Carboxypeptidase B Inhibits Over-activation of Anaphylatoxin-neutrophil Extracellular Trap Axis in COVID-19 Patients

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Research

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Abstract

Background: Thrombosis and coagulopathy are highly prevalent in severe patients with COVID-19 and increase the risk of death. Immunothrombosis has recently been demonstrated to contribute to the thrombotic events in COVID-19 patients with coagulopathy. Neutrophil extracellular traps (NETs) are primary components of immunothrombosis, whereas the mechanism of NET formation remains unclear. We aim to explore the clinical roles of NETs and the regulation of complement on the NET formation in COVID-19.

Methods: We recruited 135 COVID-19 patients and measured plasma levels of C5, C3, cell-free DNA and myeloperoxidase-DNA. We detected complement-induced NET formation by immunofluorescent staining and evaluated the cytotoxicity to vascular endothelial HUVEC cells by CCK-8 assay.

Results: We found that the plasma levels of complements (C3 and C5) and NETs were closely related to coagulopathy and multiple organ dysfunction in patients with COVID-19. By using anti-C3a and anti-C5a antibodies, we revealed that the complement component anaphylatoxins in the plasma of COVID-19 patients strongly induced NET formation. The pathological effect of the anaphylatoxin-NET axis on the damage of vascular endothelial cells could be relieved by recombinant carboxypeptidase B (CPB), a stable homolog of enzyme CPB2 which can degrade anaphylatoxins to inactive products.

Conclusions: Over-activation in anaphylatoxin-NET axis plays a pathological role in COVID-19. Early intervention in anaphylatoxins might help prevent thrombosis and disease progression in COVID-19 patients.

Background

The global outbreak of coronavirus disease 2019 (COVID-19) has posed an unprecedented health crisis and caused more than 800,000 deaths[1]. As the pandemic continues, mounting evidence implicates thrombosis and coagulopathy in a fatal outcome in COVID-19 patients[2, 3]. The latest data reported that thrombotic complications occur in up to 49% of patients with COVID-19 admitted to the intensive care unit (ICU)[4]. Autopsies further provide direct evidence of pulmonary embolism in patients with COVID-19[5, 6]. Of note, immunothrombosis, the direct interaction of activated leukocytes with platelets and plasma coagulation factors in the innate immune response[7], has been demonstrated to contribute to the thrombotic events of coagulopathy[8]. Besides, neutrophil extracellular traps (NETs), which are composed of extracellular DNA decorated with granule proteins released by activated neutrophils, was identified as a leading cause and core component of immunothrombosis [9, 10]. Hence, it is crucial to reveal the mechanisms causing NET formation in exploring more efficient therapeutic approaches to combat COVID-19.

Complement is a major component of the innate immune system involved in defending against foreign pathogens through complement fragments[11]. During the complement activation, C3 and C5 are proteolytically cleaved to generate pro-inflammatory polypeptides C3a and C5a, termed as

anaphylatoxin[12, 13]. Since the receptors to C3a and C5a are prominently expressed on the surface of neutrophils[14, 15], C3a and C5a have been recognized as potent activators for neutrophil migration, cytokine production, platelet-leukocyte aggregation, and NET release[12, 16]. Multiple studies have illustrated that complement-induced over-activation of neutrophils is involved in the pathogenesis of acute respiratory distress syndrome (ARDS) and fatal viral infections[17-19]. Thus, complement is an important soluble mediator that bridges inflammation and thrombosis in severe infections.

Of note, C3a and C5a have carboxyl-terminus containing arginine residues that are critical for optimal activity[20]. Carboxypeptidases are capable of controlling the activity of anaphylatoxins by cleaving off a C-terminal arginine residue to yield arginine derivatives ($C3a_{des\text{-}Arg}$ and $C5a_{des\text{-}Arg}$)[12]. The resulting $C5a_{des\text{-}Arg}$ retains 1-10% of the inflammatory activity of C5a, and $C3a_{des\text{-}Arg}$ is devoid of any pro-inflammatory activity[21]. Recently, Carboxypeptidase B2 (CPB2, encoded by human *CPB2* gene) was demonstrated to be an important regulator in reducing inflammatory response and organ damage by degrading plasma anaphylatoxins[22-24].

In this study, we found that the plasma levels of complements and NETs were associated with disease severity in COVID-19. More importantly, we demonstrated that recombinant CPB could reduce the NET formation by degrading anaphylatoxin C3a and C5a. These findings may shed new light on a potential therapeutic strategy for COVID-19 by targeting the anaphylatoxin-NET axis.

Methods

Patient and sample collection

The retrospective study included 135 patients with confirmed diagnosis of COVID-19 who admitted to Beijing Ditan Hospital from January 20th, 2020 to April 27th, 2020. According to the guidelines on the diagnosis and treatment of new coronavirus pneumonia (version 7) released by National Health Commission of China, the classification of COVID-19 are as follows: Mild: Clinical symptoms from mild fever, respiratory tract to pneumonia manifestation. Severe: Meeting any one of the following should be treated as severe cases, including respiratory distress, respiratory rate $\geq$ 30 breaths/min; oxygen saturation $\leq$ 93% at rest; and $PaO_2/FiO_2 \leq 300$. In severe group, 31 patients were admitted to ICU and 15 cases received mechanical ventilation. Twenty-five healthy donors matched to the age and sex of mild COVID-19 patients were enrolled. Three volunteers donated their peripheral neutrophils for *in vitro* experiments. Blood samples were collected by venipuncture into ethylenediaminetetraacetic acid tubes at the indicated timepoint and centrifuged at 450 × g for plasma separation. Samples were divided into small aliquots and stored at -80°C until the time of testing. The study was approved by Committee of Ethics at Beijing Ditan Hospital, Capital Medical University, Beijing, China. The approval number is JDLKZ(2020)D(036)-01.

Quantification of MPO-DNA and cfDNA

Cell-free DNA in plasma was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. MPO-DNA complexes were quantified similarly to what has been previously described[25]. In brief, a capture antibody against MPO was coated on a 96-well flat-bottom plate at 1:200 (Abcam, Cambridge, MA, USA), and the amount of MPO-bound DNA was quantified using the Quant-iT PicoGreen dsDNA assay as described above.

