

1 **The CXCR6/CXCL16 axis links inflamm-aging to disease severity in**
2 **COVID-19 patients**

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24 **Abstract**

25 Advancing age and chronic health conditions, significant risk factors for severe
26 COVID-19, are associated with a pro-inflammatory state, termed inflamm-aging.
27 CXCR6⁺ T cells are known to traffic to the lung and have been reported to increase with
28 age. The ligand of CXCR6, CXCL16, is constitutively expressed in the lung and
29 upregulated during inflammatory responses and the CXCR6/CXCL16 axis is associated
30 with severe lung disease and pneumonia. Genome-wide association studies have also
31 recently identified 3p21.31, encompassing the *CXCR6* gene, as a susceptibility locus for
32 severe COVID-19. We assessed numbers T cells expressing the chemokine receptor
33 CXCR6 and plasma levels of CXCL16, in control and COVID-19 patients. Results
34 demonstrated that circulating CD8⁺CXCR6⁺ T cells were significantly elevated with
35 advancing age, yet virtually absent in patients with severe COVID-19. Peripheral levels
36 of CXCL16 were significantly upregulated in severe COVID-19 patients compared to
37 either mild COVID-19 patients or SARS-CoV-2 negative controls. This study supports
38 a significant role of the CXCR6/CXCL16 axis in the immunopathogenesis of severe
39 COVID-19.

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41

42 **Introduction**

43 Coronavirus disease 2019 (COVID-19), caused by infection with severe acute
44 respiratory syndrome coronavirus-type 2 (SARS-CoV-2), encompasses clinical
45 phenotypes ranging from asymptomatic infection, through to severe disease and death.
46 The more severe end of this spectrum is often associated with respiratory pathology (1,
47 2). It is well established that the course of any infection is dependent on a number of
48 variables including pathogen virulence, environmental and host factors. The latter
49 includes variation in the immune response driven by genetics, age and the presence of
50 co-morbidities. SARS-CoV-2 infection induces both innate and adaptive immunity (3)
51 and severe COVID-19 is associated with exaggerated T cell responses producing
52 increased levels of pro-inflammatory cytokines including IL-6, TNF- α , and IL-1 (4, 5).
53 This aggressive hyper-inflammatory state results in significant lung damage and high
54 mortality (4). Post-mortem studies have shown both lymphocyte and neutrophil lung
55 infiltration, indicating that migration of pro-inflammatory cells into the lung is a key
56 step in the pathology and outcome of this infection (6, 7). Immunomodulatory therapies,
57 including the anti-IL-6 monoclonal antibody tocilizumab and the corticosteroid
58 dexamethasone, may improve outcomes, highlighting the importance of inflammatory
59 processes in COVID-19 pathology (8, 9).

60

61 Current epidemiological studies have identified advancing age, chronic health
62 conditions, such as diabetes and obesity, and certain ethnicities as risk factors for more
63 severe disease (10). Advancing age has been strongly associated with a pro-
64 inflammatory immune phenotype, so-called inflamm-aging, where T cells acquire a
65 more innate NK cell-like pro-inflammatory phenotype associated with upregulation of
66 markers of both T cell exhaustion and senescence (11). Furthermore, increased
67 expression of chemokine receptors, including CXCR6 on T cells, has been

68 demonstrated in aging animal models (12). The receptor CXCR6 (CD186) is expressed
69 on activated T cells, NK cells, NKT cells and mucosal-associated invariant T (MAIT)
70 cells (13-15).

71

72

73 **Results and Discussion**

74 We assessed the expression of CXCR6⁺ on CD4⁺ and CD8⁺ T cells in consecutive blood
75 samples to determine age-related differences and thereby support a potential role of
76 these cells in inflamm-aging and justify further assessment in the pathogenesis of
77 COVID-19 (16-18) (Figure 1).

