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1 **Angiotensin II induces reactive oxygen species, DNA damage,**
2 **and T cell apoptosis in severe COVID-19**

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31

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33 **Abstract**

34 **Background.** Lymphopenia is predictive of survival in Coronavirus disease 2019 (COVID-19)
35 patients.

36 **Objective.** The aim of this study was to understand the cause of a lymphocyte count drop in
37 severe forms of SARS-CoV-2 infection.

38 **Methods.** Monocytic production of reactive oxygen species (ROS) and T cell apoptosis were
39 measured by flow cytometry, DNA damage in peripheral mononuclear blood cells (PBMCs) by
40 immunofluorescence, and Angiotensin II (AngII) by ELISA in SARS-CoV-2-infected patients
41 upon admission to Intensive Care Units (ICU, n=29) or non-ICU (n=29), and in age- and sex-
42 matched healthy controls.

43 **Results.** We show that the monocytes of certain COVID-19 patients spontaneously released
44 ROS able to induce DNA damage and apoptosis in neighboring cells. Of note, high ROS
45 production was predictive of death in ICU patients. Accordingly, in most patients, we observed
46 the presence of DNA damage in up to 50% of their PBMCs, and T-cell apoptosis. Moreover, the
47 intensity of this DNA damage was linked to lymphopenia. SARS-CoV-2 is known to induce the
48 internalization of its receptor, Angiotensin Converting Enzyme 2, a protease able to catabolize
49 AngII. Accordingly, we observed in certain COVID-19 patients high plasma levels of AngII.
50 Looking for the stimulus responsible for their monocytic ROS production, we unveiled that
51 AngII triggers ROS production by monocytes via Angiotensin receptor I. ROS released by
52 AngII-activated monocytes induced DNA damage and apoptosis in neighboring lymphocytes.

53 **Conclusion.** We conclude that T cell apoptosis provoked via DNA damage due to the release of
54 monocytic ROS could play a major role in COVID-19 pathogenesis.

55

56 **Clinical implication.** Unveiling this new pathogenic pathway opens up new therapeutic
57 possibilities for COVID-19.

58

59 **Capsule summary.** SARS-CoV-2 may trigger a cascade of events resulting in programmed T
60 cell death and severe COVID-19 which may be prevented by an Angiotensin receptor I
61 antagonist and/or an antioxidant.

62

63 **Key words.** SARS-CoV-2, ACE2, oxidative stress, antioxidant, Angiotensin II receptor, DNA
64 oxidation, programmed cell death, lymphopenia.

65

66 **Running title:** DNA damage and T cell apoptosis in COVID-19

67

68 **Abbreviations.** SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19,
69 coronavirus disease 2019; ACE2, Angiotensin-converting enzyme 2; ICU, Intensive Care Units;
70 HD, healthy donor; RNA, ribonucleic acid; NADP, nicotinamide adenine dinucleotide
71 phosphate; ROS, reactive oxygen species; AngII, Angiotensin II; PBMCs, peripheral mononuclear
72 blood cells; DCFH-DA, dichloro-dihydro-fluorescein diacetate; DPI, diphenyleiodonium; AU,
73 arbitrary unit; NAC, N-acetylcysteine; AT1, Angiotensin receptor 1; PaO₂, arterial oxygen
74 tension; mAb, monoclonal antibody; FBS, fetal bovine serum; LPS, lipopolysaccharide.

75

76 **Conflicts of interests.** Authors declare that they have no conflicts of interests.

77 INTRODUCTION

78 COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2
79 (SARS-CoV-2). The most severe forms of COVID-19 are due to acute lung damage which is
80 strongly linked to hyperactivation of the immune system (1). A hallmark of critical COVID-19 is
81 lymphopenia (2), observed in up to 63% of COVID-19 patients, and predictive of an unfavorable
82 outcome (3). Yet, the cause of peripheral blood T-cell, B-cell, and NK-cell loss remains unclear.
83 Indeed, this loss may be the consequence of a decrease in lymphocyte production, the trapping of
84 these cells in the respiratory tract and/or a high rate of lymphocyte death. As lymphocyte counts
85 are strongly predictive of survival, understanding the causes of lymphopenia is of major
86 importance.

87 Various ribonucleic acid (RNA) viruses have been reported to induce ROS production and
88 antioxidant system depletion. For instance, the influenza virus increases the level of ROS
89 production in the host cells and decreases the concentration of antioxidants (4). Moreover, the
90 oxidative stress provoked by the virus is responsible for lung damage that may be prevented by
91 antioxidants or by targeting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2
92 (4). Likewise, respiratory syncytial virus infection causes ROS expression (5) and decreases the
93 expression of antioxidant genes, contributing to bronchiolitis (6). SARS-CoV-1 modifies the
94 oxidoreductase system of the mitochondria, via an interaction between its non-structural protein
95 10 and cytochrome oxidase II (7). In line with this mechanism, oxidative stress has been reported
96 in the lungs of SARS-CoV-1-infected mice (8). Likewise, SARS-CoV-2-infected monocytes
97 overproduce mitochondrial ROS, and an increased expression of oxidative stress-associated
98 genes has been observed in monocytes of bronchoalveolar fluid from COVID-19 patients (9). In
99 the peripheral blood of these patients, markers of NADPH oxidase-2 activation (10), impaired

100 antioxidant activity (11) and oxidative stress (12) have been revealed as possibly being linked to
101 the severity of the disease.

102 As ROS can cause DNA damage resulting in apoptosis (13), we analyzed the level of monocytic
103 ROS production in COVID-19 patients at different stages, as well as its causes and
104 consequences.

105

106 MATERIALS AND METHODS

107 **Study design.** This is an observational, monocentric, case-control study. Adults with positive
108 naso-pharyngeal swabs for SARS-CoV-2 RNA by RT-PCR were consecutively recruited at the
109 Nîmes University Hospital. Patients were either recruited on the day of their admission to an
110 intensive care unit (ICU) for oxygen saturation <90% in ambient air or <95% with 5L/mn of
111 oxygenotherapy and/or arterial oxygen tension (PaO₂) of less than 60 mm Hg or upon admission
112 to the Tropical and Infectious Diseases Department (non-ICU) for oxygen saturation <96% in
113 ambient air. No outlier was excluded. All the replicates were biological. This study was
114 approved by the French Ethics Committee, Île-de-France 1. All patients had provided written
115 informed consent, and the trial was registered (Eudract/IDRCB 2020-A00875-34 and
116 ClinicalTrials NCT04351711).

117 **Cell-sorting and co-culture.** Monocytes were sorted from peripheral mononuclear blood cells
118 (PBMCs) using CD14-coated microbeads (Miltenyi Biotech). Cells, pre-incubated or not with
119 diphenyleneiodonium (DPI) or N-acetylcysteine (NAC) for 3 hours at 37°C, were washed twice
120 and cocultured in 1µm pore-size inserts with BJ cells (fibroblasts established from skin, ATCC
121 CRL-2522) placed on coverslips in 24-well companion plates. PBMCs or monocytes and BJ
122 cells were co-cultured in 2:1 ratio in 1:1 DMEM and RPMI culture media supplemented with
123 10% heat-inactivated fetal bovine serum (FBS) for 3 days. Camptothecin (10µM) was used on BJ
124 cells for 45 min at 37°C. Lipopolysaccharide (LPS, 1µg/ml) or Angiotensin II (AngII, 75pM)
125 was added to the cells in 500µl final volume of RPMI without serum and incubated at 37°C for
126 30 minutes. Cells were washed and fixed for further staining.

