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

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Abstract

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

Introduction

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

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

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

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

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

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

The receptor for SARS-CoV-2 is angiotensin converting enzyme 2 (ACE2) which is highly expressed on alveolar epithelial type II cells of the lower respiratory tract (23). Membrane bound CXCL16 is constitutively expressed on bronchial epithelial cells and is released in metalloprotease-dependent manner in an inflammatory environment, producing a soluble form which is chemotactic for $CXCR6^+$ T-cells (24, 25). The CXCR6/CXCL16 axis mediates homing of T cells to the lungs in disease (26-28) and hyper-expression is associated with localised cellular injury (29-31). Murine studies have demonstrated that this axis is involved in lung pathology associated with other infections, including influenza, with antagonism resulting in reduced tissue inflammation (26, 29).

CXCL16 is up-regulated during viral infections and mediates $CD8^+CXCR6^+$ T-cell recruitment (32, 33). Differential expression of *CXCR6* and *CXCL16* mRNA was observed in severe COVID-19 compared to mild disease (34) and significant functional polymorphisms in *CXCR6* are linked to viral control (35). Furthermore, in HIV infection *CXCR6* polymorphisms have been linked with certain ethnicities associated with more severe lung pathology and poorer outcomes (36). We compared peripheral blood T cell populations in severe and mild COVID-19 to control samples. This revealed that absolute $CD8^+$ $CXCR6^+$ T cell populations were significantly reduced in both severe and mild COVID-19 patients compared to controls ($p<0.0001$ and $p<0.1$ respectively; Figure 3e), with significant reduction in absolute $CD4^+$ $CXCR6^+$ T cells 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).
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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 $CD161^{++}$ $CD45RA^-$ $CD27^+$ $HLA\text{-}DR^-$ $CD57^-$ (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

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

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

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

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

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

This study demonstrates an age-related increase in $CD8^{+}CXCR6^{+}$ T cells consistent with inflamm-aging in humans and that more severe outcomes in COVID-19 associate with increased peripheral CXCL16 and reduced circulating $CXCR6^{+}$ T cells, suggesting an immunopathogenic role. This may have significant implications in the stratification 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
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Methods

Samples

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

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

Flow cytometry

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

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

ELISA

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

Statistics

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

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Author contributions

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

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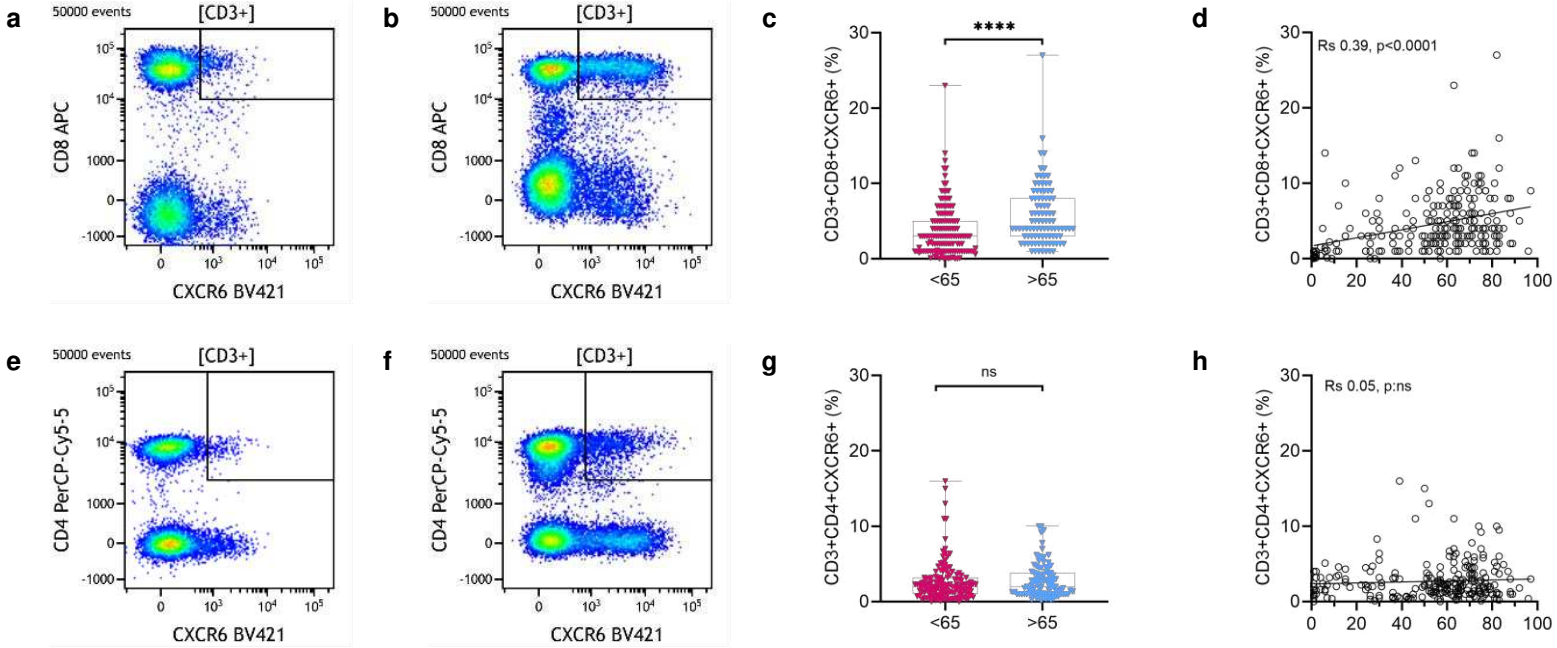