78
79 Patients over 65 years of age are known to have more severe outcomes in COVID-19
80 (19). In keeping with the inflamm-aging hypothesis, we demonstrated a highly
81 significant increase in CD8⁺CXCR6⁺ T cells in the blood of patients aged over 65 years
82 (n=96) compared to those aged under 65 years (n=137) (p<0.0001; Figure 1c). A
83 progressive increase with advancing age was also observed (Rs 0.39, p<0.0001; Figure
84 1d). There were lower proportions of CD4⁺CXCR6⁺ T cells were observed compared to
85 CD8⁺CXCR6⁺ T cells and there were no significant age-related differences (Figure 1g
86 and 1h). The increased frequency of CD8⁺CXCR6⁺ T cells in the blood of older patients
87 is supportive of a pro-inflammatory phenotype, potentially rendering this group
88 susceptible to hyper-inflammatory immune responses associated with poor outcomes in
89 COVID-19.

90
91 Peripheral blood T lymphopenia has been identified as an immunological marker for
92 SARS-CoV-1 and 2 infection with postulated mechanisms including immune-mediated
93 destruction and trafficking to pathological sites (20-22). We analysed the absolute
94 numbers and proportion of CD4⁺ T cells, CD8⁺ T cells and NK cells relative to total
95 leucocytes in controls, mild and severe COVID-19 patients. There was an absolute and
96 proportional reduction in CD4⁺ and CD8⁺ T cells in severe COVID-19 (Figure 2). This
97 was more pronounced for CD8⁺ T cells, with statistical significance in severe COVID-

98 19 compared to controls ($p < 0.001$ and $p < 0.0001$ for absolute number and proportion
99 respectively). There was no significant difference in NK cells.

100

101 The receptor for SARS-CoV-2 is angiotensin converting enzyme 2 (ACE2) which is
102 highly expressed on alveolar epithelial type II cells of the lower respiratory tract (23).

103 Membrane bound CXCL16 is constitutively expressed on bronchial epithelial cells and

104 is released in metalloprotease-dependent manner in an inflammatory environment,

105 producing a soluble form which is chemotactic for CXCR6⁺ T-cells (24, 25). The

106 CXCR6/CXCL16 axis mediates homing of T cells to the lungs in disease (26-28) and

107 hyper-expression is associated with localised cellular injury (29-31). Murine studies

108 have demonstrated that this axis is involved in lung pathology associated with other

109 infections, including influenza, with antagonism resulting in reduced tissue

110 inflammation (26, 29).

111

112 CXCL16 is up-regulated during viral infections and mediates CD8⁺CXCR6⁺ T-cell

113 recruitment (32, 33). Differential expression of *CXCR6* and *CXCL16* mRNA was

114 observed in severe COVID-19 compared to mild disease (34) and significant functional

115 polymorphisms in *CXCR6* are linked to viral control (35). Furthermore, in HIV

116 infection *CXCR6* polymorphisms have been linked with certain ethnicities associated

117 with more severe lung pathology and poorer outcomes (36). We compared peripheral

118 blood T cell populations in severe and mild COVID-19 to control samples. This

119 revealed that absolute CD8⁺ CXCR6⁺ T cell populations were significantly reduced in

120 both severe and mild COVID-19 patients compared to controls ($p < 0.0001$ and $p < 0.1$

121 respectively; Figure 3e), with significant reduction in absolute CD4⁺ CXCR6⁺ T cells

122 only between severe COVID-19 and controls ($p < 0.001$; Figure 3g). Strikingly, both

123 CD4⁺ and CD8⁺ CXCR6⁺ expressing T cells were present at extremely low proportions
124 in the blood of severe COVID-19 patients (n=12).

125

126 Studies in COVID-19 patients suggest CXCR6 correlates directly with the proportion of
127 MAIT cells and that this population is reduced in the peripheral blood in COVID-19
128 (37). Killer cell lectin like receptor subfamily B, member 1 (CD161) is a C-type lectin
129 receptor expressed on NK cells and a subset of T cells with both stimulatory and
130 inhibitory functions. Expression of this marker was assessed on CD8⁺ T cells as high
131 levels of expression have been associated with MAIT cells and Th17 responses (38).

132 We demonstrated that the majority of CD3⁺ CD8⁺ CXCR6⁺ T cells in controls and mild
133 COVID-19 cases were CD161⁺⁺ CD45RA⁻ CD27⁺ HLA-DR⁻ CD57⁻ (Figure 3 and
134 Supplementary Figure 1) suggestive of an effector memory profile. In most cases these
135 cells were also positive for CD56 and CD279 (see Supplementary Figure 1), consistent
136 with either NKT cells, invariant T cells or mucosal-associated invariant T (MAIT) cells
137 (13). CD8⁺ CXCR6⁺ CD161⁻ T cells were present in lower numbers and there was no
138 significant difference in this population between COVID-19 patients and controls.

