluor450 (455-BE6). To assess neutrophil extracellular traps (NETs), cells were stained
504 first with LIVE/DEADTM fixable Aqua, followed by staining for surface antigens as described above,
505 after which they were washed with DPBS and subsequently stained with SYTOXTM Green in DPBS
506 for 30min. To stain for extracellular MPO-DNA complexes, neutrophils were stained exactly as
507 described for NETs with the addition of the MPO staining during the surface antigen step. Cells were
508 analyzed on an Attune NxT (ThermoFisher). All antibodies and concentrations used are listed in
509 Table x. Flow cytometry data were analyzed with FlowJo (v10.2). Neutrophils and monocytes were
510 gated based on their forward- and side-scatter properties, single cells and ultimately live cells.
511 Neutrophils were characterized as CD66b⁺CD16⁺, whereas monocytes were divided into subgroup
512 based on CD14⁺CD16⁻ (classical), CD14⁺CD16⁺ (intermediate) and CD14^{dim}CD16⁺ (non-
513 classical) for further analysis.

514

515 **Microscopy and NETs quantification**

516 Neutrophils were stimulated as described above and placed within wells of a μ -slide (iBidi) and
517 centrifuged at 200 g for 2 min, after which they were challenged with *S. aureus* for 1.5h. NETs were
518 stained by directly adding SYTOXTM Green and 2 μ M Hoechst 33342 (ThermoFisher) for 30min at
519 room temperature to the wells. The confocal laser scanning microscopy images were obtained with
520 a Leica TCS SP8 inverted microscope using a 63 \times /1.4 oil immersion objective. The whole wells were
521 inspected for NETs formation and two to three representative spots per condition were imaged. The

522 obtained images were processed using Imaris 9.2.0 software (Bitplane) to obtain tifs for further
523 analysis. Other standard light microscopy images of fixed cells were obtained on a fully automated
524 Olympus IX83 with a 40X objective (UPLFLN40XPH-2) illuminated with a PE-4000 LED system
525 through a quadband filter set (U-IFCBL50). 16 positions per sample were assigned before the
526 sample was prepared to avoid potential experimenter bias. Automated NET quantification was
527 performed as described in SI Fig.4: after filtering nuclei on DAPI signal (threshold set manually for
528 each 8-samples experiment), extracellular DNA was quantified on Sytox Green signal. Images
529 containing large cell aggregates that could not be resolved were discarded. Nuclei were counted
530 after watershed segmentation on the DAPI mask. Images were processed using ImageJ software
531 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,
532 <https://imagej.nih.gov/ij/>, 1997-2018) and Matlab R2020a (MathWorks).

533

534 **Statistical analysis**

535 The number of donors is annotated in the corresponding figure legend. Differences between two
536 groups were evaluated using either Mann-Whitney test or Wilcoxon signed-rank test. Kruskal-Wallis
537 test with Dunn's multiple comparisons test was used to evaluate differences among the three groups
538 in all the analyses (GraphPad). Pearson test was used for correlations of normally distributed binary
539 data. Significance level with $p < 0.05$ are depicted in individual graphs.

540 For the statistical analyses involving several cytokines, measured cytokine values were normalized
541 based on the standard z-score formula. This allowed to compare cytokines to each other and to
542 obtain a sum of z-scores per patient.

543

544

545

546

547

548

549

550

551

552

553

554

555

556

557

558

559 **Acknowledgments**

560 Confocal laser scanning microscopy was performed with the support of the Center for Microscopy
561 and Image Analysis, University of Zurich. We thank Andrea Tarnutzer and Federica Andreoni for their
562 technical help and inputs on the manuscript.

563

564 **Funding**

565 This work was funded by the SNSF project grant 31003A_176252 (to A.S.Z), the SNF Biobanking
566 grant 31BK30_185401 (to A.S.Z), the Uniscientia Foundation Grant (to A.S.Z), by the Swedish
567 Society for Medical Research (SSMF) foundation grant P17-0179 (to S.M.S), the Promedica
568 Foundation 1449/M (to S.D.B) and unrestricted funds (to RAS).