Quantification of C5 and C3

Plasma levels of C5 (including C5 and C5a) and C3 (including C3, C3a and C3b) were detected using Human Complement C5 ELISA Kit and Human Complement C3 ELISA Kit (Abcam) according to the manufacturer's instruction.

Immunofluorescence Staining

NETs were detected by immunofluorescence staining as previously reported[26]. Neutrophils were fixed, permeabilized and blocked after 3-hour *in vitro* culture or stimulation. Cells were incubated with antibody against histone H3 citrulline R2+R8+R17 (H3Cit) and followed by secondary antibody coupled with Alexa Fluor Dyes (Invitrogen). DNA was stained using 4',6-diamidino-2-phenylindole (DAPI). Images were obtained with a confocal fluorescence microscope (Zeiss LSM 510 META; Carl Zeiss, Thornwood, NJ, USA). NETs were identified as structures positive for both histone H3Cit and DAPI staining.

Neutrophil isolation, *in vitro* culture and stimulation

Blood samples from healthy donors were collected into ethylenediaminetetraacetic acid tubes as described above for plasma separation. The anticoagulated blood was then fractionated by density-gradient centrifugation using Percoll (Stemcell Technologies, Vancouver, Canada). Neutrophils were further purified by dextran sedimentation of the red blood cell layer before lysing residual red blood cells with sodium chloride. Neutrophil preparations were at least 95% pure as confirmed by nuclear morphology.

Purified neutrophils were resuspended in RPMI-1640 medium supplemented with heat-inactivated 5% fetal bovine serum and 2 mM L-glutamine. Neutrophils were seeded into 96-well plate (5×10^4/well) for supernatant detection and 24-well plate (2×10^5/well) with polylysine-coated coverslips for NET immunofluorescence staining. Cells were rested for 1 hour at 37°C and 5% CO_2 prior to COVID-19 plasma stimulation. Recombinant CPB (YaxinBio, Shanghai, China) was used to digest C3a and C5a in the plasma at 37°C for 30 min prior to neutrophil stimulation. Anti-human C3a antibody (Merck, Darmstadt, Germany) and anti-human C5a antibody (R&D Systems, Minneapolis, MN, USA) were added into the neutrophil culture system to neutralize C3a and C5a, respectively. Three hours later, the supernatant was collected to quantify MPO-DNA content. The results were calculated by deducting the background levels of MPO-DNA in the plasma.

Preparation of NET-conditioned medium and cell viability assay

Neutrophils from healthy donors were cultured with plasma from patients with COVID-19 as described above. Three hours later, the supernatant was collected carefully and used as NET-conditioned medium. HUVEC cells (3×10^3/well) were seeded into 96-well plate and cultured for 24 hours. Cells culture media were replaced with 100 μl NET-conditioned media or new cell culture media as control for 24 hours. Cell viability assay was performed using a cell counting kit 8 (CCK-8) (Dojindo, Kumamoto, Japan) as per the manufacturer's protocol. Absorbance was detected at 590 nm using a microplate reader. The experiments were performed in sextuplicate.

Statistical Analysis

All statistical analyses were performed with the SPSS 25.0 statistical package (IBM, Armonk, NY, USA). Values are presented as the mean ± standard deviation for data that were normally distributed or median and interquartile range for data that were not normally distributed for continuous variables and number (%) for categorical variables. The Kolmogorov-Smirnov test was used to inspect the normality and homogeneity of variance of all the data. For two-group comparison, *P* values were derived from the one-way Student *t* test to determine differences between groups with normally distributed data and Mann-Whitney nonparametric test with other data. For multi-group comparison, *P* values were derived from one-way ANOVA (continuous variables) or Chi-square test (categorical variables). For all comparisons, $P < 0.05$ was considered statistically significant.

Results

COVID-19 cohort

We recruited 135 confirmed COVID-19 patients admitted to Beijing Ditan Hospital. Among them, 94 (69.6%) were mild cases and 41 (30.4%) were severe cases according to the guidelines on the diagnosis and treatment of new coronavirus pneumonia (version 7) released by the National Health Commission of China. Twenty-five age and gender-matched healthy donors (HDs) were enrolled as controls. Demographics and relevant clinical characteristics are reported in Table 1. The study was approved by the Committee of Ethics at Beijing Ditan Hospital, Capital Medical University, Beijing, China.

The elevated levels of NETs in COVID-19 patients were closely related to respiratory failure and multiple organ dysfunction

We collected plasma once a week from hospitalized patients and evaluated the levels of complements and NETs by ELISA. In comparison with HDs, COVID-19 patients had significantly higher levels of NETs in the first week of admission quantified by measuring cell-free DNA (cfDNA) and myeloperoxidase (MPO)-DNA (all $P < 0.001$; Fig. 1a). Linear regression analysis showed that severe patients had higher levels of NETs than mild cases dynamically within 60 days of hospitalization (Fig. 1b). Both cfDNA and MPO-DNA levels in severe cases showed an upward trend with the disease progression (Fig. 1b).

Previous studies have reported that NETs were associated with a variety of pathological changes such as immune status, coagulation disorder, and multiple organ dysfunction[25]. In COVID-19 patients, NET levels were positively correlated with the neutrophil count, fibrin(-ogen) degradation products (FDP), lactate dehydrogenase, and creatine kinase (Table 2). In addition, greater levels of cfDNA were detected in patients with mechanical ventilation (without vs with: 570.4 vs 897 ng/mL, $P = 0.001$; Fig. 1c) or those with sequential organ failure assessment (SOFA) scores ≥ 2 (SOFA < 2 vs SOFA ≥ 2: 554.9 vs 897 ng/mL, $P = 0.001$; Fig. 1d). These results indicated that the NET levels were closely related to the disease severity of COVID-19.