139 Proportions of both CD4⁺ CXCR6⁺ CD161⁺⁺ and CD4⁺ CXCR6⁺ CD161⁻ T cells were
140 lower compared to CD8⁺ T cells. The extended phenotype of the CD4⁺ CXCR6⁺
141 CD161⁺⁺ is suggestive of an effector/central memory population (Supplementary Figure
142 1).

143

144 Cells with this phenotype have been shown to exhibit tissue homing properties, with
145 infiltrates described in inflammatory diseases including rheumatoid arthritis, psoriasis,
146 multiple sclerosis and Crohn's disease (39). Levels of circulating and pancreatic MAIT-
147 cells in type 1 diabetes mirror the findings of our MAIT-like cells in COVID-19. In type

148 1 diabetes high levels of circulating cells are present at diagnosis with numbers falling
149 after 1 year with a concurrent increase in pancreatic numbers suggesting trafficking and
150 a potential pathogenic role (40).

151

152 To further characterise the CXCR6/CXCL16 axis in the immunopathogenesis of
153 COVID-19, plasma concentrations of CXCL16 from 28 COVID-19 patients and 12
154 controls were assessed. CXCL16 was significantly elevated in severe COVID-19
155 samples (n=10) when compared with mild COVID-19 (n=18, $p < 0.0001$) or controls
156 (n=12, $p < 0.001$; Figure 4a), which contrasts with the findings of Liao *et al* (41) where
157 *CXCL16* mRNA was more highly expressed in bronchoalveolar lavage fluid in mild
158 disease, albeit with significantly fewer patient numbers in all groups. There was an
159 inverse relationship between the concentration of blood CXCL16 and the proportion of
160 CD8⁺ and CD4⁺ CXCR6⁺ T cells in the blood in COVID-19 patients (Figures 4b and
161 4c). This suggests trafficking of CXCR6⁺ T cells to the lung drives a pro-inflammatory
162 immunopathology in severe COVID-19, with these cells infiltrating into the tissue,
163 which is supported by lower numbers of CD8⁺ T cells reported in broncho-alveolar
164 lavage in mild compared to severe COVID-19 patients (41). Furthermore, the
165 CXCR6/CXCL16 axis has been implicated in both infective (influenza) and non-
166 infective (sarcoidosis) inflammatory lung diseases (25, 26). However, this inverse
167 relationship between CXCL16 levels and CXCR6⁺ T cells and may also be explained by
168 either CXCL16 binding to CXCR6 causing receptor internalisation, epitope masking or
169 CXCL16-mediated T cell apoptosis.

170 Following infection with SARS-CoV-2, there is potential for pre-existing inflammatory
171 CXCR6⁺ populations, associated with either co-morbidity and/or inflamm-aging, to be
172 recruited from the blood to the lungs mediated by CXCL16, resulting in more severe

173 disease (42). Similarly, in other diseases characterised by a T cell infiltrate, such as type
174 1 diabetes, high expression of this chemokine receptor and ligand have been reported in
175 pancreatic tissue where they play a role in inflammation (43).

176

177 CD8⁺CD161⁺⁺CXCR6⁺ T cells, have the capacity to be cytotoxic and express the
178 transcription factor ROR γ t, which is associated with a Th17-like phenotype and a pro-
179 inflammatory cytokine profile (IFN- γ , TNF- α , IL-17 and IL-22) along with expression
180 of cytotoxic mediators such as granzyme (14, 44). These factors have all been shown to
181 be significantly elevated in severe, but not mild COVID-19 patients, despite a more
182 profound lymphopenia (45, 46). These cells have also been implicated in other lung
183 infections, with IL-17 mediated inflammation and pathogenesis reported in patients with
184 immune-mediated community-acquired pneumonia (47). A similar Th17 profile has
185 been described in patients with COVID-19(48) along with a significant reduction in
186 circulating CD161⁺⁺ cells (48, 49). It is likely that these CD161⁺⁺ cells are identical to
187 the T cells identified in this study. As well as mediating chemotaxis of inflammatory
188 cells, murine studies suggest that elevated levels of CXCL16 may directly contribute to
189 lung injury through production of reactive oxygen species and compromised epithelial
190 barrier integrity, with CXCL16 inhibitors protecting against lipopolysaccharide-
191 mediated lung injury (29).