127 **Immunofluorescence.** PBMC adherence on coverslips was obtained by using 20 µg/ml of poly-
128 lysine in serum-free RPMI for 2 hours at room temperature. Coverslips with cocultured BJ cells

129 were washed twice, fixed with 2% paraformaldehyde in PBS for 10 minutes, rinsed again with
130 PBS, and permeabilized with PBS containing 0.1% Triton-X-100 for 10 minutes at room
131 temperature. Thereafter, coverslips were washed and blocked with PBS containing 10% FBS for
132 30 minutes. Cells were then incubated with anti- γ -H2AX (Millipore, 1/500) for 1 hour or anti-
133 53BP1 (Millipore, 1/300) monoclonal antibodies (mAb) in PBS with 10% FBS. Coverslips were
134 rinsed three times with PBS and incubated with AF 546 anti-mouse IgG1 (Invitrogen, 1/2000)
135 secondary antibody for 45 minutes in PBS with 10% FBS at room temperature. After washing
136 with PBS, DNA was counter stained with DAPI (Sigma-Aldrich) for 5 minutes, and coverslips
137 were mounted in fluorescence mounting medium (Prolong gold, Invitogen). Slides were kept
138 overnight at 20°C in a dark room. Images were obtained with a Zeiss ApoTome fluorescence
139 microscope (63X magnification and 1.4 numerical aperture for BJ cells, 100X magnification and
140 1.46 numerical aperture for PBMC) with supporting software, and analyzed on Image J and FIJI
141 software systems.

142 **Flow Cytometry.** The monoclonal antibodies used for cell surface staining were: CD3-
143 APCA750, CD14-PE, CD16-APC, CD4-APC (Beckman Coulter), CD3-BV421 and CD3-AF700
144 (Biolegend). Annexin-V-PE (Biolegend) was used according to manufacturer's guidelines. For
145 ROS quantification, 10^6 PBMCs were resuspended in $1\mu\text{M}$ dichloro-dihydro-fluorescein
146 diacetate (DCFH-DA) for 25min at room temperature. Data were acquired on a Navios flow
147 cytometer (Beckman Coulter) from 20,000 gated events per sample and on a MACSQuant
148 analyser 10 (Miltenyi Biotec), and analyzed using Kaluza software.

149 **ELISA.** AngII concentrations were determined using the Angiotensin II ELISA kit (Enzo Life
150 Sciences).

151 **Statistical analyses.** No data pre-processing was performed. Statistical analyses and graphical
152 presentations were computed with GraphPad Prism version 6. D'Agostino and Pearson normality
153 test was performed. Differences between two groups were analyzed using two-sided unpaired
154 student's *t* test or Mann-Whitney test as appropriate. Differences between more than two groups
155 were analyzed using one-way ANOVA, Welch ANOVA or Kruskal-Wallis test as appropriate.
156 We used a two-sided Spearman rank test to evaluate correlations. A *p* value of <0.05 was
157 considered statistically significant.

158 **RESULTS**

159 **Patient enrollment**

160 We enrolled 29 PCR-positive SARS-CoV-2-infected patients upon admission to ICUs for an
161 oxygen saturation of less than 90% and/or PaO₂ below 60 mmHg in room air, or an oxygen
162 saturation of less than 95% while receiving 5L/min of oxygen. We also recruited 29 PCR-
163 positive SARS-CoV-2-infected patients upon admission to the Infectious Diseases Department
164 (non-ICU) for an oxygen saturation of less than 96% in room air and/or deterioration in their
165 general condition. Age- and sex-matched healthy donor (HDs: age range, 28 to 95 years) were
166 used as negative controls. The bioclinical characteristics of these patients are shown in Table 1.

167

168

Table 1. Bioclinical characteristics of the patients enrolled

169

170

		Non-ICU patients (n=29)	ICU patients (n=29)	non-ICU vs ICU
Age (years)	Mean (SD) Range	66.1 (20.9) 29.0-96.0	69.3 (13.5) 43.0-95.0	p = 0.702
Gender:				
Females	n (%)	16 (55)	13(45)	p = 0.600
Males	n (%)	13 (45)	16 (55)	
Any comorbidity	n (%)	12 (40)	12 (41)	p = 0.594
Diabetes	n (%)	7 (23)	7 (24)	p = 0.762
Cancer	n (%)	4 (13)	2 (7)	p = 0.783
Autoimmune disease	n (%)	1 (3)	0 (0)	p = 0.999
Chronic kidney failure	n (%)	0 (0)	2 (7)	p = 0.202
Duration of symptomatology (days)	Mean (SD)	6.8 (9.4)	11.8 (7.2)	p < 0.001
C-reactive protein (mg/L, normal range 0.9-1.8)	Mean (SD)	56.8 (68.1)	115.0 (81.3)	p = 0.003
Lactate dehydrogenase (IU/L, normal range 135-214)	Mean (SD)	214.8 (49.9)	416.4 (177.3)	p < 0.001
Absolute lymphocyte count (x10 ⁹ /L, normal range 0.9-1.8)	Mean (SD)	1.30 (0.53)	0.88 (0.59)	p = 0.004
Absolute monocyte count (x10 ⁹ /L, normal range 0.9-1.8)	Mean (SD)	0.70 (0.36)	0.46 (0.27)	p = 0.010

171

172

173 **Monocytes from COVID-19 patients overproduce ROS**

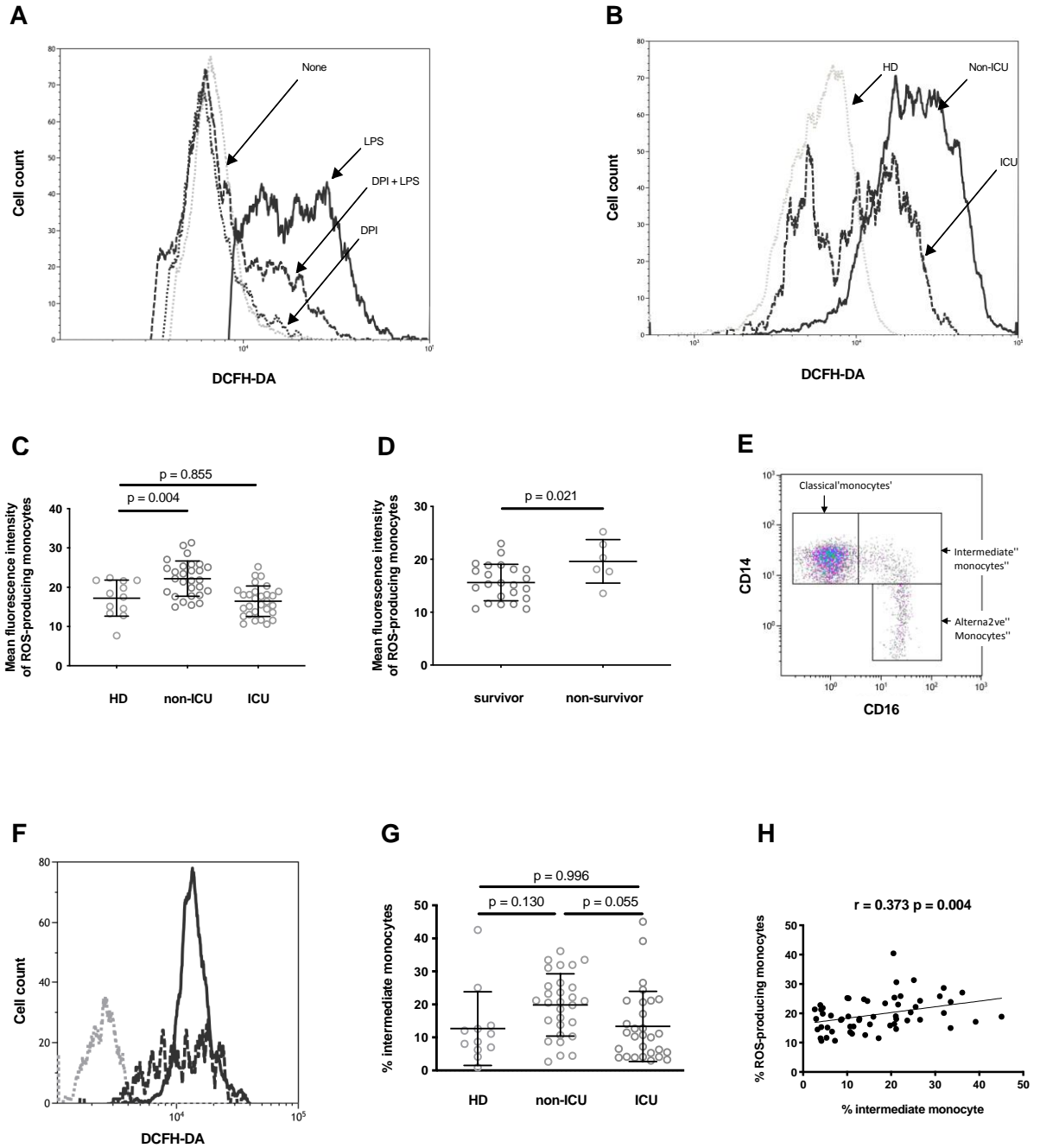
174 To test whether monocytes from COVID-19 patients produced ROS, we labeled the peripheral