The plasma levels of complement C3 and C5 were associated withdisease severity

We measured the levels of complement C3 and C5 in the plasma of patients and HDs. Within the first week of admission, both of C3 and C5 were dramatically increased in mild patients with COVID-19 compared with HDs (Median with interquartile range, C3: 0.75 [0.65, 0.79] mg/mL vs 22.61 [14.86, 44.22] mg/mL, $P < 0.001$; C5: 41.66 [30.19, 47.7] μg/mL vs 274.8 [209.3, 344.7] μg/mL, $P < 0.001$; Fig. 2a). The levels of C3 and C5 were further raised to 56.23 [31.91, 76.04] mg/mL and 326.1 [245, 388.8] μg/mL in the severe COVID-19 patients (Fig. 2a). Consistent with the longitudinal changes of NETs, linear regression analysis showed that severe patients had higher levels of C3 and C5 than mild cases as the disease progresses (Fig. 2b). There was an upward trend of C3 in patients with disease progression (Fig. 2b). Furthermore, C3 levels were positively correlated with the neutrophil count, FDP, direct bilirubin, and total bilirubin in COVID-19 patients (Table 2).

The anaphylatoxin-induced NET formation was restrained by recombinant CPB

To determine the regulation of complement anaphylatoxin C3a and C5a on NET formation, we isolated peripheral neutrophils from HCs, then cultured these cells in the presence of the plasma from severe patients with COVID-19 or the plasma from HDs. The COVID-19 plasma was capable of activating neutrophils to form NETs (Fig. 3a) and to release MPO-DNA in the media (Fig. 3b), which were significantly restrained by anti-C3a and anti-C5a neutralizing antibodies. Moreover, we added recombinant CPB, a stable homolog of enzyme CPB2 which is capable of inactivating anaphylatoxin C3a and C5a by specifically degrading their arginine residues[27]. As shown in Fig. 3a, CPB significantly reduced NET release induced by the plasma from COVID-19 patients. These data indicated that the over-production of NETs in the plasma of COVID-19 patients at least in part was induced by anaphylatoxin C3a and C5a, and the induction could be blocked by recombinant CPB.

To further investigate the pathological roles of the anaphylatoxin-NET axis, we stimulated neutrophils with COVID-19 plasma to prepare a NET-conditioned medium and then added the conditioned medium to the culture system of vascular endothelial HUVEC cells. Compare with the plasma from HDs, the plasma from mild and severe COVID-19 patients reduced cell viability of HUVEC cells (Fig. 3c). However, the cytotoxicity of COVID-19 plasma was significantly relieved after *in vitro* digestion of plasma by CPB for 30 min (Fig. 3c). Similarly, the supplement of anti-C3a antibody plus anti-C5a antibody, or the NET inhibitor Cl-amidine to the cultured neutrophils to block NET production, could also reduce the cytotoxicity

to HUVEC cells (Fig. 3c). These results suggested that recombinant CPB could protect vascular endothelial cells from damage by reducing C3a- and C5a-induced NET production in COVID-19 patients.

Discussion

As one of the major causes of mortality in severe COVID-19, thrombosis has drawn much attention[28-30], however, the formation mechanisms remain to be clarified. Recently, NETs were found in to be a key [31], which provides new clues to the pathogenesis[32, 33]. In this study, by binding *in vivo* and *in vitro* assays, we investigated the mechanism of NET formation and the pathological roles of the anaphylatoxin-NET axis in COVID-19. First, the elevated levels of NETs and complement C3 were closely related to immune status, coagulation disorders, and multiple organ dysfunction. Second, the NET formation was at least partially regulated by complement anaphylatoxin C3a and C5a. Third, we made a novel finding that recombinant CPB could effectively improve the detrimental effect of NETs on vascular endothelial cells by degrading C3a and C5a.

Consistent with our study, autopsies reported in pre-print manuscripts also revealed activation of complement C5 in lung and kidney tissues of COVID-19 patients[34, 35]. The findings from our study and these reports collectively suggested that complement activation may contribute to the development of tissue injury and organ dysfunction in patients with COVID-19. Given this, complement-blocking drugs may be a beneficial addition to the therapeutic armamentarium against COVID-19. Thus, several clinical trials were launched just recently in order to prevent ARDS and mortality of COVID-19 by C5a inhibitor (Zilucoplan, NCT04382755) or anti-C5a antibody (Eculizumab, NCT04288713). Considering that in addition to C5a, a significant elevation of C3a was also observed in the patients with COVID-19, we highly recommend recombinant CPB as a potential choice for simultaneously degrading both C3a and C5a[12]. It is worthy of expectation for preclinical studies on recombinant CPB to suppress the unrestrained inflammation and reduce the clinical severity of COVID-19. Notably, the endogenous CPB2 was also known as thrombin-activatable fibrinolysis inhibitor (TAFI) to inhibit fibrinolysis and thereby reduce the binding of plasminogen to the fibrin clot[23]. An excessive supplement of recombinant CPB may upset the balance between coagulation and fibrinolysis. Therefore, an appropriate dosage should be carefully considered in further studies.

We noticed that the NET production induced by plasma from severe COVID-19 patients could not be completely inhibited by either neutralizing antibodies or recombinant CPB (Fig. 3a), which implied that in addition to anaphylatoxins, there were other inducers of NETs in the plasma of severe patients. Many studies have reported an increase in IL-6, IL-1β, and CXCL-8 in severe patients with COVID-19[36-38], which are also important factors that induce granulocyte activation and NET release. These pro-inflammatory cytokines may also contribute to the over-production of NETs in severe COVID-19 patients. Anaphylatoxins are leading mediators for rapidly inducing the synthesis of pro-inflammatory cytokines[39, 40]. Thus, complement activation may be a pivotal link in amplifying inflammatory response in early infection. Consistently, we found that compared with HDs, complement C3 and C5 increased remarkably in patients with mild symptoms. Although the complement component

anaphylatoxins may contribute to increased disease severity following SARS-CoV-2 infection, complement activation is necessary for the development of a protective humoral response. In this respect, early intervention in anaphylatoxins without affecting complement cascade activation in COVID-19 patients might help prevent thrombosis and disease progression.

Our results demonstrated that the increased complement component plays an important role in promoting the formation of NETs in patients with COVID-19. It is different from our previous findings in the infection of severe fever with thrombocytopenia syndrome virus (SFTSV). The patients with SFTS had significantly higher levels of NETs but comparable levels of C3 and C5 to the healthy controls[25]. The difference in complement activation between COVID-19 and SFTS might be related to differences in clinical manifestations. Pulmonary thrombosis appears to be frequent in COVID-19 pneumonia, while the patients with SFTS have a marked propensity for bleeding with a rare thrombus. Thus, the mechanism and function of NETs may be different in these two viral infections associated with coagulation abnormalities.