569 The MicrobiotaCOVID prospective cohort study was approved by the local ethics committee of the
570 Canton of Zurich, Switzerland (Kantonale Ethikkommission Zurich BASEC ID 2020 - 00646)

571

572 **Author Contributions**

573 **Conceptualization:** SMS, SDB and ASZ

574 **Investigation:** SMS, AGM, TAS, SDB and ASZ

575 **Experimental design:** SMS, AGM, TAS, MH and ASZ

576 **Methodology:** SMS, AGM, TAS, MH, CCC, CA, CV, NM, DAH, PBK and SDB

577 **Data curation:** SMS, AGM, TAS, MH, CV, CA, JBP, MPH, PDWG and SDB

578 **Formal Analysis:** SMS, AGM, TAS, MH, CA, JBP, and CV

579 **Funding acquisition:** SDB, PBK, RAS, and ASZ

580 **Visualization:** SMS, AGM, TAS, JBP and CV

581 **Resources:** PBK, SDB, RAS and ASZ

582 **Writing – original draft:** SMS, AGM, TAS and MH

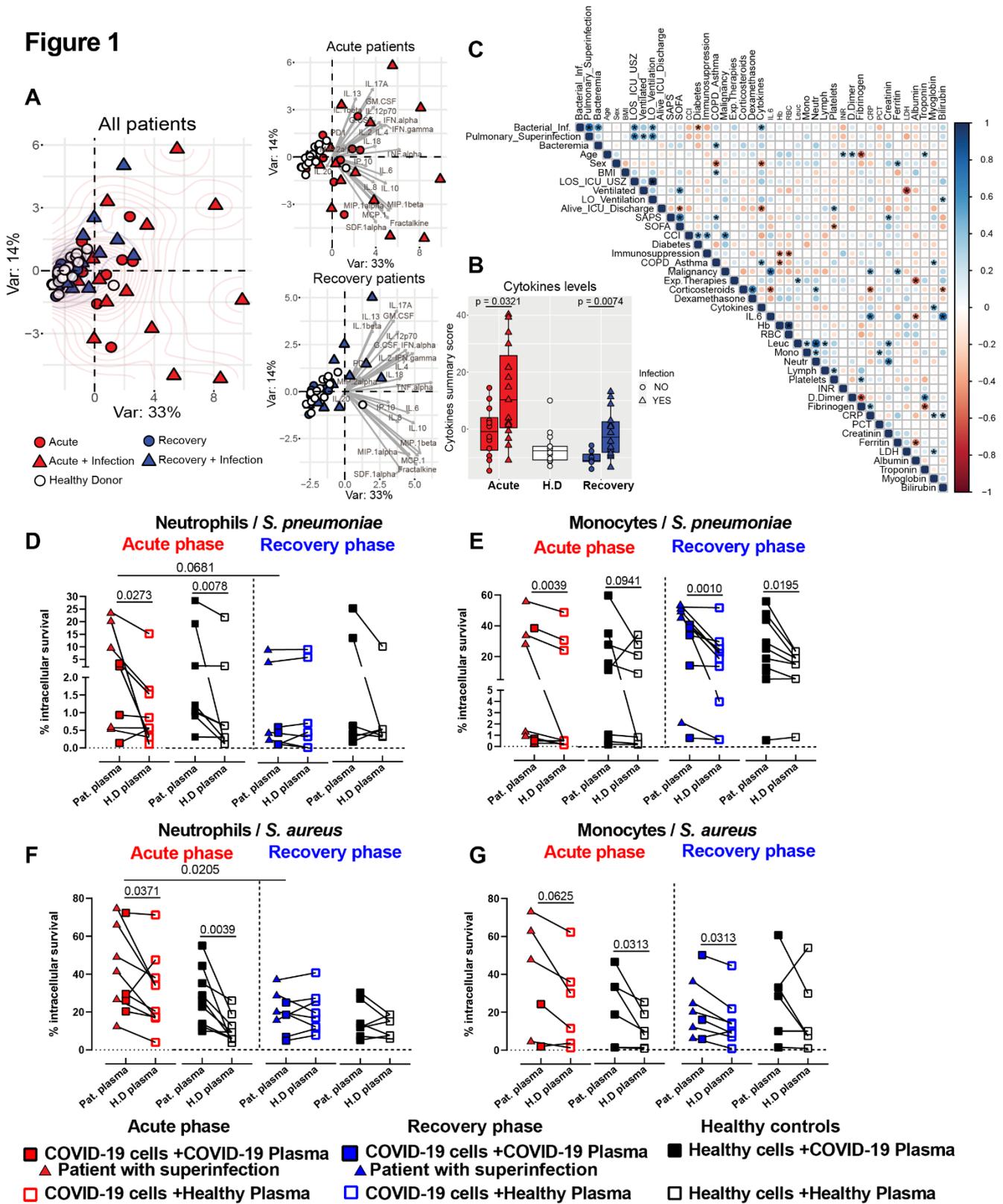
583 **Writing – review & editing:** SMS, AGM, TAS, MH, CCC, CA, JBP, CV, DAH, PBK, RAS, SDB
584 and ASZ