175 PBMCs of SARS-CoV-2-infected individuals with DCFH-DA, which reacts with ROS to give a

176 fluorescent product. Figure 1A shows that monocytes from HDs become fluorescent when they

177 are incubated with DCFH-DA and exposed to LPS, used as a positive control. This ROS

178 production was prevented by pre-incubation with the NADPH oxidase inhibitor
179 diphenyleneiodonium (DPI, Fig. 1A). By contrast, the spontaneous fluorescence of monocytes
180 from HDs incubated with DCFH-DA was not reduced in the presence of DPI (Fig. 1A).
181 Monocytes from certain COVID-19 patients became more fluorescent than monocytes from HDs
182 after being exposed to DCFH-DA (Fig. 1B and 1C). Figure 1C shows the intensity of
183 spontaneous monocytic ROS production in HDs, ICU and non-ICU patients. Non-ICU patients
184 produced more ROS than HDs (22.2 ± 4.5 vs. 17.2 ± 4.6 mean \pm standard deviation (SD)
185 arbitrary units of mean fluorescence intensity (AU), t test $p = 0.004$) whereas ICU patients did
186 not (16.4 ± 3.9 vs. 17.2 ± 4.6 mean \pm SD AU, t test $p = 0.855$). Yet, the ICU patients who
187 survived presented lower monocytic ROS production than those who did not (15.6 ± 3.4 vs. 19.6
188 ± 4.1 mean \pm SD AU, t test $p = 0.021$, Fig. 1D). To identify the monocyte subpopulations
189 responsible for ROS production, we labeled the PBMCs exposed to DCFH-DA with anti-CD14
190 and anti-CD16 antibodies to identify classical (CD14^{hi}CD16^{lo}), intermediate (CD14⁺CD16⁺),
191 and alternative (CD14^{lo}CD16^{hi}) monocytes (Fig. 1E). Figure 1F shows that the intermediate and
192 classical monocytes produced the highest amount of ROS. Compared with ICU, non-ICU
193 participants had a higher percentage of intermediate monocytes (20.7 ± 13.8 vs. 10.7 ± 16.2
194 median \pm interquartile range (IQR), Mann-Whitney $p = 0.055$, Fig. 1G). Logically, the
195 proportions of intermediate monocytes and ROS-producing monocytes were correlated in
196 COVID-19 patients ($r = 0.373$, $p = 0.004$, Fig. 1H).



197

198 **Figure 1. The monocytes from certain COVID-19 patients spontaneously produce ROS.** (A) Fluorescence in
 199 monocytes from a healthy donor, pre-incubated (DPI + LPS, ---) or not (LPS, —) with DPI, exposed to DCFH-DA,
 200 and stimulated with lipopolysaccharide. As negative controls, fluorescence in the same monocytes pre-incubated
 201 (DPI, ---) or not (None, ---) with NADPH oxidase inhibitor DPI and exposed to DCFH-DA was analyzed. (B)
 202 Fluorescence in monocytes from a healthy donor (HD, ---), a non-ICU patient (non-ICU, —), and an ICU patient
 203 (ICU, ---) exposed to DCFH-DA. (C) Mean fluorescence intensity of ROS-producing monocytes from healthy

204 donors (HD), non-ICU patients (non-ICU), and ICU patients (ICU) exposed to DCFH-DA. One-way ANOVA test p
205 < 0.001. (D) Mean fluorescence intensity of ROS-producing monocytes from ICU patients who survived or not. (E)
206 Identification of the classical, intermediate, and alternative monocyte subpopulations by flow cytometry. (F)
207 Fluorescence in CD14^{hi}CD16^{lo} (---), CD14⁺CD16⁺ (—), and CD14^{lo}CD16^{hi} (··) monocytes from an ICU patient
208 exposed to DCFH-DA. (G) Percentages of CD14-CD16⁺ monocytes circulating in healthy (HDs), ICU and non-ICU
209 donors. One-way ANOVA test p = 0.032. (H) Correlation between the proportions of intermediate and ROS-
210 producing monocytes in ICU and non-ICU patients.