Figure 1. Age-related percentage of CXCR6⁺ T cells in consecutive samples

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

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 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 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 0.39, p<0.0001, suggesting a trend to increase with age.

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 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 was used to compare populations, ns: not significant. **h:** Correlation of $CD3^+CD4^+CXCR6^+$ with age in 233 peripheral blood samples: R_s 0.05, p: ns.

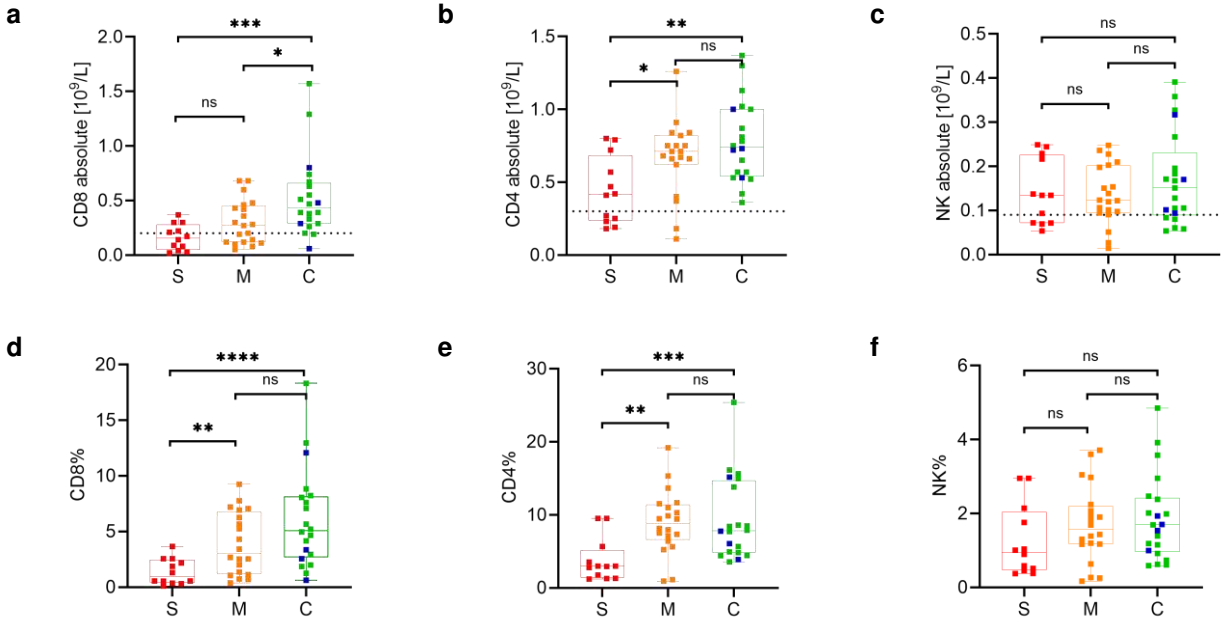

Figure 2. Reduced absolute and total T-cell numbers in severe COVID-19 patients

(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, orange). **(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 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.

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 $CD4^+$ 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.