We acknowledged that our study has several limitations. First, the CPB2 levels in the plasma of COVID-19 patients were not available because it is unstable in physiological condition with a half-life of 10 min at 37°C. Second, for the same reason, we used recombinant pancreatic enzyme CPB instead in *in vitro* study, which is a stable homolog of CPB2. Third, compared with MPO-DNA, a specific marker of NETs, cfDNA could also be released from other leukocytes and damaged endothelial cells following cellular death. We were not able to accurately determine the cellular origins of peripheral cfDNA in the present study. As there were higher degrees of correlation of cfDNA with clinical parameters than MPO-DNA (Table 2), it may be directly related to leukopenia and tissue damage in patients with COVID-19.

Conclusions

In conclusion, our study offers new insights into the immunological pathogenesis of COVID-19. Based on these findings, degrading the over-generated C3a and C5a by recombinant CPB to restrain the downstream NET production might be a promising approach to prevent thrombosis and reduce the clinical severity of COVID-19.

Declarations

Ethics approval and consent to participate

This study was approved by the Committee of Ethics at Beijing Ditan Hospital, Capital Medical University, Beijing, China, and each patient gave written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YZ performed the experiments, analyzed the data and did the statistical analysis. KH collected samples from patients. CD extracted clinical information. RL participated in sample collection and experiments. JL participated in patient recruitment and clinical data collection. HZ recruited the patients and revised the manuscript. LZ designed the study, performed the experiments and wrote the manuscript. AL recruited the patients and participated in the study design. All authors read and approved the final manuscript.

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References

1. WHO: **WHO coronavirus disease (COVID-19) dashboard**. *WHO* 2020, **https://covid19.who.int/**
2. Klok FA, Kruip M, van der Meer NJM, Arbous MS, Gommers D, Kant KM, Kaptein FHJ, van Paassen J, Stals MAM, Huisman MV *et al*: **Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: An updated analysis**. *Thrombosis research* 2020, **191**:148-150.
3. Tang N, Li D, Wang X, Sun Z: **Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia**. *Journal of thrombosis and haemostasis : JTH* 2020, **18**(4):844-847.

4. Wiersinga WJ, Rhodes A, Cheng AC, Peacock SJ, Prescott HC: **Pathophysiology, Transmission, Diagnosis, and Treatment of Coronavirus Disease 2019 (COVID-19): A Review**. *Jama* 2020.
5. Wichmann D, Sperhake JP, Lutgehetmann M, Steurer S, Edler C, Heinemann A, Heinrich F, Mushumba H, Kniep I, Schroder AS *et al*: **Autopsy Findings and Venous Thromboembolism in Patients With COVID-19**. *Ann Intern Med* 2020.
6. Grimes Z, Bryce C, Sordillo EM, Gordon RE, Reidy J, Paniz Mondolfi AE, Fowkes M: **Fatal Pulmonary Thromboembolism in SARS-CoV-2-Infection**. *Cardiovasc Pathol* 2020, **48**:107227.
7. Schrottmaier WC, Mussbacher M, Salzmann M, Assinger A: **Platelet-leukocyte interplay during vascular disease**. *Atherosclerosis* 2020, **307**:109-120.
8. Middleton EA, He XY, Denorme F, Campbell RA, Ng D, Salvatore SP, Mostyka M, Baxter-Stoltzfus A, Borczuk AC, Loda M *et al*: **Neutrophil Extracellular Traps (NETs) Contribute to Immunothrombosis in COVID-19 Acute Respiratory Distress Syndrome**. *Blood* 2020.
9. Palankar R, Greinacher A: **Challenging the concept of immunothrombosis**. *Blood* 2019, **133**(6):508-509.
10. Cerletti C, Tamburrelli C, Izzi B, Gianfagna F, de Gaetano G: **Platelet-leukocyte interactions in thrombosis**. *Thrombosis research* 2012, **129**(3):263-266.
11. Stepniewska J, Dolegowska B, Golembiewska E, Marchelek-Mysliwiec M, Domanski M, Ciechanowski K, Zair L: **The activation of complement system in different types of renal replacement therapy**. *J Physiol Pharmacol* 2020, **71**(2).
12. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J: **The role of the anaphylatoxins in health and disease**. *Molecular immunology* 2009, **46**(14):2753-2766.
13. Verschoor A, Karsten CM, Broadley SP, Laumonnier Y, Kohl J: **Old dogs-new tricks: immunoregulatory properties of C3 and C5 cleavage fragments**. *Immunological reviews* 2016, **274**(1):112-126.
14. Schreiber A, Xiao H, Jennette JC, Schneider W, Luft FC, Kettritz R: **C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis**. *J Am Soc Nephrol* 2009, **20**(2):289-298.
15. Wu MC, Brennan FH, Lynch JP, Mantovani S, Phipps S, Wetsel RA, Ruitenberg MJ, Taylor SM, Woodruff TM: **The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization**. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**(23):9439-9444.
16. Sadik CD, Miyabe Y, Sezin T, Luster AD: **The critical role of C5a as an initiator of neutrophil-mediated autoimmune inflammation of the joint and skin**. *Semin Immunol* 2018, **37**:21-29.
17. Garcia CC, Weston-Davies W, Russo RC, Tavares LP, Rachid MA, Alves-Filho JC, Machado AV, Ryffel B, Nunn MA, Teixeira MM: **Complement C5 activation during influenza A infection in mice contributes to neutrophil recruitment and lung injury**. *PloS one* 2013, **8**(5):e64443.
18. Palmer LJ, Damgaard C, Holmstrup P, Nielsen CH: **Influence of complement on neutrophil extracellular trap release induced by bacteria**. *Journal of periodontal research* 2016, **51**(1):70-76.