211

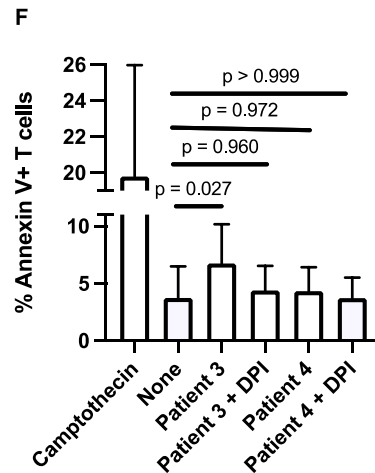
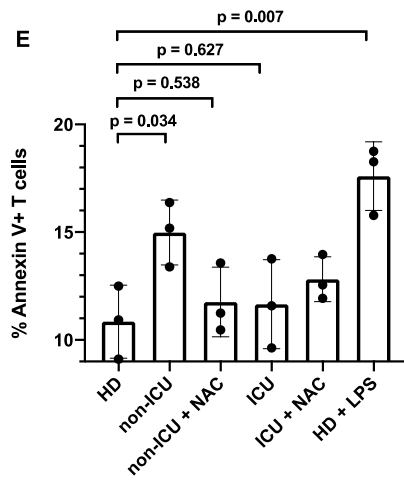
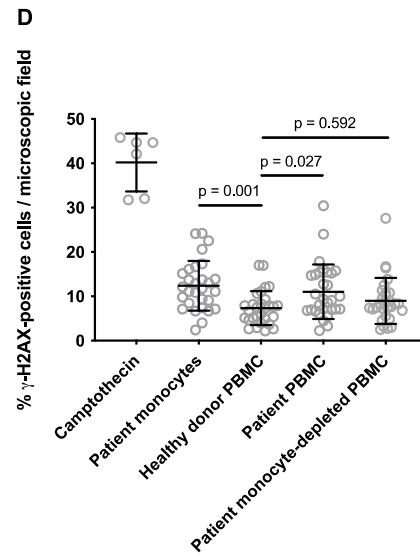
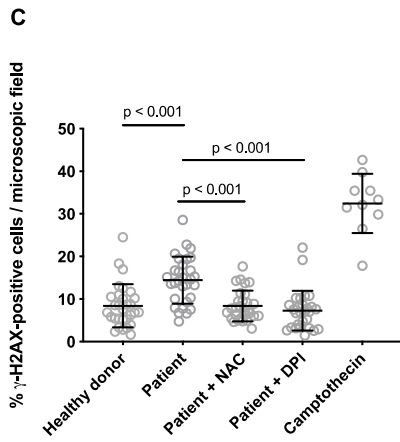
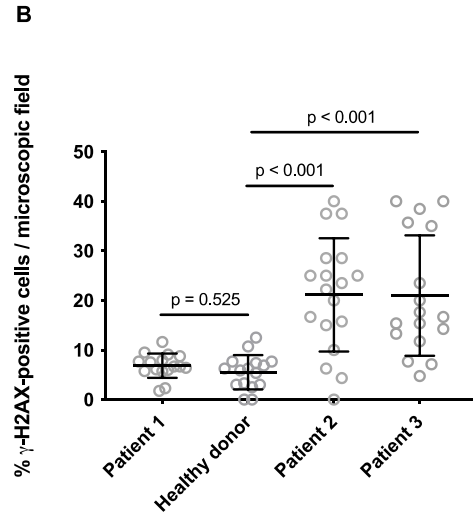
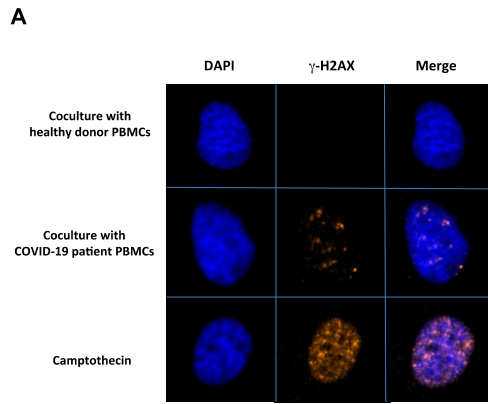
212 **Monocytes from COVID-19 patients induce DNA damage via ROS**

213 ROS can oxidize proteins, lipids or DNA. We searched for the effect of monocytic ROS
214 production on the DNA of bystander cells. For this purpose, we probed the presence of the
215 phosphorylated form of the histone variant H2AX (γ -H2AX), a hallmark of chromosome breaks
216 and DNA replication stress (14), in primary BJ fibroblasts co-cultured with PBMCs from
217 COVID-19 patients. In this assay, PBMCs were co-cultured in transwells, i.e. with no cell-to-cell
218 contact with BJ cells. Camptothecin, a topoisomerase I inhibitor which induces replication-
219 dependent DNA lesions, was used as a positive control, whereas PBMCs from healthy donors
220 were included as negative controls. We found that PBMCs in 8 out of the 25 patients we tested
221 (32%), induced γ -H2AX nuclear foci in bystander BJ cells as exemplified in Figure 2A and 2B.
222 Figure 2C shows that the formation of these foci was prevented by pre-incubating PBMCs with
223 the ROS scavenger, NAC, or the NADPH oxidase inhibitor, DPI. This establishes that the
224 γ -H2AX foci are indeed induced by ROS. To make really sure that the sources of DNA cells
225 damaging ROS were monocytes, we repeated the experiment after depleting patient PBMCs of
226 monocytes using CD14-coated magnetic beads. Figure 2D shows that, whereas PBMCs and

227 monocytes from the patient we analyzed induced DNA damage, monocyte-depleted PBMCs
228 from the same patient did not.

229 **PBMC DNA damage results in T-cell apoptosis during severe SARS-CoV-2 infection**

230 ROS-induced DNA damage may provoke apoptosis (13). Therefore, we tested whether co-
231 culturing with COVID-19 PBMCs might trigger apoptosis in HD PBMCs. Indeed, HD T-cells
232 presented more phosphatidylserine at their surface, a marker of apoptosis, as measured by
233 Annexin V labelling on day 6 when they were exposed to COVID-19 PBMCs able to induce
234 DNA damage than when they were exposed to another healthy volunteer PBMCs (15.0 ± 1.5 vs.
235 10.8 ± 1.7 % mean \pm SD, *t* test *p* = 0.034, Fig. 2E). This programmed cell death provoked by
236 COVID-19 PBMCs was entirely mediated by ROS, since the presence of NAC reduced
237 apoptosis to the background level observed in the presence of HD PBMCs (11.8 ± 1.6 vs. $10.8 \pm$
238 1.7 % mean \pm SD, *t* test *p* = 0.939, Fig. 2E). By contrast, co-culturing with COVID-19 PBMCs
239 unable to induce DNA damage or with HD PBMCs resulted in the same level of apoptosis (11.7
240 ± 2.1 vs. 10.8 ± 1.7 % mean \pm SD, *t* test *p* = 0.959, Fig. 2E). As a positive control, we used LPS-
241 stimulated PBMCs, which triggered apoptosis in HD PBMCs (17.6 ± 1.6 vs 10.8 ± 1.7 % mean \pm
242 SD, *t* test *p* = 0.001, Fig. 2E). We obtained the same results when we co-cultured purified
243 COVID-19 monocytes able to cause DNA damage with HD PBMCs (Fig. 2F). The monocytes of
244 a patient (patient 3) known to induce γ -H2AX foci in neighbouring cells provoked apoptosis in
245 co-cultured PBMCs ($6.8 \pm 3.4\%$ vs. $3.7 \pm 2.8\%$ mean \pm SD, *t* test *p* = 0.027), prevented by DPI
246 ($4.4 \pm 2.1\%$ vs. $3.7 \pm 2.8\%$ mean \pm SD, *t* test *p* = 0.980), whereas monocytes of a patient (patient
247 4) unable to induce γ -H2AX foci in neighbouring cells did not ($4.3 \pm 2.1\%$ vs. $3.7 \pm 2.8\%$ mean
248 \pm SD, *t* test *p* = 0.972).