585

586 **Declaration of Interests**

587 The authors declare no competing interests

Figure 1



588

589

590

591

592

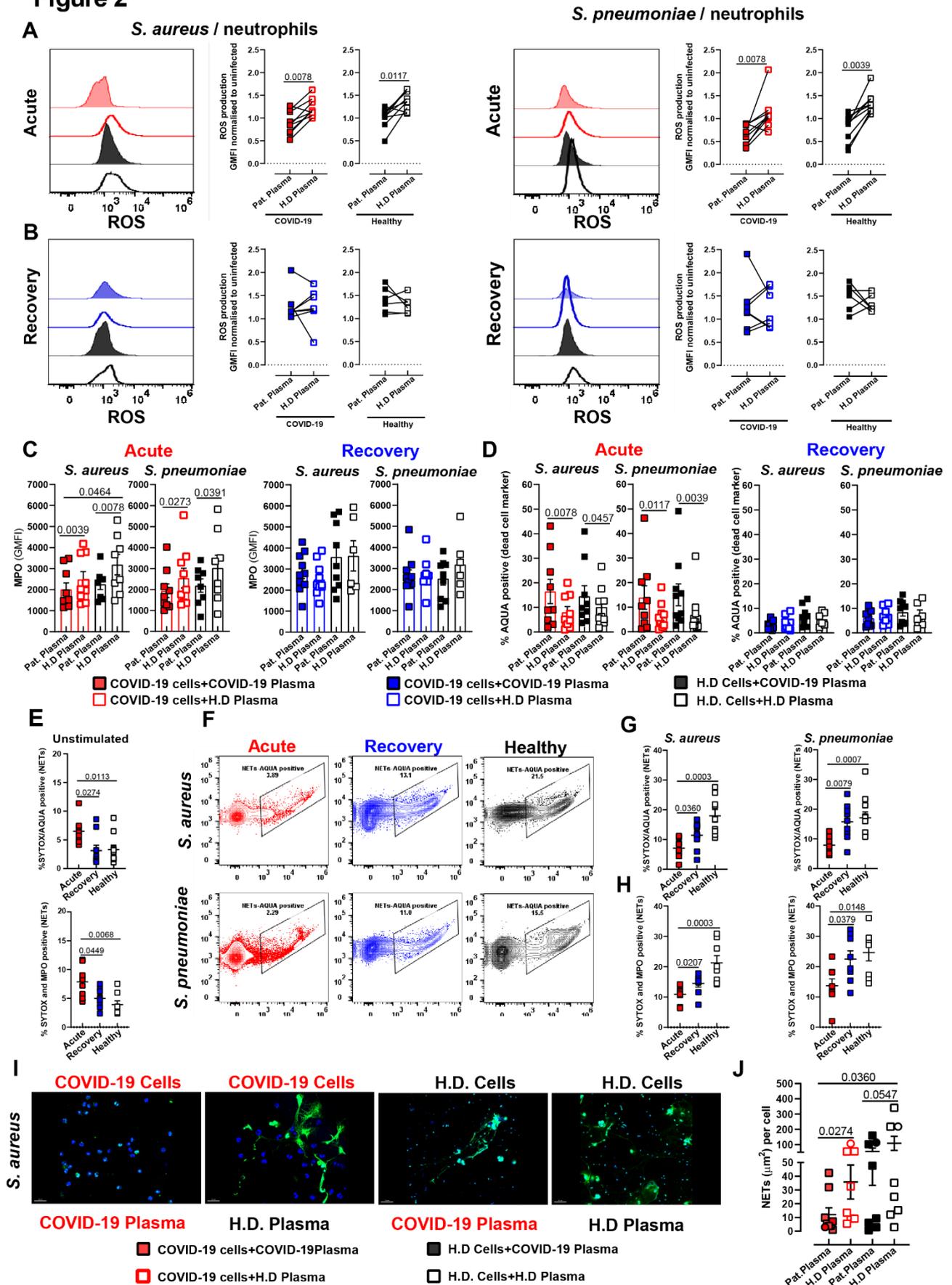
593

594

595 **Figure 1. Characterization of inflammatory mediators in COVID-19 plasma and impaired**
596 **bactericidal capacity of innate immune cells**

597 **(A)** PCA of healthy donors (white) vs acute (red) and rec (blue)–COVID-19 patients grouping the
598 plasma cytokine levels and status of secondary bacterial infections. Patients with secondary bacterial
599 infection are depicted as triangle and patients without superinfection as circle symbols. **(B)**
600 Normalised cytokine values (sum of Z-scores) in the plasma of acute (red), rec (blue) patients with
601 or without bacterial superinfection and healthy donors (white) **(C)** Integrated correlation clustering
602 map of relevant clinical parameters; circle-color indicates positive (blue) and negative (red)
603 correlations, color intensity represents correlation strength as measured by the Pearson's correlation
604 coefficient. **(D-E)** Intracellular killing capacity of COVID-19 patient (acute-red and rec-blue)
605 neutrophils (left) and monocytes (right) (n=8-10) pre-exposed to plasma from patients (solid
606 symbols) vs healthy plasma (open symbols) upon infection with SP **(D)** or SA **(E)**.

