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LETTER TO THE EDITOR

Proteomic analysis of neutrophils from patients with COVID-19

It was rapidly recognised, after the start of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic in December 2019, that only a minority of patients with coronavirus disease 2019 (COVID-19) develop severe or critical disease, while most SARS-CoV-2-infected patients are either asymptomatic or exhibit mild symptoms.[1] Patients with severe COVID-19 may present life-threatening acute respiratory distress syndrome (ARDS) resulting from lower respiratory tract disease, associated with excessive inflammatory response, high pro-inflammatory cytokine production (cytokine storm) and coagulopathy.[2] Several comorbidities, including hypertension, diabetes, cardiovascular disease and respiratory disease, have been associated with poorer prognosis and increased mortality.[3]

Increasing evidence suggests that neutrophils, a principal effector of the immune defence in airway infections, may play a major role in severe COVID-19 disease pathophysiology. Indeed, increased neutrophil-to-lymphocyte ratio, in association with high D-dimer levels,[1] the presence of abnormal immature neutrophils,[4] marked infiltration of neutrophils and deposits of neutrophil extracellular traps (NETs) in the pulmonary microvasculature[5] have been reported in patients. To gain further insight into the involvement of neutrophils in COVID-19 clinical expression, we performed quantitative proteomic analysis of neutrophils from patients with COVID-19 and from two non-COVID-19 groups composed of healthy subjects and patients with ARDS hospitalised in an intensive care unit (ICU). All patients were from a homogeneous population who attended the University Hospital of Guadeloupe.

A total of 14 patients with COVID-19 were included in the study, six hospitalised in the ICU (three of them in an ARDS condition) and eight in other hospital departments. In addition, four uninfected patients with ARDS and five healthy controls were included. Table S1 presents a summary of clinical data. Diagnosis of SARS-CoV-2 infection was established by reverse-transcriptase polymerase chain reaction, in accordance with current standards.

Neutrophils were isolated from EDTA-blood samples, within 1 h of sampling, using the MACSexpress whole blood neutrophil isolation kit and MACSexpress erythrocyte depletion kit (Miltenyl Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Quantitative proteomics was performed as described in Data S1.

Neutrophil proteomic analysis identified 6205 proteins. Principal component analysis (PCA) (Figure 1) revealed striking differential segregation among the three groups (COVID-19 and non-COVID-19 patients with ARDS and healthy controls), with the exception of one non-COVID-19 patient with ARDS who had repeatedly tested negative for SARS-CoV-2. Importantly, no distinct cluster was observed for ICU-hospitalised patients with COVID-19, as opposed to those in other hospital departments. Moreover, no difference was detected regarding haematological and biochemical parameters, comorbidities and survival rate between these two groups (Table S1). Altogether, these data strongly suggest that these patients exhibited similar clinical severity. Considering Guadeloupe as one of the major COVID-19 hot spots in France, with limited hospital capacity, we speculate that local medical support capacity was overridden. Regardless of causes, and based on medical outcomes, we grouped these patients with COVID-19 for further analyses.

Cluster analysis of differentially expressed proteins in neutrophils of the three patient groups did not provide evidence of a common biological pathway for down-regulated proteins in patients with COVID-19 (*data not shown*). A two-dimensional enrichment analysis test using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and keyword annotation databases[6] using (PubMed unique identifier [PMID]: 23176165) identified several pathways for up-regulated proteins in the COVID-19 group, compared to the two others. Indeed, neutrophil proteins from patients with COVID-19 were significantly enriched for biological processes associated with type I interferon (IFN) signalling, antigen processing and presentation, and neutrophil aggregation, as shown in Figure 2, panels A, B and C respectively. In addition, we detected higher levels of proteins involved in chromosome condensation and segregation belonging to the condensin and the structural maintenance of chromosome (SMC) protein complexes in patients with COVID-19 and patients with non-COVID-19 ARDS, compared to healthy controls (Figure 2D).