192

193 This study demonstrates an age-related increase in CD8⁺CXCR6⁺ T cells consistent
194 with inflamm-aging in humans and that more severe outcomes in COVID-19 associate
195 with increased peripheral CXCL16 and reduced circulating CXCR6⁺ T cells, suggesting
196 an immunopathogenic role. This may have significant implications in the stratification
197 of the risk for patients infected with SARS-CoV-2 and raises the possibility of novel

198 therapeutic agents targeting this axis in severe COVID-19. Studies on CXCR6
199 expression on T-cells and levels of CXCL16 in dexamethasone and tocilizumab treated
200 patients will provide further insight into the pathogenesis and putative mechanisms of
201 therapy. This axis may also be relevant in other infections associated with lung
202 pathology such as influenza.
203
204

205 **Methods**

206 *Samples*

207 *Consecutive samples:* CXCR6 expression was assessed on CD4+ and CD8+ T cells as
208 part of routine diagnostics on samples taken between 30th March and 1st July 2020
209 from patients not tested for SARS-CoV-2. Samples consisted of 233 peripheral blood
210 (PB) aliquots, (age range 1 to 97 years, median 60; 135 male, 98 female).

211 *COVID-19 study samples:* With the exceptions of age and gender investigators were
212 blinded to all other demographics due to ethical constraints. Samples were less than 24
213 hours old and obtained from patients admitted to Leeds Teaching Hospitals NHS Trust
214 between 7th April and 16th July 2020. Samples were collected from 52 patients: 1)
215 (Mild), 20 samples from mild cases of COVID-19, defined by positive RT-PCR for
216 SARS-CoV-2 and not requiring Intensive Care Unit (ITU) support (age range 20 to 90
217 years, median 73; 13 males, 7 females. 2) (Severe) 12 samples from severe cases of
218 COVID-19, defined by positive RT-PCR for SARS-CoV-2 and requiring ITU support
219 (age range 44 to 82 years, median 64; 6 males, 6 females). 3) (Control) 20 control
220 samples from patients with no features of COVID-19 and negative RT-PCR for SARS-
221 CoV-2, 16/20 were not on ITU, 4/20 were on ITU (age range 20 to 91 years, median 57;
222 11 males, 9 females). Flow cytometry was performed, and plasma extracted using
223 standard methods and stored at -20°C. CXCL16 ELISA was performed on 40 samples
224 (12 C [including 2 ITU patients], 10 S, 18 M).

225 *Flow cytometry*

226 Samples were analysed using 2 phenotyping panels (Supplementary Table 1).
227 Consecutive sample analyses were performed using a FACS Canto II flow cytometer
228 (BD Biosciences) verified through daily calibration with CS&T beads (BD Biosciences)

229 and 8 peak Rainbow beads (Spherotech) utilising a 7-parameter panel (CD8-FITC,
230 CD16-PE, CD4-PerCP-Cy5.5, CD3-PE-Cy7, CD8-APC, CD45-APC-Cy7, CD186-
231 BV421 [Becton Dickinson]). Samples from COVID-19 patients and controls were
232 assessed on a Cytoflex LX cytometer (Beckman Coulter), verified through daily
233 calibration with CytoFLEX Daily QC Fluorospheres (Beckman Coulter), using a 12-
234 parameter panel (CD57-FITC, CD4-PE, CD161-PC7, CD8-KrO, CD279-PC5.5,
235 CD45RA-A700, CD3-APC-A750 [Beckman Coulter] and CD56-BV605, CD45-PerCP,
236 CD186-BV421, CD27-BV786, HLA-DR-BUV395 [Becton Dickinson]). A minimum of
237 50,000 CD3⁺ T cell events were assessed and analysed using standard methods. All
238 analyses were only performed once due to the volume of sample available. Data was
239 analysed with Kaluza Analysis software version 2.1 (Beckman Coulter) and Cytobank
240 software (Beckman Coulter) Representative gating strategy can be seen in
241 Supplementary figure 1.