Figure 2

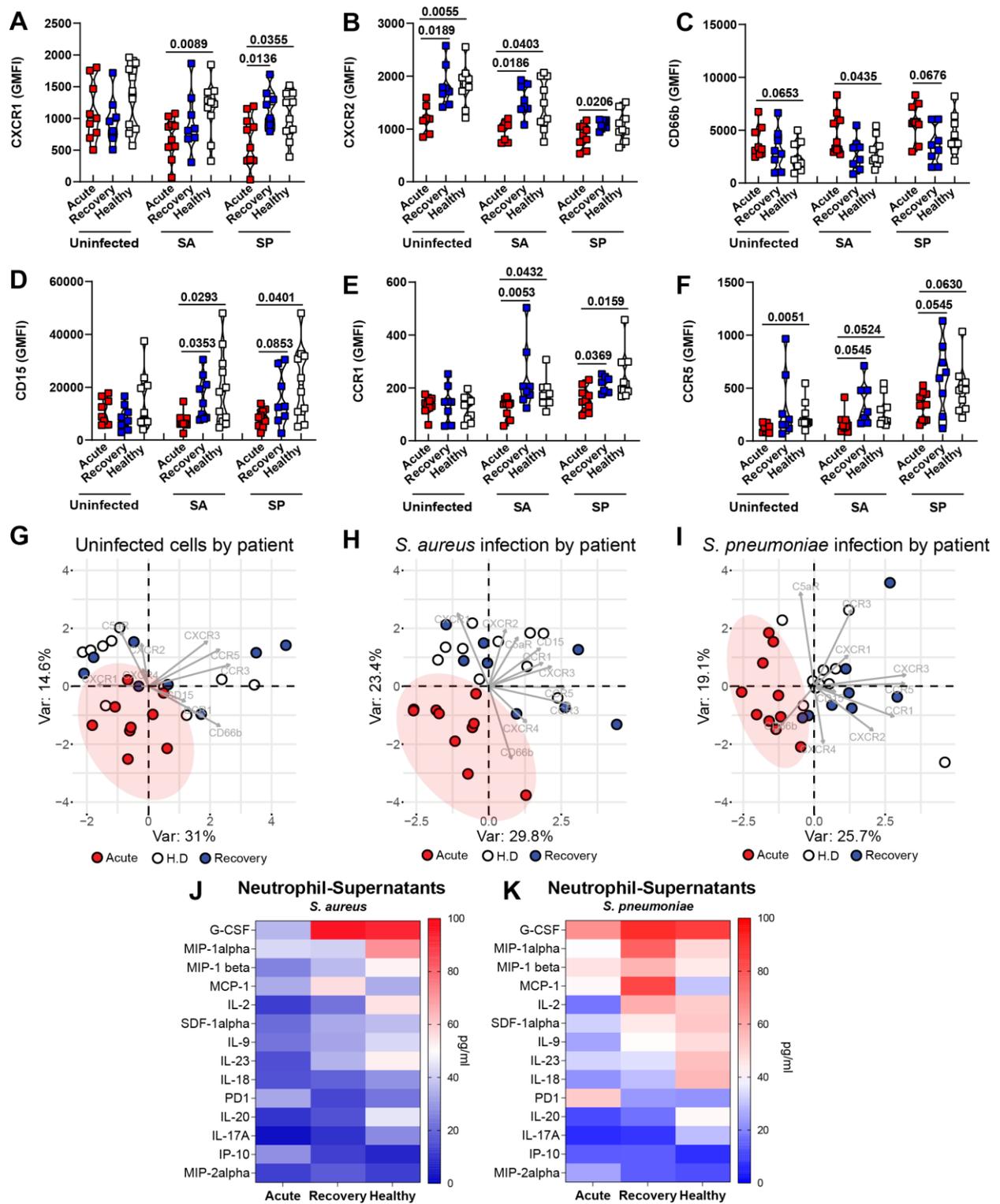


607
608
609

610 **Figure 2. Impaired neutrophil effector response against bacterial challenge in acute COVID-**
611 **19 patients**