250 **Figure 2. COVID-19 patient monocytes may induce DNA damage via ROS.** (A) Detection of γ -H2AX foci by
251 immunofluorescence in BJ cells co-cultured with PBMCs from a healthy donor or from a COVID-19 patient.
252 Healthy donor's PBMCs treated with camptothecin were used as positive controls. (B) Quantification of the
253 γ -H2AX foci induced in BJ fibroblasts by PBMCs from COVID-19 patients. The proportion of BJ cells presenting
254 at least 5 foci per nucleus was quantified under microscopy. Each point represents one microscope field. Welch
255 ANOVA test $p < 0.001$. (C) γ -H2AX foci induced in BJ cells by COVID-19 patient's PBMCs are prevented by pre-
256 incubating PBMCs with N-acetylcysteine (NAC) or diphenyleneiodonium (DPI). Each point represents one
257 microscope field. Kruskal-Wallis test $p < 0.001$. (D) Monocytes isolated from the PBMCs of a COVID-19 patient
258 are able to induce DNA damage in BJ cells. The ability to induce γ -H2AX foci in the BJ fibroblasts of PBMCs from
259 a COVID-19 patient, of the same PBMCs depleted of monocytes, and of monocytes isolated from these PBMCs was
260 tested. Each point represents one microscope field. Kruskal-Wallis test $p < 0.001$. (E) Intensity of
261 phosphatidylserine expression at the surface of healthy donor PBMCs cocultured with non-ICU PBMCs able to
262 induce DNA damage treated (non-ICU + NAC) or not (non-ICU) with N-acetylcysteine, or with ICU PBMCs
263 unable to induce DNA damage treated (ICU + NAC) or not (ICU) with N-acetylcysteine, as detected by flow
264 cytometry at day 6. Healthy donor PBMCs co-cultured with another healthy donor PBMCs (HD) or treated with
265 lipopolysaccharide (HD + LPS) were used as negative and positive controls, respectively. One-way ANOVA test p
266 = 0.002. (F) Intensity of phosphatidylserine expression at the surface of healthy donor PBMCs cocultured with
267 COVID-19 monocytes able (patient 3) or not (patient 4) to induce DNA damage and treated (+ DPI) or not with
268 DPI, as detected by flow cytometry at day 6. Camptothecin was used as a positive control.

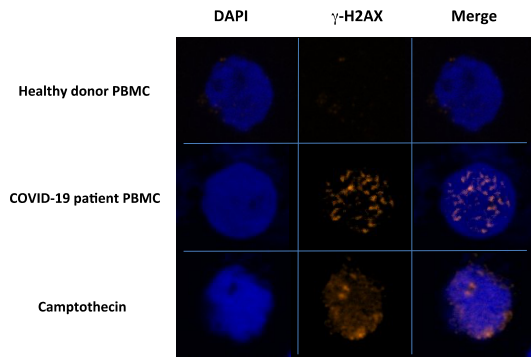
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270 **COVID-19 patient PBMCs present DNA damage**

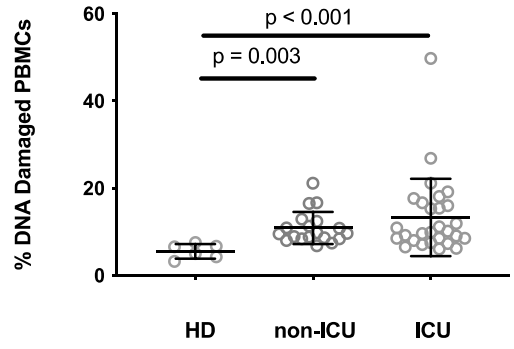
271 As the monocytes of certain COVID-19 patients release ROS which are able to cause DNA
272 damage to neighboring cells, we analyzed whether the PBMCs of these patients presented DNA
273 damage. To do this, we looked for the presence of γ -H2AX nuclear foci in their PBMCs. Figure
274 3A shows an example of a COVID-19 patient whose PBMCs harbor such DNA damage markers.
275 Globally, the proportion of DNA-damaged PBMCs was higher in the 19 non-ICU patients ($9.7 \pm$

276 4.0 % vs. 5.8 ± 2.9 % median \pm IQR, Mann-Whitney $p = 0.003$), and the 28 ICU patients (10.0
277 ± 8.4 % vs. 5.8 ± 2.9 % median \pm IQR, Mann-Whitney $p < 0.001$) than in age-matched healthy
278 donors we analyzed (Fig. 3B). COVID-19 patient PBMCs also harboured DNA double-strand
279 breaks, as revealed by the labelling with an antibody specific for 53BP1, a protein known to
280 aggregate at double-strand ends (15). In the example shown in Figure 3C, 16.8 ± 3.4 % of the
281 patient PBMCs presented 53BP1 foci, a higher proportion than in the healthy donor (6.9 ± 1.6 %
282 mean \pm SD, t test $p = 0.011$). Next, we quantified CD4⁺ T-cell and CD8⁺ T-cell apoptosis in the
283 participant peripheral blood. Figure 3D shows that Annexin V expression at the surface of both
284 lymphocyte subpopulations, particularly on CD8⁺ T-cells, was more frequent in COVID-19
285 patients than in controls. We also tested whether the phenomenon we describe could result in
286 lymphopenia in COVID-19 patients. To this aim, we looked for a correlation between the
287 intensity of DNA damage in PBMCs and lymphopenia. As shown in Figure 3E, we observed an
288 inverse correlation between the percentage of PBMCs with γ -H2AX foci and lymphocyte count
289 in the patients and healthy volunteers we analyzed ($r = -0.341$, $p = 0.025$).

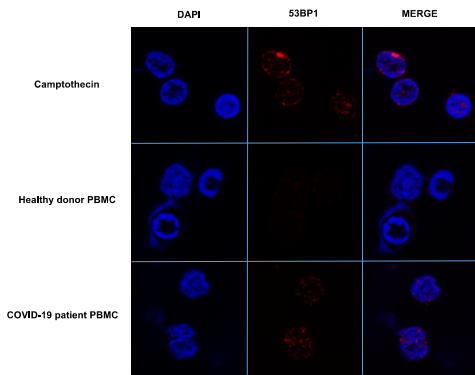
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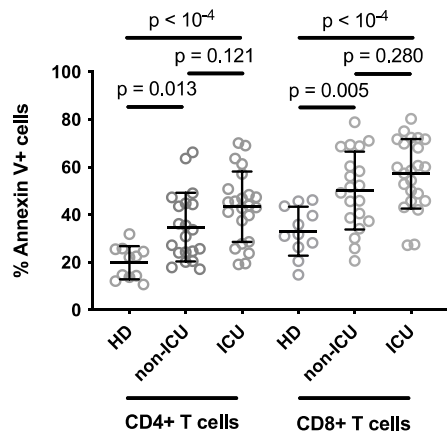
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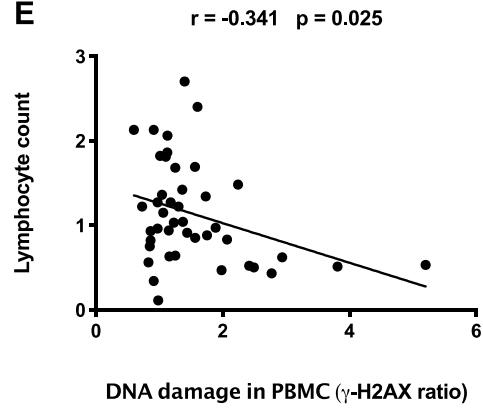
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295 **Figure 3. DNA damage in COVID-19 PBMCs.** (A) PBMCs from a COVID-19 patient whose monocytes induce
296 DNA damage in bystander BJ cells spontaneously present with γ -H2AX foci. PBMCs from a healthy donor treated
297 or not with camptothecin were used as positive and negative controls, respectively. (B) Percentages of PBMCs
298 harboring γ -H2AX foci in healthy donors (HD), non-ICU patients (non-ICU), and ICU patients (ICU). Kruskal-
299 Wallis test $p = 0.002$. (C) PBMCs from a COVID-19 patient whose monocytes induce DNA damage in bystander BJ
300 cells spontaneously present with 53BP1 foci. PBMCs from a healthy donor treated or not with camptothecin were
301 used as positive and negative controls, respectively. (D) Annexin V expression on peripheral blood CD4+ T-cells
302 and CD8+ T-cells of healthy donors (HD), non-ICU patients (non-ICU), and ICU patients (ICU). One-way ANOVA
303 test $p < 0.001$ for CD4+ T-cells and $p < 0.001$ for CD8+ T-cells. (E) Correlation between the intensity of DNA
304 damage in PBMC and lymphocyte counts. The intensity of DNA damage in PBMCs is expressed as the ratio (%
305 patient PBMCs presenting γ -H2AX foci) : (% HD PBMCs presenting γ -H2AX foci).