19. Kambas K, Markiewski MM, Pneumatikos IA, Rafail SS, Theodorou V, Konstantonis D, Kourtzelis I, Doumas MN, Magotti P, Deangelis RA *et al*: **C5a and TNF-alpha up-regulate the expression of tissue factor in intra-alveolar neutrophils of patients with the acute respiratory distress syndrome**. *Journal of immunology* 2008, **180**(11):7368-7375.
20. Schatz-Jakobsen JA, Yatime L, Larsen C, Petersen SV, Klos A, Andersen GR: **Structural and functional characterization of human and murine C5a anaphylatoxins**. *Acta Crystallogr D Biol Crystallogr* 2014, **70**(Pt 6):1704-1717.
21. Matthews KW, Mueller-Ortiz SL, Wetsel RA: **Carboxypeptidase N: a pleiotropic regulator of inflammation**. *Molecular immunology* 2004, **40**(11):785-793.
22. Morser J, Shao Z, Nishimura T, Zhou Q, Zhao L, Higgins J, Leung LLK: **Carboxypeptidase B2 and N play different roles in regulation of activated complements C3a and C5a in mice**. *Journal of thrombosis and haemostasis : JTH* 2018, **16**(5):991-1002.
23. Foley JH, Conway EM: **Basic weapons to degrade C3a and C5a**. *Journal of thrombosis and haemostasis : JTH* 2018, **16**(5):987-990.
24. Foley JH, Kim PY, Hendriks D, Morser J, Gils A, Mutch NJ, Subcommittee on F: **Evaluation of and recommendation for the nomenclature of the CPB2 gene product (also known as TAFI and proCPU): communication from the SSC of the ISTH**. *Journal of thrombosis and haemostasis : JTH* 2015, **13**(12):2277-2278.
25. Zhang Y, Song R, Shen Y, Zhao Y, Zhao Z, Fan T, Yang X, Wang L, Zhang W, Chen C *et al*: **High Levels of Circulating Cell-free DNA Are Associated With a Poor Prognosis in Patients With Severe Fever With Thrombocytopenia Syndrome**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2020, **70**(9):1941-1949.
26. Zhu L, Liu L, Zhang Y, Pu L, Liu J, Li X, Chen Z, Hao Y, Wang B, Han J *et al*: **High Level of Neutrophil Extracellular Traps Correlates With Poor Prognosis of Severe Influenza A Infection**. *The Journal of infectious diseases* 2018, **217**(3):428-437.
27. Song JJ, Hwang I, Cho KH, Garcia MA, Kim AJ, Wang TH, Lindstrom TM, Lee AT, Nishimura T, Zhao L *et al*: **Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis**. *J Clin Invest* 2011, **121**(9):3517-3527.
28. Singh B, Aly R, Kaur P, Gupta S, Vasudev R, Virk HS, Shamoon F, Bikkina M: **COVID-19 Infection and Arterial Thrombosis: Report of three cases**. *Ann Vasc Surg* 2020.
29. Manenti A, Farinetti A, Manco G, Mattioli A: **Vasculitis and aortitis: Covid-19 challenging complications**. *J Vasc Surg* 2020.
30. Graziani A, Domenicali M, Zanframundo G, Palmese F, Caroli B, Graziani L: **Pulmonary artery thrombosis in COVID-19 patients**. *Pulmonology* 2020.
31. Noubouossie DF, Reeves BN, Strahl BD, Key NS: **Neutrophils: back in the thrombosis spotlight**. *Blood* 2019, **133**(20):2186-2197.
32. Leppkes M, Knopf J, Naschberger E, Lindemann A, Singh J, Herrmann I, Sturzl M, Staats L, Mahajan A, Schauer C *et al*: **Vascular occlusion by neutrophil extracellular traps in COVID-19**. *EBioMedicine*

2020, **58**:102925.

33. Barnes BJ, Adrover JM, Baxter-Stoltzfus A, Borczuk A, Cools-Lartigue J, Crawford JM, Dassler-Plenker J, Guerci P, Huynh C, Knight JS *et al*: **Targeting potential drivers of COVID-19: Neutrophil extracellular traps**. *The Journal of experimental medicine* 2020, **217**(6).
34. Gao T, Hu M, Zhang X, Li H, Zhu L, Liu HW, Dong Q, Zhang Z, Wang Z, Hu Y *et al*: **Highly pathogenic coronavirus N protein aggravates lung injury by MASP-2-mediated complement over-activation**. *medRxiv* 2020.
35. Diao B, Wang C, Wang R, Feng Z, Tan Y, Wang H, Wang C, Liu L, Liu Y, Liu Y *et al*: **Human Kidney is a Target for Novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection**. *medRxiv* 2020.
36. Xu X, Yu MQ, Shen Q, Wang LZ, Yan RD, Zhang MY, Liu JY, Qu YQ: **Analysis of inflammatory parameters and disease severity for 88 hospitalized COVID-19 patients in Wuhan, China**. *Int J Med Sci* 2020, **17**(13):2052-2062.
37. Wilson JG, Simpson LJ, Ferreira AM, Rustagi A, Roque J, Asuni A, Ranganath T, Grant PM, Subramanian A, Rosenberg-Hasson Y *et al*: **Cytokine profile in plasma of severe COVID-19 does not differ from ARDS and sepsis**. *JCI insight* 2020, **5**(17).
38. Riva G, Nasillo V, Tagliafico E, Trenti T, Comoli P, Luppi M: **COVID-19: more than a cytokine storm**. *Critical care* 2020, **24**(1):549.
39. Ohta R, Torii Y, Imai M, Kimura H, Okada N, Ito Y: **Serum concentrations of complement anaphylatoxins and proinflammatory mediators in patients with 2009 H1N1 influenza**. *Microbiology and immunology* 2011, **55**(3):191-198.
40. Basta M, Van Goor F, Luccioli S, Billings EM, Vortmeyer AO, Baranyi L, Szebeni J, Alving CR, Carroll MC, Berkower I *et al*: **F(ab)'2-mediated neutralization of C3a and C5a anaphylatoxins: a novel effector function of immunoglobulins**. *Nature medicine* 2003, **9**(4):431-438.

Tables

Table 1. Demographics and clinical characteristics of healthy donors and 2 groups of COVID-19 patients.