612 Functional characterization of neutrophils pre-exposed to plasma from COVID-19 acute (red) or rec
613 (blue) patients (solid symbols) vs healthy plasma (open symbols) upon challenge with either SA or
614 SP. Neutrophil functionality was assessed by quantification of ROS (**A- acute**) (**B- rec**) (n=7-8),
615 Intracellular MPO (**C**) (right–acute) (left -rec) and cell viability (**D**) (right - acute) (left - rec) (n=7-9).
616 The ability to produce NETs was also measured by flow-cytometry. **E**) SYTOX and AQUA positive
617 cells (top) and MPO-SYTOX positive cells (bottom) under unstimulated COVID-19 conditions. (**F, G**
618 **and H**) SYTOX and AQUA positive cells (top) and MPO-SYTOX positive cells (bottom) upon bacterial
619 challenge (n=6-8). **I**) Representative confocal images on NETs formation upon challenge with SA
620 using HOECHST (nuclei; blue), SYTOX (staining extracellular-DNA; green). **J**) Quantification of
621 NETs using fluorescence microscopy (squares, n= [226 - 8898]) and confocal microscopy (circles,
622 n= [19 - 113]) nuclei per point (n=7-8).

Figure 3



623

624

625

626

627

628 **Figure 3. Expression of surface markers and secretion of cytokines in neutrophils upon**
629 **bacterial challenge**

630 Expression of key surface markers in COVID-19 acute (red), rec (blue) and healthy donors' (white)
631 neutrophils (**A-F**) (n=8-10). PCA of cell surface phenotype of COVID-19 patients acute (red), rec
632 (blue) and healthy donors' (white) neutrophils at the basal level without bacterial challenge (COVID-
633 19 status) (**G**), upon SA infection (**H**) or SP infection (**I**). Heat map of cytokine secreted by neutrophils
634 from COVID-19 patients (acute and rec) and healthy controls after bacterial challenge with SA (left)
635 or SP (right) (n=3-4) (**J-K**).

640 **Figure 4. Phenotypic characterization of surface markers and the secretion of cytokines in**
641 **monocytes upon bacterial challenge**

642 Expression of key surface markers in COVID-19 acute (red), rec (blue) and in healthy donors' (white)
643 classical (**A-C**) and non-classical (**D-F**) monocytes (n=9-11). PCA of cell surface phenotype of
644 COVID-19 patients acute (red), rec (blue) and in healthy donors (white) of classical monocytes at
645 the basal level without bacterial challenge (COVID-19 status) (**G**), upon SA infection (**H**) or SP
646 infection (**I**). Heat map of cytokine secreted by monocytes from COVID-19 patients (acute and rec)
647 and healthy controls after bacterial challenge with SA (left) or SP (right) (n=2-3) (**J-K**).