306

307 **Angiotensin II induces monocytic ROS production**

308 SARS-CoV-2 downregulates the cell surface expression of Angiotensin-converting enzyme 2
309 (ACE2), its main receptor, via ACE2 co-internalization and cleavage by the serine protease
310 TMPRSS2 (16). Knowing that ACE2 converts AngII into Angiotensin 1-7, this should result in
311 an increase in AngII concentration (16). As AngII has been shown to induce ROS production in
312 human mesangial cells (17), we tested whether this peptide was also able to provoke the release
313 of ROS by human monocytes. Indeed, we observed that, like lipopolysaccharide, AngII
314 increased the fluorescence of HD monocytes preincubated with DCFH-DA (Fig. 4A). This effect
315 was completely prevented by 1-hour preincubation with DPI ($91,7 \pm 15.3\%$, Fig. 4B) or the
316 Angiotensin receptor 1 (AT1) antagonist losartan at $10\mu\text{g/mL}$ ($98,7 \pm 4.5\%$, Fig. 4C). Next, we
317 checked to see whether the peripheral blood concentration of AngII was actually high in
318 COVID-19 patients. Figure 4D shows that plasma levels of AngII in non-ICU patients ($72.3 \pm$
319 68.6 vs. 54.5 ± 73.3 pg/mL median \pm IQR , Mann-Whitney test $p = 0.017$), but not in ICU

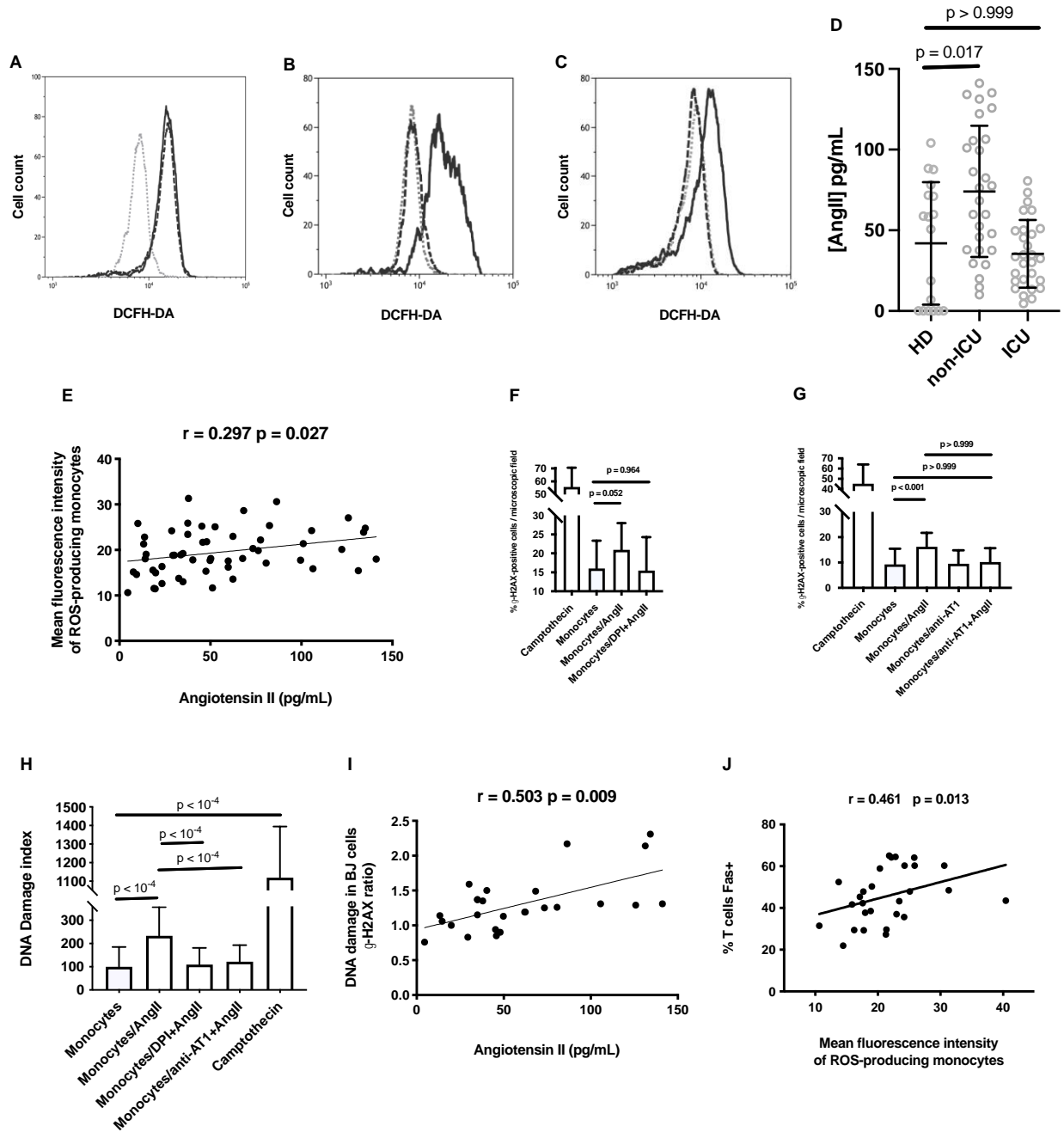
320 patients (33.2 ± 31.5 vs. 54.5 ± 73.3 pg/mL median \pm IQR , Mann-Whitney test $p > 0.999$) were
321 higher than those of normal volunteers. The lower level of AngII in ICU patients as compared
322 with non-ICU patients might be the consequence of the increase in ACE2 expression reported in
323 severe COVID-19 (18), driven by interferon (19), and/or reoxygenation (20). To test the
324 hypothesis that AngII might be involved in the monocytic ROS overproduction that we had
325 unveiled in certain patients, we looked for a link between AngII plasma levels and the intensity
326 of ROS synthesis in HD, ICU and non-ICU participants. Figure 4E shows a clear correlation
327 between these two parameters ($r = 0.299$, $p = 0.027$). This explains the fact that ROS expression
328 was less intense in ICU patients than in non-ICU patients Thereafter, we checked whether AngII-
329 stimulated monocytes could induce DNA damage in BJ cells. Indeed, this was the case, and the
330 DNA damage was prevented by DPI (Fig. 4F) and the AT1 antagonist losartan (Fig. 4G). We
331 repeated the experiment with HD PBMCs instead of BJ cells (Fig. 4H). Again, we observed that
332 AngII-activated monocytes were able to cause a DNA damage which was prevented by losartan
333 or DPI. Furthermore, circulating levels of AngII strongly correlated with the ability of patient
334 PBMCs to induce DNA damage in BJ cells ($r = 0.704$, $p = 0.005$, Fig. 4I).