Characteristic	HD	Severity		*P* value (HD vs. Mild)	*P* value (Mild vs. Severe)
		Mild	Severe		
Patient amount	25	94	41	-	-
Age (years)	42 (32, 69)	34.9±15.0	61.8±16.7	**0.001**	**<0.001**
Male sex, n (%)	15 (60)	43 (45.7)	26 (63.4)	0.607	0.059
Complications, n (%)					
Hypertension	0	6 (6.4)	16 (39.0)	-	**<0.001**
Cardiovascular disease	0	0	5 (12.2)	-	**0.001**
Chronic pulmonary disease	0	2 (2.1)	9 (22.0)	-	**<0.001**
Diabetes	0	1 (1.1)	10 (24.4)	-	**<0.001**
Hyperlipemia	0	1 (1.1)	2 (4.9)	-	0.167
Chronic kidney disease	0	1 (1.1)	4 (9.8)	-	**0.014**
Immune disorders	0	0	3 (7.3)	-	**0.008**
Others	0	1 (1.1)	1 (2.4)	-	0.543
Hospitalization Period (days)	0	25.5 (17.3, 35.7)	36 (22, 43)	-	**0.004**
SOFA score	-	-	2 (0, 3.5)	-	**-**
APACHE II score	-	-	14 (9, 15)	-	**-**
Laboratory data at admission					
WBC ($\times 10^9$/L)	6.1±1.1	4.8 (4.1, 6.5)	6.7 (4.8, 8.9)	**0.007**	**<0.001**
Lymphocyte ($\times 10^9$/L)	2.2 (2.0, 2.6)	1.6 (1.2, 2.0)	1.0 (0.8, 1.4)	**<0.001**	**<0.001**
Neutrophil ($\times 10^9$/L)	3.4±0.8	2.7 (2.1, 3.8)	5.2 (3.3, 7.9)	**0.045**	**<0.001**
Monocyte ($\times 10^9$/L)	0.4 ±0.1	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	**0.006**	0.429
Hemoglobin (g/L)	140.6±13.4	137.5±14.4	121.4±18.9	0.431	**<0.001**
Platelets ($\times 10^9$/L)	248.1±41	230.5 (182, 278.3)	214 (153, 296)	0.180	0.277
D-dimer (mg/L)	-	0.3□0.1, 0.4□	1.1 (0.6, 2.2)	-	**<0.001**

CRP (mg/L)	-	1.6 (0.6, 9.4)	47.1 (24.7, 105.3)	-	**<0.001**
LDH (U/L)	-	195.5 (172.8, 227.6)	341.1 (296.3, 474.5)	-	**<0.001**
CK (U/L)	-	69.1 (46.4, 105.4)	110.1 (48.1, 175.4)	-	**0.049**
ALT (U/L)	-	19.5 (14, 30.2)	30.4 (17.8, 47.5)	-	**0.008**
AST (U/L)	-	20.2 (15.7, 27)	33.7 (22.1, 51.5)	-	**<0.001**
TBIL (umol/L)	-	9.6 (7.4, 14.3)	11.7 (8, 17.5)	-	0.171
DBIL (umol/L)	-	3.4 (2.4, 4.7)	4.6 (3.5, 8.7)	-	**0.001**
BUN (mmol/L)	-	4.3±1.1	4.4 (3.7, 6.3)	-	0.439
sCr (umol/L)	-	66.1 (53.4, 78.2)	69.0 (52.8, 84.5)	-	0.470
GFR (ml/min/1.73m^2)	-	110.9 (99.1, 126.9)	94.2±21.8	-	**<0.001**

Data were presented as mean ± standard deviation or as median (interquartile range) for continuous variables and number (%) for categorical variables. The different characteristics between two groups were tested by one-way *t*-tests (normally distributed continuous variables), Mann Whitney nonparametric test (nonnormally distributed variables) or Chi-square test (categorical variables). A two-sided α of less than 0.05 was considered statistically significant

Abbreviations: SOFA score, sequential organ failure assessment score; APACHE II score, acute physiology and chronic health evaluation II score; WBC, white blood cells; CRP, C-reactive protein; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; BUN, blood urea nitrogen; sCr, serum creatinine; GFR, glomerular filtration rate.

Table 2. Correlations between laboratory parameters and the plasma levels of cfDNA, MPO-DNA, C3 and C5 in COVID-19 patients.

Parameter	cfDNA		MPO-DNA		C3		C5	
	R	*P*	R	*P*	R	*P*	R	*P*
Immunological parameters[a]								
WBC (×10^9/L)	0.367	**<0.001**	0.183	0.036	0.190	0.037	0.102	0.265
Neutrophil (×10^9/L)	0.439	**<0.001**	0.305	**0.001**	0.318	**0.001**	0.129	0.159
CRP (U/L)	0.374	**<0.001**	0.065	0.515	0.194	0.040	0.205	0.029
Coagulation parameters[b]								
D-dimer (g/L)	0.347	**0.007**	0.2442	0.043	0.240	0.024	0.139	0.196
PT (s)	0.615	**<0.001**	0.073	0.484	-0.0043	0.686	0.139	0.190
FDP (μg/mL)	0.474	**<0.001**	0.316	**0.002**	0.300	**0.005**	0.068	0.526
PTA (%)	-0.585	**<0.001**	-0.0725	0.4851	0.041	0.704	-0.148	0.164
INR	0.588	**<0.001**	0.073	0.480	-0.042	0.695	0.144	-.175
Tissue damage parameters[a]								
LDH (U/L)	0.379	**<0.001**	0.306	**0.003**	0.162	0.100	0.143	0.149
DBIL (μmol/L)	0.496	**<0.001**	0.009	0.367	0.303	**0.004**	-0.034	0.755
TBIL (μmol/L)	0.360	**<0.001**	0.054	0.599	0.304	**0.005**	-0.072	0.507
CK(U/L)	0.309	**<0.001**	0.308	**0.004**	0.074	0.506	-0.104	0.349
GFR (g/L)	-0.389	**<0.001**	-0.187	0.165	-0.333	0.002	-0.129	0.236

Correlations were calculated by Spearman correlation analysis (r). a, case number = 130; b, case number = 60.