648

649

650

651

652

653

654

655

656

657

658

659

660

661

662

663

664

665

666

667

668

669

670

671

672

673

674

675

676 References

- 677 Arunachalam, P.S., Wimmers, F., Mok, C.K.P., Perera, R., Scott, M., Hagan, T., Sigal, N., Feng, Y., Bristow, L.,
678 Tak-Yin Tsang, O., *et al.* (2020). Systems biological assessment of immunity to mild versus severe COVID-19
679 infection in humans. *Science* 369, 1210-1220.
- 680 Blanco-Melo, D., Nilsson-Payant, B.E., Liu, W.C., Uhl, S., Hoagland, D., Møller, R., Jordan, T.X., Oishi, K.,
681 Panis, M., Sachs, D., *et al.* (2020). Imbalanced Host Response to SARS-CoV-2 Drives Development of COVID-
682 19. *Cell* 181, 1036-1045.e1039.
- 683 Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and
684 Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science* 303, 1532.
- 685 Buehler, P.K., Zinkernagel, A.S., Hofmaenner, D.A., Wendel Garcia, P.D., Acevedo, C.T., Gomez-Mejia, A.,
686 Mairpady Shambat, S., Andreoni, F., Maibach, M., Bartussek, J., *et al.* (2020). Bacterial pulmonary
687 superinfections are associated with unfavourable outcomes in critically ill COVID-19 patients. medRxiv,
688 2020.2009.2010.20191882.
- 689 Castellani, S., D'Oria, S., Diana, A., Polizzi, A.M., Di Gioia, S., Marigiò, M.A., Guerra, L., Favia, M., Vinella,
690 A., Leonetti, G., *et al.* (2019). G-CSF and GM-CSF Modify Neutrophil Functions at Concentrations found in
691 Cystic Fibrosis. *Scientific Reports* 9, 12937.
- 692 Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T., Zhang, X., Chen, H., Yu, H., *et al.* (2020).
693 Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 130,
694 2620-2629.
- 695 Chevrier, S., Zurbuchen, Y., Cervia, C., Adamo, S., Raeber, M.E., de Souza, N., Sivapatham, S., Jacobs, A.,
696 Bächli, E., Rudiger, A., *et al.* (2020). A distinct innate immune signature marks progression from mild to severe
697 COVID-19. bioRxiv, 2020.2008.2004.236315.
- 698 Coperchini, F., Chiovato, L., Croce, L., Magri, F., and Rotondi, M. (2020). The cytokine storm in COVID-19: An
699 overview of the involvement of the chemokine/chemokine-receptor system. *Cytokine & Growth Factor Reviews*
700 53, 25-32.
- 701 Cummings, C.J., Martin, T.R., Frevert, C.W., Quan, J.M., Wong, V.A., Mongovin, S.M., Hagen, T.R., Steinberg,
702 K.P., and Goodman, R.B. (1999). Expression and function of the chemokine receptors CXCR1 and CXCR2 in
703 sepsis. *J Immunol* 162, 2341-2346.
- 704 Evrard, M., Kwok, I.W.H., Chong, S.Z., Teng, K.W.W., Becht, E., Chen, J., Sieow, J.L., Penny, H.L., Ching,
705 G.C., Devi, S., *et al.* (2018). Developmental Analysis of Bone Marrow Neutrophils Reveals Populations
706 Specialized in Expansion, Trafficking, and Effector Functions. *Immunity* 48, 364-379.e368.
- 707 Fischer-Riepe, L., Daber, N., Schulte-Schrepping, J., Véras De Carvalho, B.C., Russo, A., Pohlen, M., Fischer,
708 J., Chasan, A.I., Wolf, M., Ulas, T., *et al.* CD163 expression defines specific, IRF8-dependent, immune-
709 modulatory macrophages in the bone marrow. *Journal of Allergy and Clinical Immunology*.
- 710 Giamarellos-Bourboulis, E.J., Netea, M.G., Rovina, N., Akinosoglou, K., Antoniadou, A., Antonakos, N.,
711 Damoraki, G., Gkavogianni, T., Adami, M.-E., Katsaounou, P., *et al.* (2020a). Complex Immune Dysregulation
712 in COVID-19 Patients with Severe Respiratory Failure. *Cell Host & Microbe* 27, 992-1000.e1003.
- 713 Giamarellos-Bourboulis, E.J., Netea, M.G., Rovina, N., Akinosoglou, K., Antoniadou, A., Antonakos, N.,
714 Damoraki, G., Gkavogianni, T., Adami, M.E., Katsaounou, P., *et al.* (2020b). Complex Immune Dysregulation
715 in COVID-19 Patients with Severe Respiratory Failure. *Cell Host Microbe* 27, 992-1000.e1003.
- 716 Guan, W.J., Ni, Z.Y., Hu, Y., Liang, W.H., Ou, C.Q., He, J.X., Liu, L., Shan, H., Lei, C.L., Hui, D.S.C., *et al.*
717 (2020). Clinical Characteristics of Coronavirus Disease 2019 in China. *N Engl J Med* 382, 1708-1720.
- 718 Hadjadj, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Pere, H., Charbit, B., Bondet, V., Chenevier-
719 Gobeaux, C., Breillat, P., *et al.* (2020). Impaired type I interferon activity and exacerbated inflammatory
720 responses in severe Covid-19 patients. medRxiv, 2020.2004.2019.20068015.
- 721 He, Y., Li, W., Wang, Z., Chen, H., Tian, L., and Liu, D. (2020). Nosocomial infection among patients with
722 COVID-19: A retrospective data analysis of 918 cases from a single center in Wuhan, China. *Infect Control*
723 *Hosp Epidemiol* 41, 982-983.
- 724 Hu, S., He, W., Du, X., Yang, J., Wen, Q., Zhong, X.-P., and Ma, L. (2017). IL-17 Production of Neutrophils
725 Enhances Antibacteria Ability but Promotes Arthritis Development During *Mycobacterium*
726 *tuberculosis* Infection. *EBioMedicine* 23, 88-99.
- 727 Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., *et al.* (2020). Clinical
728 features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497-506.
- 729 Hughes, S., Troise, O., Donaldson, H., Mughal, N., and Moore, L.S.P. (2020). Bacterial and fungal coinfection
730 among hospitalized patients with COVID-19: a retrospective cohort study in a UK secondary-care setting. *Clin*
731 *Microbiol Infect* 26, 1395-1399.
- 732 Kuri-Cervantes, L., Pampena, M.B., Meng, W., Rosenfeld, A.M., Ittner, C.A.G., Weisman, A.R., Agyekum, R.S.,
733 Mathew, D., Baxter, A.E., Vella, L.A., *et al.* (2020). Comprehensive mapping of immune perturbations
734 associated with severe COVID-19. *Science Immunology* 5, eabd7114.
- 735 Lansbury, L., Lim, B., Baskaran, V., and Lim, W.S. (2020). Co-infections in people with COVID-19: a systematic
736 review and meta-analysis. *J Infect* 81, 266-275.