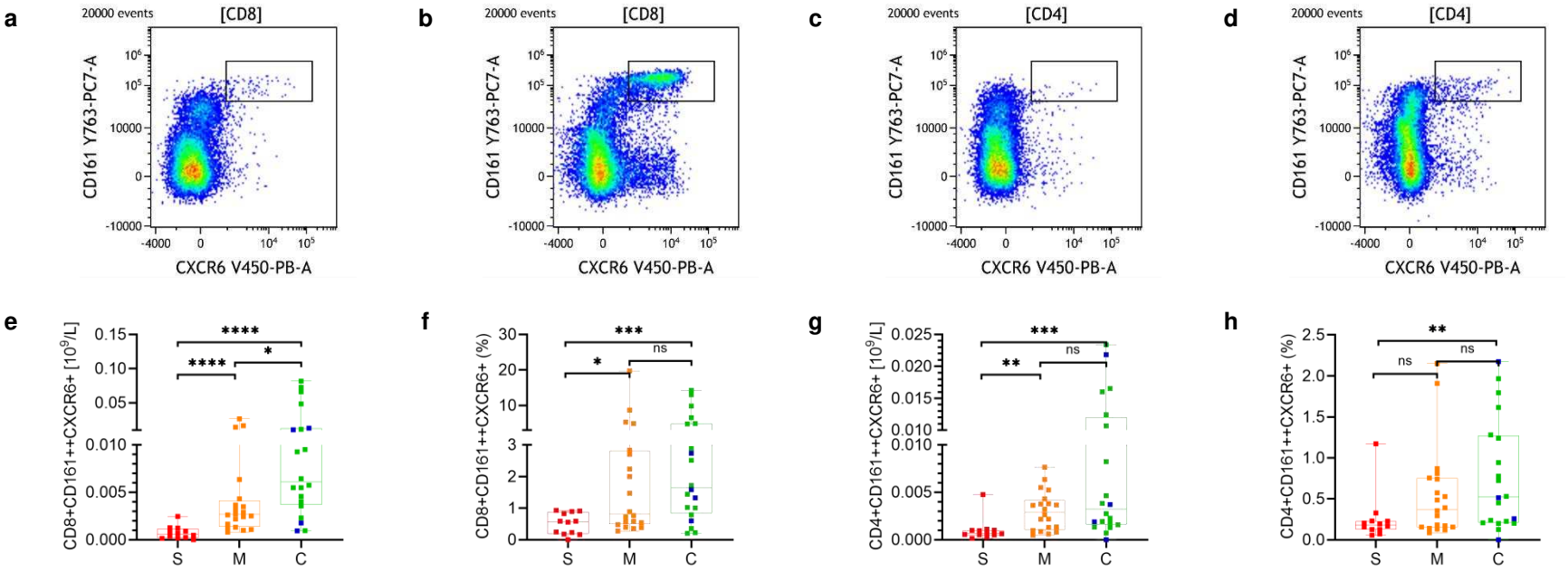

Figure 3. Reduced CXCR6⁺T cells in severe COVID-19 patients

Gated on CD3⁺CD8⁺ T cells, **a:** illustrates reduced CXCR6⁺CD161⁺⁺ cells in severe COVID-19 when compared with **b:** control sample. Gated on CD3⁺CD4⁺ T cells, **c:** illustrates reduced CXCR6⁺CD161⁺⁺ cells in severe COVID-19 when compared with **d:** control sample. **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 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-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-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 populations. **** p<0.0001 *** p<0.001 ** p<0.01 * p<0.1 ns = not significant

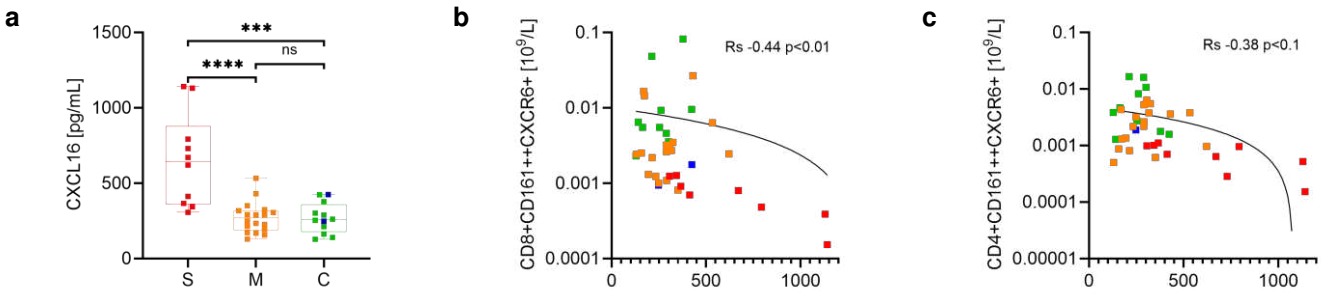

Figure 4. Plasma concentrations of CXCL16 in COVID-19 compared with controls

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-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). (**** $p<0.0001$ *** $p<0.001$ ns = not significant) Significantly increased levels of CXCL16 are present in the plasma of severe COVID-19 patients. **b** and **c:** $CD8^+$ and $CD4^+$ $CD161^{++}CXCR6^+$ T cell count falls (log values) as plasma concentration of CXCL16 increases, SARS-CoV-2 RT-PCR positive patients on ITU (n=10, red), 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 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