Activation of the type I IFN pathway in the COVID-19 group is demonstrated by the high expression level of major INF signalling proteins (ISP) such as IFIT1, IFIT2, IFIT3, OAS1, OAS2 and OAS3, confirming previous observations of the significance of this pathway in the innate immune response during viral infection. Interestingly, we also detected

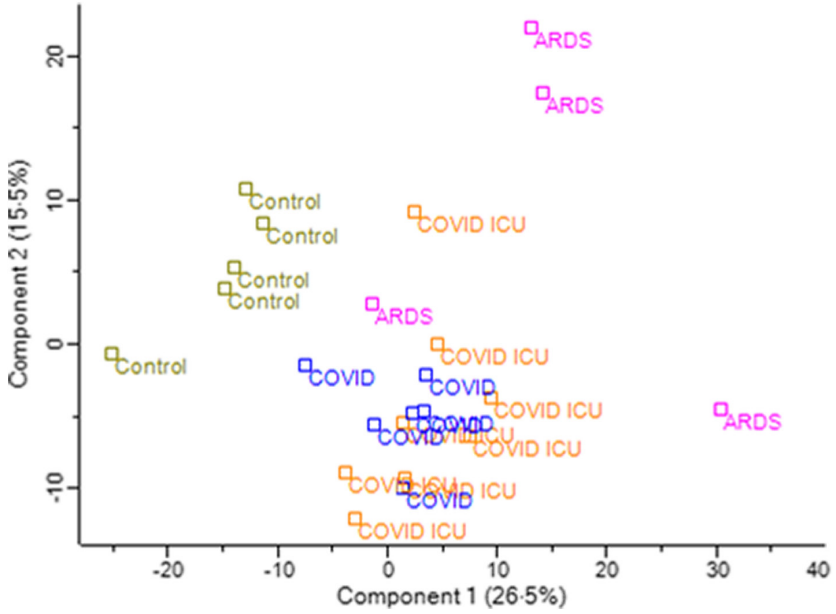

FIGURE 1 Overall proteomic analysis of neutrophils isolated from healthy controls (controls), patients with non-COVID-19 ARDS (ARDS), patients with COVID-19 hospitalised in the ICU (COVID-ICU) and patients with COVID-19 hospitalised in other medical departments (COVID). ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; ICU, intensive care unit.

an increased level of transporter associated with antigen processing (TAP) proteins, known to be up-regulated by IFN and involved in antigen transport into the endoplasmic reticulum for loading onto class I major histocompatibility complex molecules. Although originally thought to be protective, these new data suggest a more balanced impact of the type I IFN pathway on the COVID-19 clinical course. While mutated proteins involved in the type I IFN pathway[7] or production of anti-IFN-I autoantibodies[8] have been linked to severe forms of COVID-19, an early IFN-I production was recently shown to be protective, whereas a late and intense IFN-I response leads to an exacerbated and deleterious pro-inflammatory state.[9] As regards the potential therapeutic use of IFN-I in patients with COVID-19, further studies are warranted to better describe the activation time course of this pathway.

Consistent with our results, neutrophils from patients with COVID-19 reportedly exhibit an increase in proteins associated with fibrin clot formation, such as fibrinogen alpha and fibrinogen beta,[5] and display augmented aggregate formation between neutrophils and platelets. Such aggregates could directly contribute to vascular damage by impairing neutrophil transmigration and promoting microthrombi formation in patients' lung vasculature.

The abnormal expression of proteins involved in chromosome condensation and segregation observed in the neutrophils of patients with COVID-19 and patients with non-COVID-19 ARDS was puzzling, as dysregulation of these proteins in terminally differentiated cells is unexpected; however, two studies have previously reported the presence of a low-density inflammatory neutrophil (LDN) population in patients with these two conditions.[5,10] LDNs, considered as immature neutrophils, exhibit several abnormal properties, including abundant spontaneous formation of NETs.[10] It may be postulated that this high expression level of proteins regulating the organisation of genetic material could be linked to the unravelling of DNA during NET formation. We determined the plasma concentration of nucleosome, a known marker of NETosis, in 13 controls, 33 patients with COVID-19 and four patients with ARDS. As shown in Figure 2E, higher nucleosome concentrations were detected in patients with COVID-19 and non-COVID-19 ARDS, compared to controls. Further experiments are clearly warranted to confirm these relationships and to decipher the molecular mechanism involved.

In conclusion, although our patient populations were small, our quantitative proteomic study of neutrophils confirms the activation of the type I IFN pathway and identifies targets of IFN, such as TAP proteins, specifically in patients with COVID-19, but not in patients with non-COVID-19 ARDS, and describes modifications of the neutrophil proteome potentially associated with ARDS.

AUTHOR CONTRIBUTIONS

Caroline Le Van Kim, Marc Romana and Véronique Baccini designed the study. Marc Romana isolated the neutrophils and prepared the cellular extracts. Marjorie Leduc, Johanna Bruce and Emilie-Fleur Gautier performed the proteomic analysis. Frédéric Martino and Véronique Baccini enrolled the patients. Patricia Hermand and Yohann Garnier performed nucleosome profile analysis. All authors discussed the data, revised, and approved the manuscript.