- 737 Leung, B.P., Culshaw, S., Gracie, J.A., Hunter, D., Canetti, C.A., Campbell, C., Cunha, F., Liew, F.Y., and
738 McInnes, I.B. (2001). A Role for IL-18 in Neutrophil Activation. *The Journal of Immunology* 167, 2879.
- 739 Lucas, C., Wong, P., Klein, J., Castro, T.B.R., Silva, J., Sundaram, M., Ellingson, M.K., Mao, T., Oh, J.E.,
740 Israelow, B., *et al.* (2020). Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature*
741 584, 463-469.
- 742 MacParland, S.A., Liu, J.C., Ma, X.Z., Innes, B.T., Bartczak, A.M., Gage, B.K., Manuel, J., Khuu, N., Echeverri,
743 J., Linares, I., *et al.* (2018). Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage
744 populations. *Nat Commun* 9, 4383.
- 745 Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D., and
746 Anderson, P. (2001). Intranasal immunization with killed unencapsulated whole cells prevents colonization and
747 invasive disease by capsulated pneumococci. *Infect Immun* 69, 4870-4873.
- 748 Middleton, E.A., He, X.-Y., Denorme, F., Campbell, R.A., Ng, D., Salvatore, S.P., Mostyka, M., Baxter-Stoltzfus,
749 A., Borczuk, A.C., Loda, M., *et al.* (2020). Neutrophil extracellular traps contribute to immunothrombosis in
750 COVID-19 acute respiratory distress syndrome. *Blood* 136, 1169-1179.
- 751 Morrissey, S.M., Geller, A.E., Hu, X., Tieri, D., Cooke, E.A., Ding, C., Woeste, M., Zhange, H.-g., Cavallazi, R.,
752 Clifford, S.P., *et al.* (2020). Emergence of Low-density Inflammatory Neutrophils Correlates with
753 Hypercoagulable State and Disease Severity in COVID-19 Patients. medRxiv, 2020.2005.2022.20106724.
- 754 Radermecker, C., Detrembleur, N., Guiot, J., Cavalier, E., Henket, M., d'Emal, C., Vanwinge, C., Cataldo, D.,
755 Oury, C., Delvenne, P., *et al.* (2020). Neutrophil extracellular traps infiltrate the lung airway, interstitial, and
756 vascular compartments in severe COVID-19. *Journal of Experimental Medicine* 217.
- 757 Schulte-Schrepping, J., Reusch, N., Paclik, D., Baßler, K., Schlickeiser, S., Zhang, B., Krämer, B., Krammer,
758 T., Brumhard, S., Bonaguro, L., *et al.* (2020). Severe COVID-19 Is Marked by a Dysregulated Myeloid Cell
759 Compartment. *Cell* 182, 1419-1440.e1423.
- 760 Shi, S., Qin, M., Shen, B., Cai, Y., Liu, T., Yang, F., Gong, W., Liu, X., Liang, J., Zhao, Q., *et al.* (2020).
761 Association of Cardiac Injury With Mortality in Hospitalized Patients With COVID-19 in Wuhan, China. *JAMA*
762 *Cardiol* 5, 802-810.
- 763 Silvin, A., Chapuis, N., Dunsmore, G., Goubet, A.-G., Dubuisson, A., Derosa, L., Almiré, C., Hénon, C.,
764 Kosmider, O., Droin, N., *et al.* (2020). Elevated Calprotectin and Abnormal Myeloid Cell Subsets Discriminate
765 Severe from Mild COVID-19. *Cell* 182, 1401-1418.e1418.
- 766 Swamydas, M., Gao, J.-L., Break, T.J., Johnson, M.D., Jaeger, M., Rodriguez, C.A., Lim, J.K., Green, N.M.,
767 Collar, A.L., Fischer, B.G., *et al.* (2016). CXCR1-mediated neutrophil degranulation and fungal killing promote
768 Candida clearance and host survival. *Science Translational Medicine* 8, 322ra310.
- 769 Tan, L., Wang, Q., Zhang, D., Ding, J., Huang, Q., Tang, Y.Q., Wang, Q., and Miao, H. (2020). Lymphopenia
770 predicts disease severity of COVID-19: a descriptive and predictive study. *Signal Transduct Target Ther* 5, 33.
- 771 The, A.D.T.F. (2012). Acute Respiratory Distress Syndrome: The Berlin Definition. *JAMA* 307, 2526-2533.
