SUPPLEMENTAL METHODS

1.1 Serum and plasma collection

To isolate serum, venous blood was collected in appropriate vacutainer tubes (BD Vacutainer® Plus Plastic Serum Tubes, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). After centrifugation at 500x g for 15 min, the serum was collected.

Venous blood collected in BD Vacutainer® citrate tubes was used to isolate either platelet-poor plasma (PPP) or platelet-rich plasma (PRP). For PPP, tubes were centrifuged at 500x g for 15 min. For PRP, tubes were centrifuged at 150x g for 10 min. In both cases, serum/plasma samples were stored at -80°C until analyzed (1,2).

1.2 Neutrophil isolation

Peripheral blood neutrophils were isolated from heparinized blood by Histopaque double-gradient density centrifugation (11191 and 10771, Sigma-Aldrich, St Louis, MO, USA; 30 min, at 700x g, at 20-25°C) according to the manufacturer's instructions (3). The neutrophils were then washed once with phosphate-buffered saline (PBS-1x) and centrifuged for 10 min at 200x g before being cultured. The cell purity was ≥98%.

1.3 Human aortic endothelial cell culture

Human aortic endothelial cells (HAEC) were purchased from PromoCell (C-12271). The cells were cultured in 5% CO_2 at 37°C in endothelial cell growth medium MV2 (C-22020, PromoCell, Heidelberg, Germany) and passaged after reaching confluence by using trypsin/EDTA. Only cells from passages 3–6 were used for this study.

1.4 Stimulation and inhibition studies

1.4.1 Neutrophils isolated from either patients or healthy individuals ("control neutrophils") were cultured at 37^{o}C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) medium (21875, Thermo Fisher Scientific, Carlsbad, SA, USA) supplemented with 2% heterologous healthy donor serum.

To reproduce the ex vivo findings, control neutrophils were stimulated with PRP, PPP or serum derived from patients with COVID-19 ("COVID-19 serum") at a final concentration of 5% in RPMI.

In view of our first findings suggesting that the generation of TF-expressing NETs in SARS-CoV-2 infection is a "double-hit phenomenon" requiring both platelets and a disease-related inflammatory environment, subsequent inhibition studies were performed in the presence of PRP. Therefore, to block protease-activated receptor-1 (PAR-1) signaling, control neutrophils were pre-treated with the FLLRN peptide (500 mM, AS-60678, Anaspec, Fremont, California, USA) for 30 min (1). To inhibit thrombin, PRP was treated with dabigatran (provided by Dr Ashley Goss - Boehringer Ingelheim International GmbH, Ingelheim am Rhein, Germany) for 30 min (1). To inhibit the autophagic machinery, control neutrophils were pre-incubated for 30 min with a late-stage autophagy inhibitor, hydroxychloroquine sulfate (HCQ; 50μM, H0915, Sigma-Aldrich) (2). To evaluate the role of complement in COVID-19 pathogenesis, control neutrophils were pre-treated for 30 min at 10 μM with a specific C5a receptor antagonist (C5aRa/PMX-53), the cyclic hexapeptide acetylated phenylalanine–[ornithyl-proline-(D)cyclohexylalanine-tryptophyl-arginine] (Ac-F[OP(D)Cha-WR])), to attenuate C5a signaling. An inactive, scrambled peptide based

on the PMX-53 sequence was used as a negative control and showed no effect, as expected (4,5). The cyclic hexapeptide C5aRa/PMX-53 and the scrambled control peptide were synthesized by Fmoc solid-phase methodology and purified by RP-HPLC as described previously (5).

In order to study the activation of the complement cascade upstream of C5 and to further evaluate its role in TF induction we developed a serum co-incubation system. Since complement had been found to be fully activated in COVID-19 patient-derived serum, control serum (as a source of non-activated complement) was pretreated (or left untreated) for 30 min with the compstatin analog Cp40 [D]Tyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH2 at 20 μM to block C3 activation. This Cp40-treated (or untreated) serum was then incubated with COVID-19-derived serum at a final concentration of 10%, for an additional 30 min in order to activate C3. Subsequently, this mixed serum was used to stimulate control neutrophils, being compared to COVID-19 serum alone. An inactive, scrambled peptide based on the Cp40 sequence served as the negative control, and did not exhibit any effect (6). Cp40 was synthesized by Fmoc solid-phase methodology and purified by RP-HPLC as described previously (6).

In all in vitro stimulations, control neutrophils were cultured for 60 min or 3 h, in order to study the expression of TF (mRNA levels) and to assess NET formation, respectively.