242 ***ELISA***

243 Plasma CXCL16 levels were measured in duplicate using a commercial ELISA
244 (ThermoFisher Scientific), with a coefficient of variation of less than 10%, according to
245 the manufacturer's specifications.

246 ***Statistics***

247 Data was analysed with GraphPad Prism version 9.0.0 (GraphPad software).
248 Categorical data was compared with Mann-Whitney statistical analyses. Spearman
249 Rank and regression analysis was used to assess data correlation, $p < 0.05$ was
250 considered significant.

251 *Study approval*

252 Local and National ethical approval (IRAS: 284369) allowed collection of anonymised
253 excess peripheral blood from patients tested for SARS-CoV-2 infection.

254

255 **Author contributions**

256 DP carried out the experiments, planned the study, analysed data, performed statistical
257 analysis and wrote the manuscript. SD, RL acquired data. RP, SG, CM, GC, SR were
258 involved in the design of the study. PH, TM, LA, KR, CM, SP were involved in the
259 identification and collection of patient samples. RB and DN planned the study,
260 interpreted the data and drafted the manuscript. All authors critically reviewed the
261 manuscript.

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270

271 **References**

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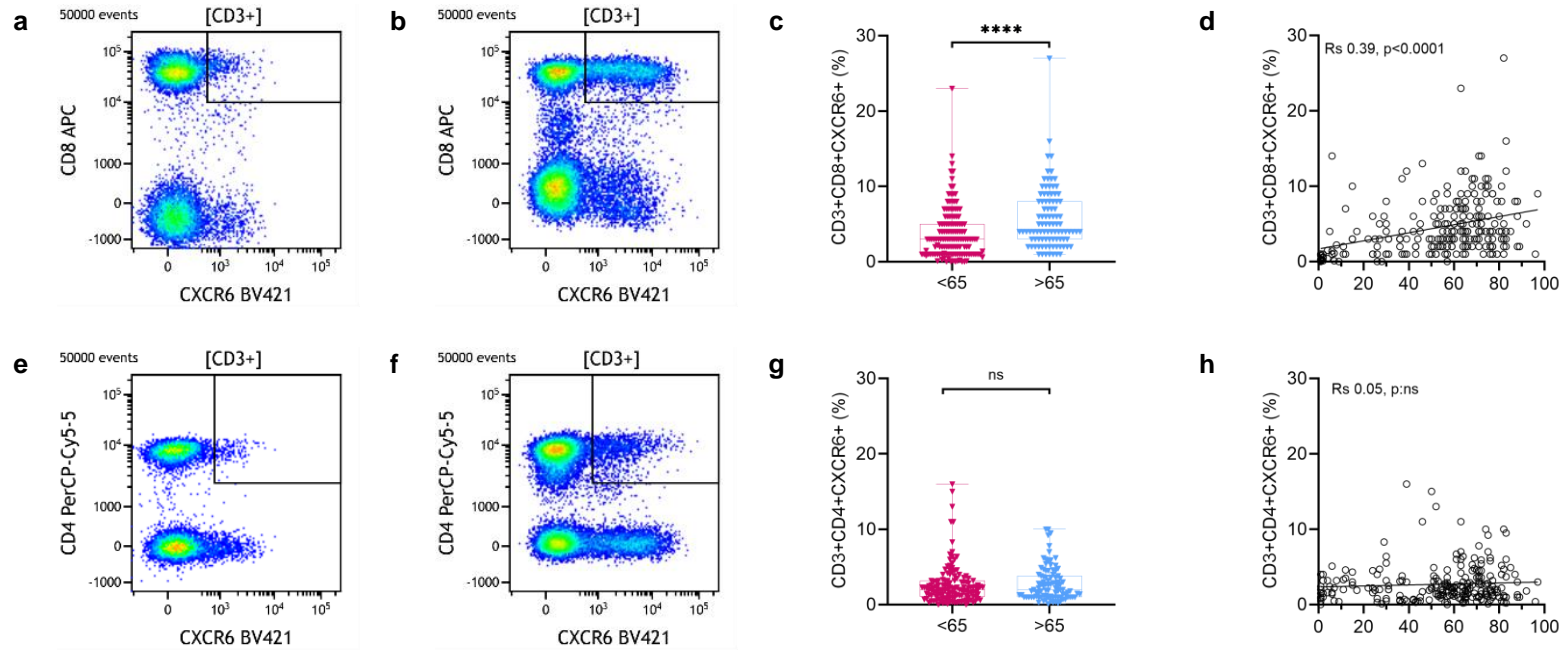
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421 **Display items**