- 772 Thevarajan, I., Nguyen, T.H.O., Koutsakos, M., Druce, J., Caly, L., van de Sandt, C.E., Jia, X., Nicholson, S.,
773 Catton, M., Cowie, B., *et al.* (2020). Breadth of concomitant immune responses prior to patient recovery: a
774 case report of non-severe COVID-19. *Nat Med* 26, 453-455.
- 775 Varga, Z., Flammer, A.J., Steiger, P., Haberecker, M., Andermatt, R., Zinkernagel, A.S., Mehra, M.R.,
776 Schuepbach, R.A., Ruschitzka, F., and Moch, H. (2020). Endothelial cell infection and endotheliitis in COVID-
777 19. *The Lancet* 395, 1417-1418.
- 778 Veglia, F., Perego, M., and Gabrilovich, D. (2018). Myeloid-derived suppressor cells coming of age. *Nat*
779 *Immunol* 19, 108-119.
- 780 Venet, F., Demaret, J., Gossez, M., and Monneret, G. (2020). Myeloid cells in sepsis-acquired
781 immunodeficiency. *Annals of the New York Academy of Sciences* *n/a*.
- 782 Veras, F.P., Pontelli, M.C., Silva, C.M., Toller-Kawahisa, J.E., de Lima, M., Nascimento, D.C., Schneider, A.H.,
783 Caetité, D., Tavares, L.A., Paiva, I.M., *et al.* (2020). SARS-CoV-2-triggered neutrophil extracellular traps
784 mediate COVID-19 pathology. *Journal of Experimental Medicine* 217.
- 785 Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., Wang, B., Xiang, H., Cheng, Z., Xiong, Y., *et al.* (2020).
786 Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in
787 Wuhan, China. *JAMA* 323, 1061-1069.
- 788 Wen, W., Su, W., Tang, H., Le, W., Zhang, X., Zheng, Y., Liu, X., Xie, L., Li, J., Ye, J., *et al.* (2020). Immune
789 cell profiling of COVID-19 patients in the recovery stage by single-cell sequencing. *Cell Discovery* 6, 31.
- 790 Wilk, A.J., Rustagi, A., Zhao, N.Q., Roque, J., Martínez-Colón, G.J., McKechnie, J.L., Ivison, G.T., Ranganath,
791 T., Vergara, R., Hollis, T., *et al.* (2020). A single-cell atlas of the peripheral immune response in patients with
792 severe COVID-19. *Nature Medicine* 26, 1070-1076.
- 793 Woytschak, J., Keller, N., Krieg, C., Impellizzeri, D., Thompson, Robert W., Wynn, Thomas A., Zinkernagel,
794 Annelies S., and Boyman, O. (2016). Type 2 Interleukin-4 Receptor Signaling in Neutrophils Antagonizes Their
795 Expansion and Migration during Infection and Inflammation. *Immunity* 45, 172-184.
- 796 Wu, C., Chen, X., Cai, Y., Xia, J., Zhou, X., Xu, S., Huang, H., Zhang, L., Zhou, X., Du, C., *et al.* (2020). Risk
797 Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus
798 Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med* 180, 934-943.

799 Wu, Z., and McGoogan, J.M. (2020). Characteristics of and Important Lessons From the Coronavirus Disease
800 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for
801 Disease Control and Prevention. *JAMA* 323, 1239-1242.

802 Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., Liu, S., Zhao, P., Liu, H., Zhu, L., *et al.* (2020).
803 Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *The Lancet*
804 *Respiratory Medicine* 8, 420-422.

805 Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., *et al.* (2020). Clinical
806 course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort
807 study. *Lancet* 395, 1054-1062.

808 Zuo, Y., Yalavarthi, S., Shi, H., Gockman, K., Zuo, M., Madison, J.A., Blair, C., Weber, A., Barnes, B.J., Egeblad,
809 M., *et al.* (2020). Neutrophil extracellular traps in COVID-19. *JCI Insight* 5.

810