Abbreviations: WBC, white blood cells; CRP, C-reactive protein; PT, thrombin time; FDP, fibrin(-ogen) degradation products; PTA, prothrombin activity; INR, international normalized ratio; LDH, lactate dehydrogenase; DBIL, direct bilirubin; TBIL, total bilirubin; CK, creatine kinase; GFR, glomerular filtration rate.

Figures

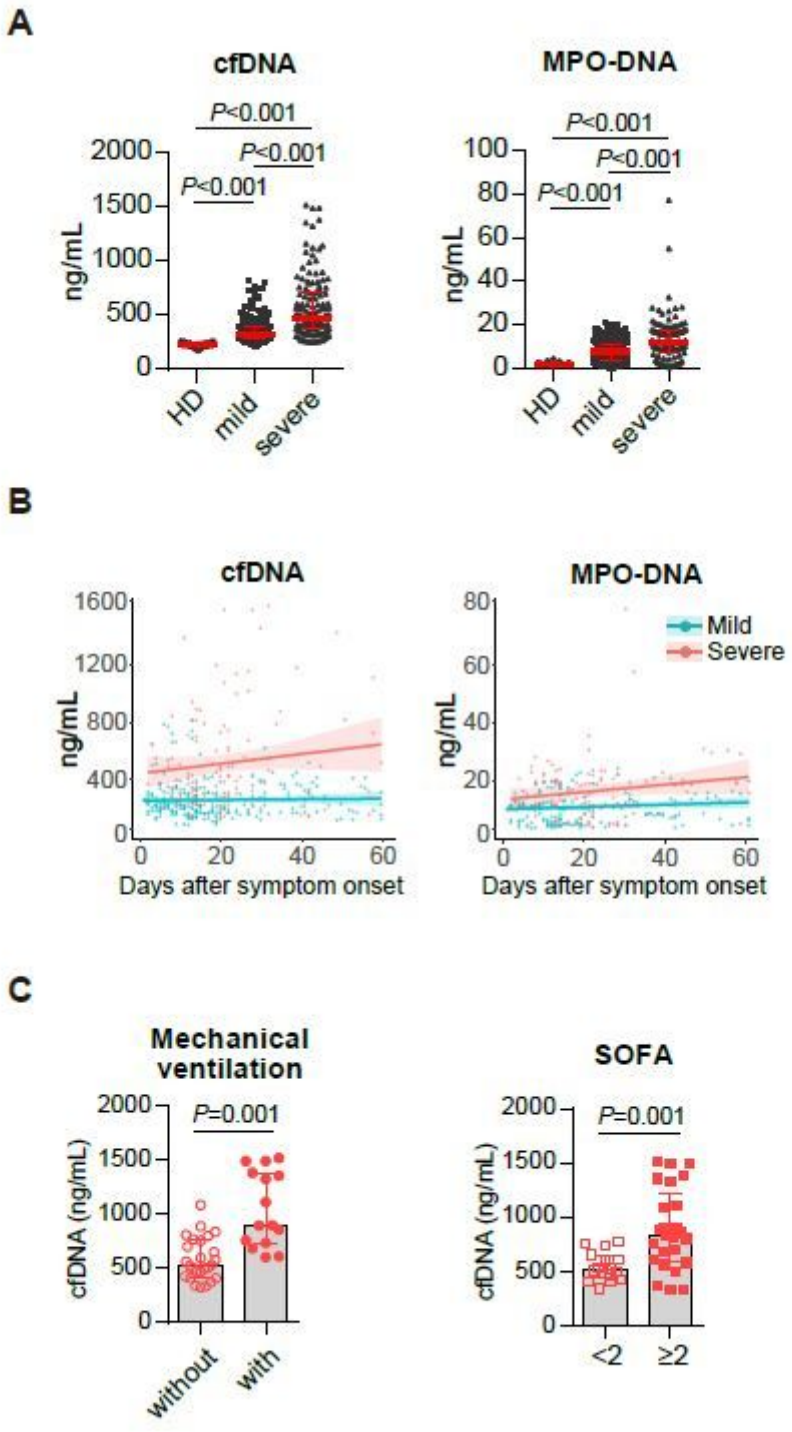

Figure 1

The plasma levels of NETs were associated with severity of COVID-19. a Plasma levels of cfDNA and MPO-DNA. HD, n = 25; mild, n = 94; severe, n = 41. b Longitudinal dynamics of cfDNA and MPO-DNA in COVID-19 patients with 60 days of hospitalization. Confidence interval at 95% was indicated as a colored shadow. c The cfDNA levels in the patients with/without requirement of mechanical ventilation (without, n = 12; with, n = 15), and the patients with different SOFA scores (< 2, n = 22; ≥ 2, n = 19) who were

admitted to ICU. Data are presented as median (interquartile range). P values were obtained by Mann-Whitney U test or Student t test.