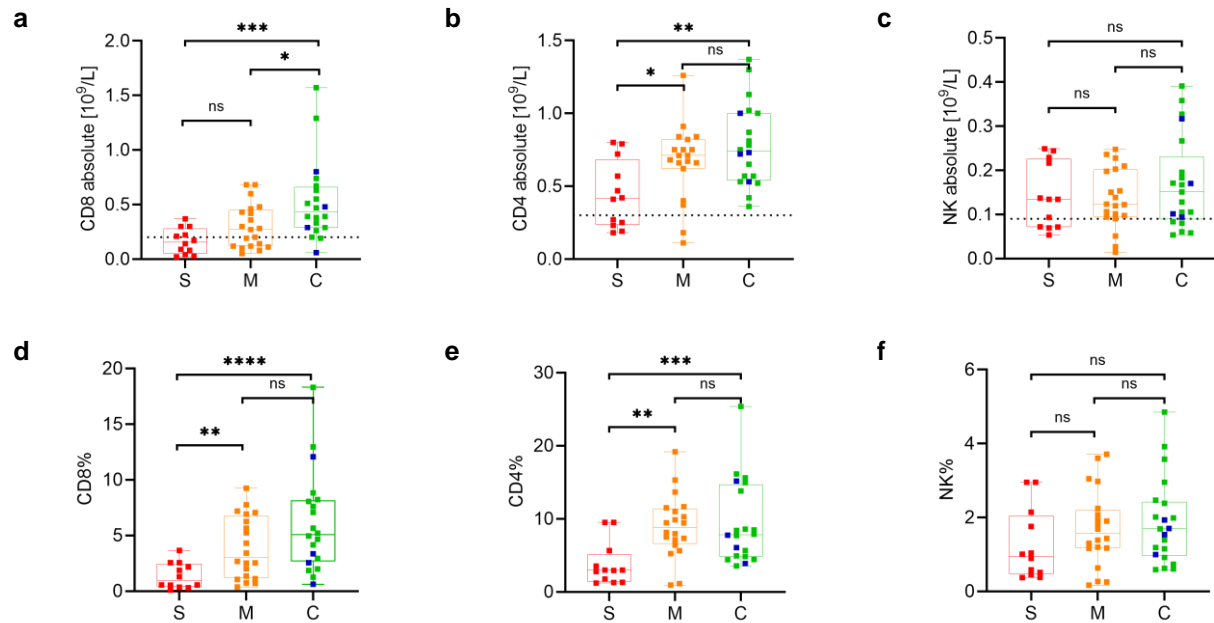


422 **Figure 1. Age-related percentage of CXCR6⁺ T cells in consecutive samples**