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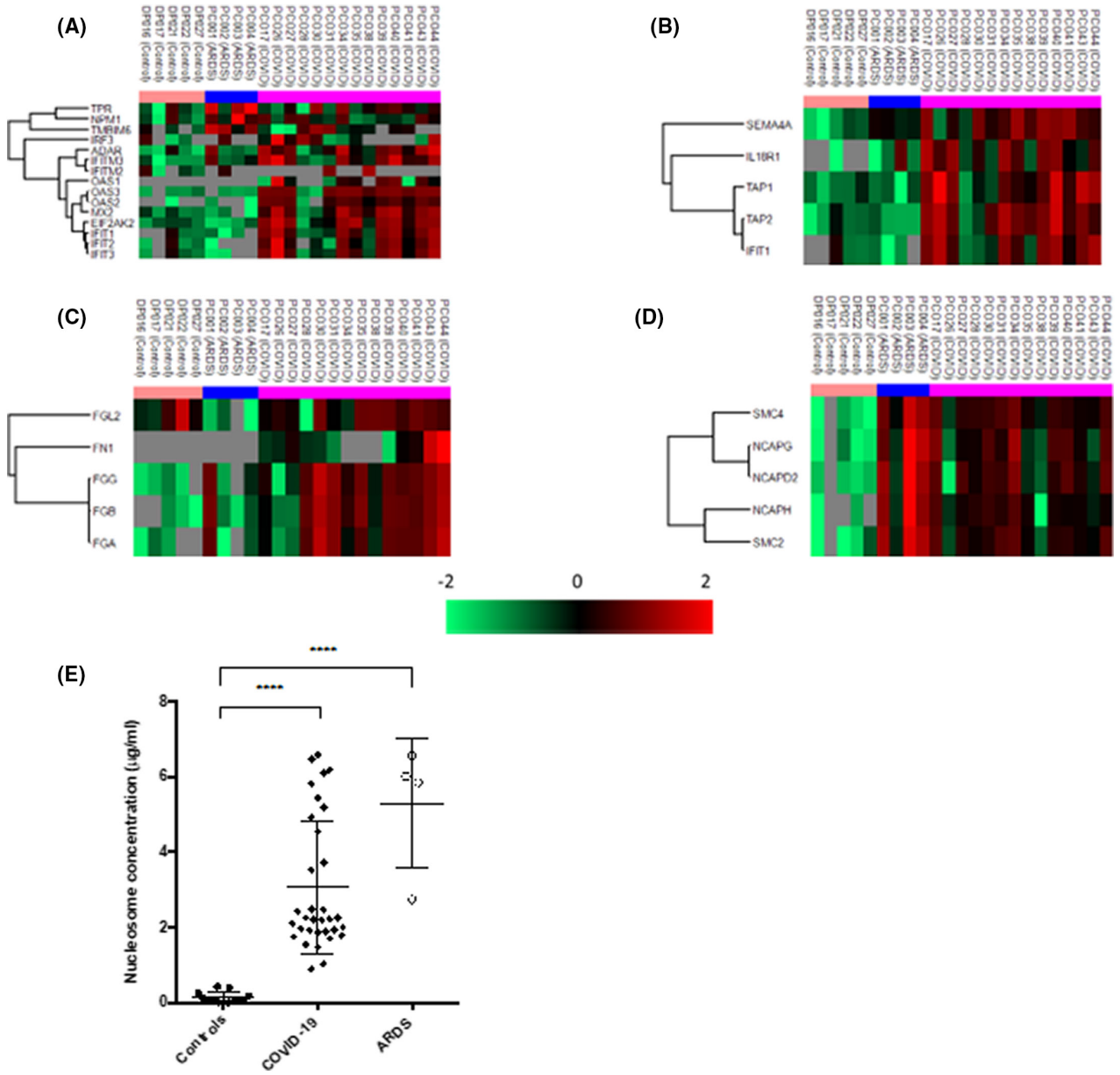

FIGURE 2 (A–D) Heatmaps for proteins overexpressed in the neutrophils of patients with COVID-19, as highlighted by two-dimensional enrichment analysis. Annotations out of the diagonal corresponding to differential expression in healthy controls (controls), patients with non-COVID-19 ARDS (ARDS) or patients with COVID-19 (COVID) are indicated. The colour scale indicates the level of protein expression. (E) Plasma nucleosome concentrations were compared between the three groups (controls, patients with COVID-19, and patients with ARDS) using Kruskal–Wallis test. ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; ****$p < 0.0001$.

participating in this study. For those who lost a family member, we express our deep regret.

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DATA SHARING STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[1] partner repository with the dataset identifier PXD033293.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.