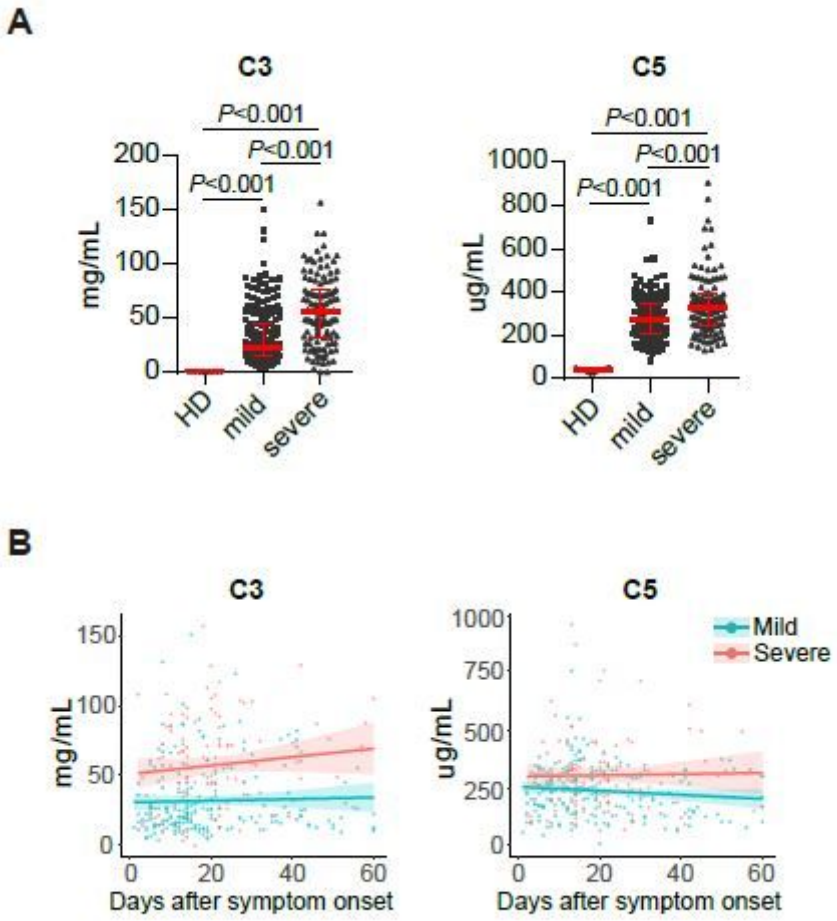

Figure 2

The plasma levels of complement C3 and C5. a Plasma levels of C3 and C5. HD, n = 25; mild, n = 94; severe, n = 41. b Longitudinal dynamics of C3 and C5 in COVID-19 patients with 60 days of hospitalization. Confidence interval at 95% was indicated as a colored shadow. P values were obtained by Mann-Whitney U test or Student t test.

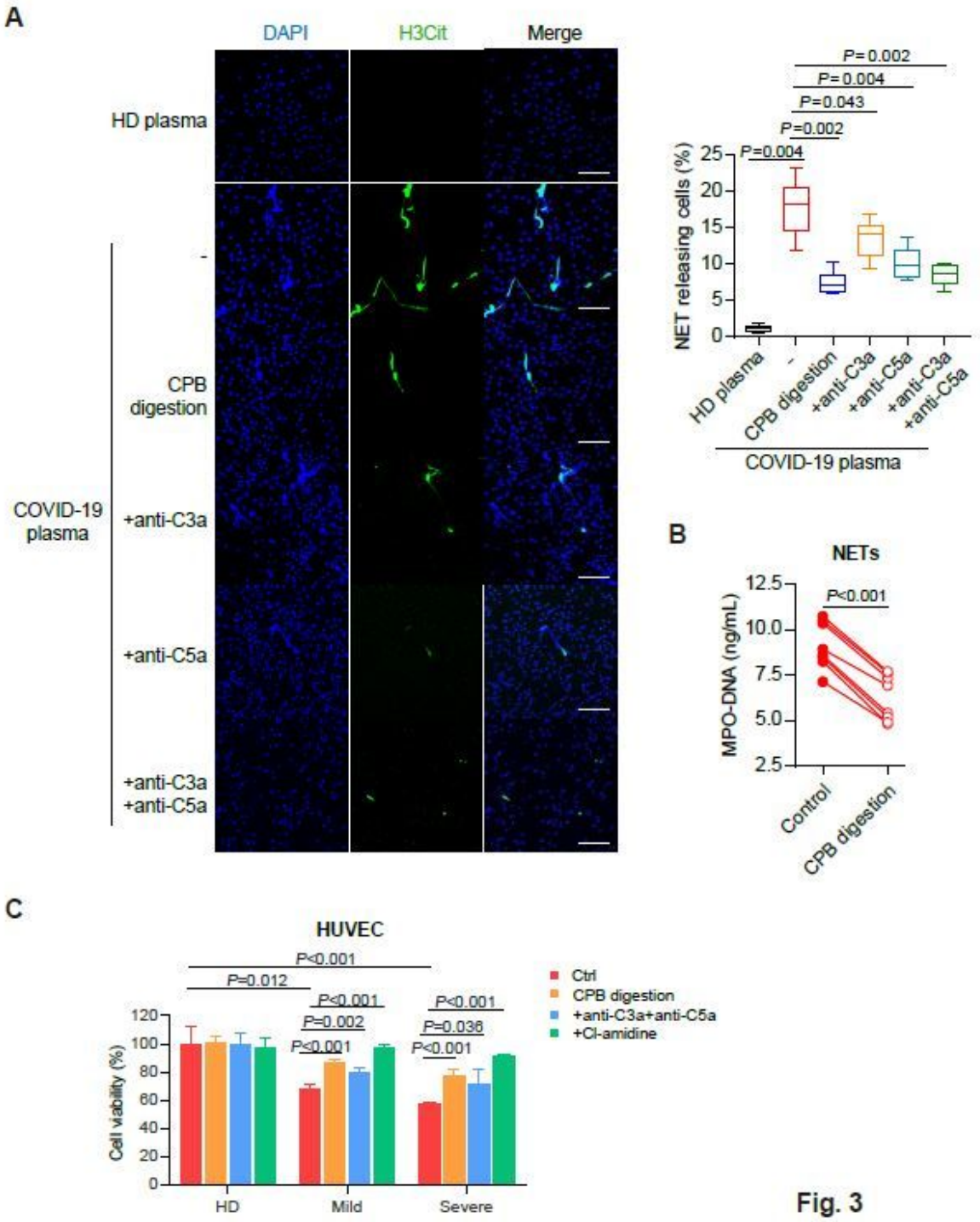

Fig. 3

Figure 3

Anaphylatoxin-induced NET release was inhibited by recombinant CPB. a Immunofluorescent staining for NET formation. Neutrophils from HDs were cultured with plasma from HDs or severe COVID-19 patients with different treatment. The extracellular histone of NETs was stained with anti-histone H3 citrulline (H3Cit) antibody (green) and DNA was stained with DAPI (blue). Figure shows a representative image from 1 of 3 patients. Scale bar = 100 μm. The percentage of NETs was calculated as an average of 5-10

fields normalized to the total number of neutrophils, and results are expressed as mean ±SEM. b NET release as indicated by MPO-DNA concentration in the media. c Cell viability of HUVEC cultured with NET-conditioned medium was measured by CCK-8 assay. Cells cultured with HD plasma-conditioned medium were taken as control and normalized as 100%. Data are presented as mean ±SEM. Data are presented as median (interquartile range). P values were obtained by Mann-Whitney U test or Student t test.