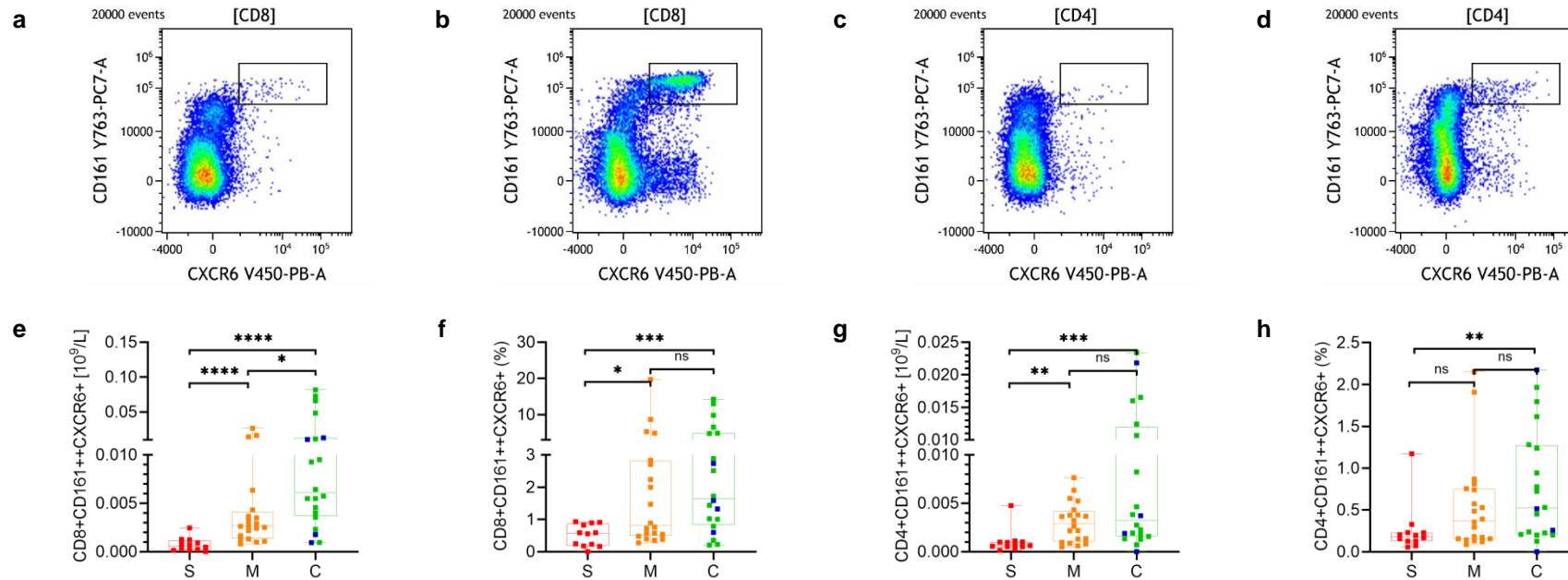
423 SARS-CoV-2 status of these samples was undetermined. Data presented as a percentage of CD3⁺ gated T cells.

424 Dot plots illustrate differences observed in proportion of CD3⁺CD8⁺CXCR6⁺ cells **a**: 15 year old **b**: 83 year old. **c**: Levels of CD3⁺CD8⁺CXCR6⁺ cells in peripheral
 425 blood of different age groups (**<65**) <65 years old (n=137). (**>65**) >65 years old (n=96). Median, maximum, and minimum values shown. Mann Whitney was
 426 used to compare populations, **** p<0.0001;. **d**: Correlation of CD3⁺CD8⁺CXCR6⁺ with age in 233 peripheral blood samples. Spearman rank correlation: R_s
 427 0.39, p<0.0001, suggesting a trend to increase with age.

428 Dot plots illustrate differences observed in proportion of CD3⁺CD4⁺CXCR6⁺ cells **e**: 15 year old and **f**: 83 year old. **g**: Levels of CD3⁺CD4⁺CXCR6⁺ cells in
 429 peripheral blood of different age groups (**<65**) <65 years old (n=137). (**>65**) >65 years old (n=96). Median, maximum, and minimum values shown. Mann Whitney
 430 was used to compare populations, ns: not significant. **h**: Correlation of CD3⁺CD4⁺CXCR6⁺ with age in 233 peripheral blood samples: R_s0.05, p: ns.



432 **Figure 2. Reduced absolute and total T-cell numbers in severe COVID-19 patients**
 433 **(S)evere COVID-19;** SARS-CoV-2 RT-PCR positive patients on ITU (n=12, red). **(M)ild COVID-19;** SARS-CoV-2 RT-PCR positive patients non-ITU (n=20,
 434 **(C)ontrols;** SARS-CoV-2 RT-PCR negative non-ITU (n=16, green). **on ITU** (n=4, blue). maximum, and minimum values shown, dotted line shows lower
 435 end of absolute reference range. Mann Whitney was used to compare populations; **** p<0.0001 *** p<0.001 ** p<0.01 * p<0.1 ns = not significant.
 436 **a** and **d**: Significantly reduced absolute and percentage CD8⁺ T cells in when comparing S to M or C. **b** and **e**: Significantly reduced absolute and percentage
 437 CD4⁺ T cells in when comparing S to M or C. **c** and **f**: No significant difference in NK cells was observed when comparing S, M to C.
 438