335

336 **T cell surface Fas expression is linked to ROS production**

337 Our data are compatible with a model where ROS-induced DNA damage provokes T cell
338 apoptosis. We have previously observed in severe COVID-19 that programmed T cell death is
339 also linked to T cell surface expression of the death receptor Fas (CD95) (21). ROS are known to
340 increase Fas expression in kidney cells (22), intestinal cells (23), myogenic cells (24), and
341 neurons (25). Conversely, in chronic granulomatous disease, characterized by a defect in ROS
342 production, patients express low T cell surface levels of Fas (26). Therefore, we searched for an

343 association between monocytic ROS production and Fas expression on T cells in COVID-19
 344 patients. Figure 4J shows a strong link between these two parameters ($r = 0.461$, $p = 0.013$).
 345 Thus, ROS released by monocytes could provoke apoptosis in T cells not only by breaking their
 346 DNA, but also by inducing Fas expression at their surface.
 347



349 **Figure 4. Angiotensin II induces ROS monocytic production and DNA damage.** (A) Fluorescence in monocytes
350 from a healthy donor, pre-incubated or not (○) with LPS (---) or AngII (—), and exposed to DCFH-DA. (B)
351 Fluorescence in monocytes from a healthy donor, pre-incubated or not (○) with AngII (—) or with DPI and AngII (--
352 -), and exposed to DCFH-DA. (C) Fluorescence in monocytes from a healthy donor, pre-incubated or not (○) with
353 AngII (—), or with losartan and AngII (---), and exposed to DCFH-DA. (D) Plasma levels of AngII in patients and
354 controls. Kruskal-Wallis test $p = 0.001$. (E) Correlation between plasma levels of AngII and mean fluorescence
355 intensity of ROS-producing monocytes exposed to DCFH-DA in patients and controls. (F, G, H) AngII-activated
356 monocytes induce DNA damage in neighbouring cells. Ability of healthy donor monocytes stimulated
357 (Monocytes/AngII) or not (Monocytes) by AngII to cause γ -H2AX foci in bystander BJ cells (F, G) and HD PBMCs
358 (H). The effect of DPI (Monocytes/DPI+AngII, f) or AT1 antagonist (Monocytes/anti-AT1+AngII, g) preincubation
359 on the ability of AngII-stimulated monocytes to induce DNA damage is shown. BJ cells (F, G) or PBMCs (H)
360 exposed to camptothecin were used as a positive control (Camptothecin). F, Welch ANOVA $p < 0.001$; G and H,
361 Kruskal-Wallis test $p < 0.001$. (I) Correlation between plasma levels of AngII and the ability of patient PBMCs to
362 induce γ -H2AX foci in bystander BJ cells. This ability is expressed as the ratio (% BJ cells presenting γ -H2AX foci
363 in presence of patient PBMCs) : (% BJ cells presenting γ -H2AX foci in presence of HD PBMCs). (J) Correlation
364 between mean fluorescence intensity of ROS-producing monocytes exposed to DCFH-DA and the percentage of T
365 lymphocytes expressing Fas.

DISCUSSION

In this study, we discovered a new pathogenic mechanism, DNA damage and T cell surface Fas overexpression due to AngII-driven ROS production by the monocytes of certain COVID-19 patients, and resulting in PBMC apoptosis (Fig. S1). Of note, ICU patients exhibit more T cell apoptosis and lymphopenia than non-ICU patients whereas their plasma level of AngII and their monocytic ROS production are lower. The explanation to this apparent paradox might lie in the delay of a few days between DNA damage and apoptosis (speculative scenario shown in Fig. S2). This delay could be due to the fact that cells first try to repair the damage, and thereafter, in case of failure, trigger apoptosis (27). Accordingly, we observed apoptosis in PBMCs co-cultured with patient monocytes only after 6 days (Fig. 2, E and F). Non-ICU patients are at Day 7 of the disease (Table 1). SARS-CoV-2 has replicated, internalized ACE2, and thereby increased Angiotensin II plasma level (Fig. 4D). Angiotensin II has induced monocytic ROS production (Fig. 1, B and C) responsible for DNA damage in T cells (Fig. 3B). At that time, T lymphocytes are possibly trying to repair this damage, an hypothesis accounting for the fact that the lymphopenia is not yet major. In this scenario, it is only a few days later that the consequence of this irreparable injury would appear clearly; lymphopenia in ICU patients who are at Day 12 of the disease (Table 1). In ICU patients, ROS expression was less intense than in non-ICU patients, probably due to the lower AngII plasma level in the former than in the latter. This decrease in AngII concentration over time might be the consequence of the increase in ACE2 expression reported in severe COVID-19 (18), driven by interferon (19), and/or reoxygenation (20), as well as the decrease in viral load (28). The amount of ROS released by monocytes would then be insufficient to provoke T cell apoptosis.

388 ROS-induced PBMC programmed cell death may have various deleterious effects. First, it may
389 result in an immune deficiency favoring coinfections with other viruses (29), bacteria (30) or
390 mycoses (31), and in a poor immunological memory paving the way for SARS-CoV-2
391 reinfection. Second, regulatory T-cell apoptosis may account for the Treg deficiency observed in
392 severe forms of COVID-19 (32), favoring immune activation. Third, CD8⁺ T-cell and NK cell
393 loss due to programmed cell death might contribute to a cytokine storm. Indeed, these cytotoxic
394 lymphocytes have been found to be involved in the downregulation of immune activation in the
395 course of infections via their ability to kill T-cells, NK, and antigen presenting cells (33, 34).
396 Accordingly, in primary hemophagocytic lymphohistiocytosis, mutations resulting in cytolytic
397 deficiency may provoke cytokine storms (35). Thus, the programmed death of CD8⁺ T-cells and
398 NK cells could impair a negative feedback on immune activation. Fourth, CD4⁺ T lymphocyte
399 apoptosis, particularly follicular helper T- cell apoptosis which may account for the depletion of
400 this subpopulation (36) might explain the poor isotype switch and B memory observed in severe
401 forms (37).

402 The release of ROS could have direct effects in addition to these indirect effects. As ROS are
403 known to activate the pro-inflammatory transcription factor NFκB (38) and the NLRP3
404 inflammasome (39), they could favor a cytokine storm in severe forms. Locally, the numerous
405 monocytes/macrophages in the low respiratory tract could also participate in endothelial cell,
406 alveolar and vascular damage via ROS (40).

407 As we found, *in vitro*, that ROS released by COVID-19 monocytes induce DNA damage and
408 apoptosis, as the proportion of DNA-damaged PBMCs we measured in patients correlated with
409 their lymphopenia (a major pronostic marker in COVID-19), and as we found a link between the
410 level of monocytic ROS expression in ICU patients and their survival, our data and the well-

411 documented proinflammatory effect of ROS argue for a role of this pathogenic pathway in the
412 outcome of this disease. They could explain also why older people, males, patients with diabetes
413 or prior cardiovascular diseases, who express low levels of ACE2 (41), present more often severe
414 forms of COVID-19.

415 The mechanism we uncovered may also explain why SARS-CoV-2 variants with an enhanced
416 affinity for their ACE2 receptor may be more pathogenic. Actually, these variants should
417 provoke an increased ACE2 internalization, a higher level of AngII, a greater monocytic ROS
418 production, and thereby more inflammation and more DNA damage resulting in lymphopenia
419 and immune deficiency.

420 From a therapeutic viewpoint, our data may explain the beneficial effects of AT1 antagonists
421 (42) and antioxidants (43) on COVID-19 observed in certain clinical trials. Given all the
422 potential consequences of ROS release in severe COVID-19, therapeutic strategies aimed at
423 reducing AngII signaling via AT1, ROS production, and apoptosis deserve more consideration
424 (Fig. S1).