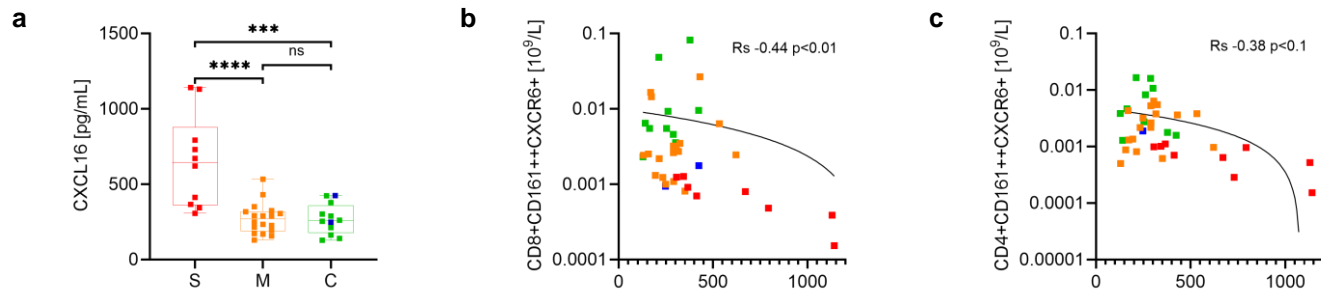


441 **Figure 3. Reduced CXCR6⁺T cells in severe COVID-19 patients**

442 Gated on CD3⁺CD8⁺ T cells, **a**: illustrates reduced CXCR6⁺CD161⁺⁺ cells in severe COVID-19 when compared with **b**: control sample.

443 Gated on CD3⁺CD4⁺ T cells, **c**: illustrates reduced CXCR6⁺CD161⁺⁺ cells in severe COVID-19 when compared with **d**: control sample.

444 **e**: Significantly reduced absolute and **f**: percentage of CD8⁺CD161⁺⁺CXCR6⁺ cells in severe COVID-19 compared to mild COVID-19 and controls. **e**: Significantly
 445 reduced absolute and **f**: percentage of CD4⁺CD161⁺⁺CXCR6⁺ cells in severe COVID-19 compared to mild COVID-19 and controls. **(S)**evere COVID-19; SARS-
 446 CoV-2 RT-PCR positive patients on ITU (n=12, red). **(M)**ild COVID-19; SARS-CoV-2 RT-PCR positive patients non-ITU (n=20, orange). **(C)**ontrols; SARS-CoV-
 447 2 RT-PCR negative non-ITU (n = 16, green), on ITU (n=4, blue). Median, maximum, and minimum values shown. Mann Whitney was used to compare graphed
 448 populations. **** p<0.0001 *** p<0.001 ** p<0.01 * p<0.1 ns = not significant



450 **Figure 4. Plasma concentrations of CXCL16 in COVID-19 compared with controls**

451 **a:** Median, maximum, and minimum values shown. **(S)evere COVID-19;** SARS-CoV-2 RT-PCR positive patients on ITU (n=10, red). **(M)ild COVID-19;** SARS-
 452 CoV-2 RT-PCR positive patients non-ITU (n=18, orange). **(C)ontrols;** SARS-CoV-2 RT-PCR negative non-ITU (n=10, green). on ITU (n=2, blue). (**** p<0.0001
 453 *** p<0.001 ns = not significant) Significantly increased levels of CXCL16 are present in the plasma of severe COVID-19 patients. **b** and **c:** CD8⁺ and CD4⁺
 454 CD161⁺⁺CXCR6⁺ T cell count falls (log values) as plasma concentration of CXCL16 increases, SARS-CoV-2 RT-PCR positive patients on ITU (n=10, red),
 455 SARS-CoV-2 RT-PCR positive patients non-ITU (n=18, orange), SARS-CoV-2 RT-PCR negative non-ITU (n=10, green). on ITU (n=2, blue), line logistical
 456 regression shown. Spearman rank correlation: $R_s = -0.44$, $p < 0.01$ and $R_s = -0.38$, $p < 0.1$ for CD8⁺ and CD4⁺ cells respectively