425 **Acknowledgments**

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- 545

546 **Figure legends**547 **Figure 1. The monocytes from certain COVID-19 patients spontaneously produce ROS.** (A)

548 Fluorescence in monocytes from a healthy donor, pre-incubated (DPI + LPS, ---) or not (LPS,
549 —) with DPI, exposed to DCFH-DA, and stimulated with lipopolysaccharide. As negative
550 controls, fluorescence in the same monocytes pre-incubated (DPI, ⋯) or not (None, ⋯) with
551 NADPH oxidase inhibitor DPI and exposed to DCFH-DA was analyzed. (B) Fluorescence in
552 monocytes from a healthy donor (HD, ⋯), a non-ICU patient (non-ICU, —), and an ICU patient
553 (ICU, ---) exposed to DCFH-DA. (C) Mean fluorescence intensity of ROS-producing monocytes
554 from healthy donors (HD), non-ICU patients (non-ICU), and ICU patients (ICU) exposed to
555 DCFH-DA. One-way ANOVA test $p < 0.001$. (D) Mean fluorescence intensity of ROS-
556 producing monocytes from ICU patients who survived or not. (E) Identification of the classical,
557 intermediate, and alternative monocyte subpopulations by flow cytometry. (F) Fluorescence in
558 CD14^{hi}CD16^{lo} (---), CD14⁺CD16⁺ (—), and CD14^{lo}CD16^{hi} (⋯) monocytes from an ICU
559 patient exposed to DCFH-DA. (G) Percentages of CD14⁺CD16⁺ monocytes circulating in
560 healthy (HDs), ICU and non-ICU donors. One-way ANOVA test $p = 0.032$. (H) Correlation
561 between the proportions of intermediate and ROS-producing monocytes in ICU and non-ICU
562 patients.

563 **Figure 2. COVID-19 patient monocytes may induce DNA damage via ROS.** (A) Detection of

564 γ -H2AX foci by immunofluorescence in BJ cells co-cultured with PBMCs from a healthy donor
565 or from a COVID-19 patient. Healthy donor's PBMCs treated with camptothecin were used as
566 positive controls. (B) Quantification of the γ -H2AX foci induced in BJ fibroblasts by PBMCs
567 from COVID-19 patients. The proportion of BJ cells presenting at least 5 foci per nucleus was
568 quantified under microscopy. Each point represents one microscope field. Welch ANOVA test p

569 < 0.001. (C) γ -H2AX foci induced in BJ cells by COVID-19 patient's PBMCs are prevented by
570 pre-incubating PBMCs with N-acetylcysteine (NAC) or diphenyleneiodonium (DPI). Each point
571 represents one microscope field. Kruskal-Wallis test $p < 0.001$. (D) Monocytes isolated from the
572 PBMCs of a COVID-19 patient are able to induce DNA damage in BJ cells. The ability to induce
573 γ -H2AX foci in the BJ fibroblasts of PBMCs from a COVID-19 patient, of the same PBMCs
574 depleted of monocytes, and of monocytes isolated from these PBMCs was tested. Each point
575 represents one microscope field. Kruskal-Wallis test $p < 0.001$. (E) Intensity of
576 phosphatidylserine expression at the surface of healthy donor PBMCs cocultured with non-ICU
577 PBMCs able to induce DNA damage treated (non-ICU + NAC) or not (non-ICU) with N-
578 acetylcysteine, or with ICU PBMCs unable to induce DNA damage treated (ICU + NAC) or not
579 (ICU) with N-acetylcysteine, as detected by flow cytometry at day 6. Healthy donor PBMCs co-
580 cultured with another healthy donor PBMCs (HD) or treated with lipopolysaccharide (HD +
581 LPS) were used as negative and positive controls, respectively. One-way ANOVA test $p = 0.002$.
582 (F) Intensity of phosphatidylserine expression at the surface of healthy donor PBMCs cocultured
583 with COVID-19 monocytes able (patient 3) or not (patient 4) to induce DNA damage and treated
584 (+ DPI) or not with DPI, as detected by flow cytometry at day 6. Camptothecin was used as a
585 positive control.

586 **Figure 3. DNA damage in COVID-19 PBMCs.** (A) PBMCs from a COVID-19 patient whose
587 monocytes induce DNA damage in bystander BJ cells spontaneously present with γ -H2AX foci.
588 PBMCs from a healthy donor treated or not with camptothecin were used as positive and
589 negative controls, respectively. (B) Percentages of PBMCs harboring γ -H2AX foci in healthy
590 donors (HD), non-ICU patients (non-ICU), and ICU patients (ICU). Kruskal-Wallis test $p =$
591 0.002. (C) PBMCs from a COVID-19 patient whose monocytes induce DNA damage in

592 bystander BJ cells spontaneously present with 53BP1 foci. PBMCs from a healthy donor treated
593 or not with camptothecin were used as positive and negative controls, respectively. (D) Annexin
594 V expression on peripheral blood CD4⁺ T-cells and CD8⁺ T-cells of healthy donors (HD), non-
595 ICU patients (non-ICU), and ICU patients (ICU). One-way ANOVA test $p < 0.001$ for CD4⁺ T-
596 cells and $p < 0.001$ for CD8⁺ T-cells. (E) Correlation between the intensity of DNA damage in
597 PBMC and lymphocyte counts. The intensity of DNA damage in PBMCs is expressed as the
598 ratio (% patient PBMCs presenting γ -H2AX foci) : (% HD PBMCs presenting γ -H2AX foci).

599 **Figure 4. Angiotensin II induces ROS monocytic production and DNA damage.** (A)
600 Fluorescence in monocytes from a healthy donor, pre-incubated or not (---) with LPS (---) or
601 AngII (—), and exposed to DCFH-DA. (B) Fluorescence in monocytes from a healthy donor,
602 pre-incubated or not (·) with AngII (—) or with DPI and AngII (---), and exposed to DCFH-DA.
603 (C) Fluorescence in monocytes from a healthy donor, pre-incubated or not (·) with AngII (—),
604 or with losartan and AngII (---), and exposed to DCFH-DA. (D) Plasma levels of AngII in
605 patients and controls. Kruskal-Wallis test $p = 0.001$. (E) Correlation between plasma levels of
606 AngII and mean fluorescence intensity of ROS-producing monocytes exposed to DCFH-DA in
607 patients and controls. (F, G, H) AngII-activated monocytes induce DNA damage in
608 neighbouring cells. Ability of healthy donor monocytes stimulated (Monocytes/AngII) or not
609 (Monocytes) by AngII to cause γ -H2AX foci in bystander BJ cells (F, G) and HD PBMCs (H).
610 The effect of DPI (Monocytes/DPI+AngII, f) or AT1 antagonist (Monocytes/anti-AT1+AngII, g)
611 preincubation on the ability of AngII-stimulated monocytes to induce DNA damage is shown. BJ
612 cells (F, G) or PBMCs (H) exposed to camptothecin were used as a positive control
613 (Camptothecin). F, Welch ANOVA $p < 0.001$; G and H, Kruskal-Wallis test $p < 0.001$. (I)
614 Correlation between plasma levels of AngII and the ability of patient PBMCs to induce γ -H2AX

615 foci in bystander BJ cells. This ability is expressed as the ratio (% BJ cells presenting γ -H2AX
616 foci in presence of patient PBMCs) : (% BJ cells presenting γ -H2AX foci in presence of HD
617 PBMCs). (J) Correlation between mean fluorescence intensity of ROS-producing monocytes
618 exposed to DCFH-DA and the percentage of T lymphocytes expressing Fas.