1.4.2 To investigate the cross-talk between the platelet/neutrophil/TF axis and endothelial cells, HAEC were treated with PRP (5%) or NET structures (0.5 μg DNA/ml) plus 1% control plasma (7). NET structures were generated in vitro by treating control neutrophils with PRP derived from four COVID-19 patients ("COVID-19 PRP"). NETs generated from control neutrophils after incubation with

phorbol 12-myristate 13-acetate (PMA; 40ng/ml, P1585, Sigma-Aldrich), a generic inducer of NETs, were used as a positive marker (8). For mRNA studies, HAEC were analyzed after 3 h of incubation with PRP, NET structures, or PMA-treated NETs at 37°C. For thrombin-antithrombin (TAT) complex ELISAs, endothelial cells were treated for 6 h, and cell culture supernatants were then collected. Before TAT assay, the supernatants were centrifuged to remove debris.

Of note, the concentrations and time points used to test neutrophils and HAEC were optimized before the experiments. All substances used in this study were endotoxin-free, as determined by a Limulus amebocyte assay (E8029, Sigma-Aldrich).

1.5 Immunofluorescent staining of isolated peripheral neutrophils

Isolated peripheral neutrophils were seeded onto lysine-coated glass coverslips (Neuvitro; H-12-1.5-PDL) to evaluate their capacity to form NETs. The neutrophils were fixed with 4% paraformaldehyde for 30 min at room temperature. Nonspecific binding sites were blocked with 6% normal goat serum (31872, Invitrogen, Carlsbad, SA, USA). The samples were stained using a mouse anti-TF monoclonal antibody (mAb) (1:200 dilution, no.4509, clone IIID8, Sekisui Diagnostics, Burlington, Massachusetts, USA) or a rabbit anti-neutrophil elastase (NE) mAb (1:200 dilution, sc-25621, polyclonal-rabbit IgG, Santa Cruz Biotechnology, Inc, Dallas, Texas, USA). A rabbit IgG polyclonal antibody (Isotype control, ab171870, Abcam, Cambridge, UK) or a mouse IgG polyclonal (Isotype control, ab37355, Abcam) were used as controls. A polyclonal goat anti-rabbit IgG AlexaFluor 647 antibody (A27040, Invitrogen) or a polyclonal rabbit anti-mouse IgG AlexaFluor 488 antibody (A27023, Invitrogen) was utilized as the secondary antibody. DAPI (D9542, Sigma-Aldrich) was used for DNA staining (1,2,8).

Visualization was performed using either a confocal microscope (Spinning Disk Andor Revolution Confocal System, Ireland) with PLAPON 606O/TIRFM-SP, NA 1.45 and UPLSAPO 100XO, NA 1.4 objectives (Olympus) or a fluorescence microscope (OLYMPUS BX51) with a fixed NIKON camera (model DS-Fi1).

1.6 NET isolation

A total of 1.5×10^6 neutrophils were cultured in RPMI medium for 3 h (5% CO_2 at 37°C). The medium was then removed, and the cells were washed with pre-warmed RPMI medium. After vigorous agitation of the culture plate and centrifugation at 20× g for 5 min, NET structures were collected in the supernatant phase (9).

1.7 MPO/DNA complex ELISA

To quantify NETs, MPO/DNA complexes were measured in (a) ex vivo citrated plasma (PPP) obtained from COVID-19 patients and control individuals and (b) NET structures isolated from $1.5x10^6$ neutrophils. In brief, NETs were captured by human anti-MPO antibody (1:500 dilution, HM2164, clone 6G3-mouse IgG1, Hycult Biotech, Uden, Netherlands), and an anti-double-stranded DNA antibody was used for DNA detection (Cell Death Detection ELISA Kit, 11544675001, Merck, Kenilworth, New Jersey, USA). Absorbance was measured at 405 nm (10,11).

1.8 Thrombin-anti-thrombin (TAT) complex ELISA

The thrombin concentration was measured in: (a) ex vivo citrated plasma from COVID-19 patients and control individuals, (b) NET structures isolated from $1.5x10^6$ neutrophils and (c) supernatants (contain 1% control plasma) collected from HAEC cultures. The TAT complex concentrations were determined according to the

manufacturer's instructions (ET1020-1, Assaypro, St Charles, MO, USA). In the case of NET structures, NETs were introduced, at a final concentration of 20%, in citrated PPP isolated from control individuals, and incubated for 10 min at 37^{o} C. After incubation, they were immediately placed on ice to stop further thrombin activation (1).

1.9 Human terminal complement complex (TCC) ELISA

Human soluble terminal complement complexes (sTCC), i.e. C5b-9 complexes, were measured in ex vivo citrated plasma (PPP) from COVID-19 patients and control individuals by using a commercially available ELISA kit (HK328-02, Hycult Biotech).

1.10 RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (RT-qPCR)

RNA isolation, cDNA synthesis and RT-qPCR were conducted as described previously (1,2,8). The expression of TF was examined in neutrophils, using GAPDH as an internal control gene. To evaluate the activation of HAEC, the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) was examined. TF expression was also studied in HAEC. To normalize the expression of the abovementioned genes in HAEC, ribosomal protein L13a (RPL13a) was used as an internal control gene. Further details regarding the primers and conditions of qPCR used are given in Supplemental Table S3. The data were analyzed using the $2^{-\Delta\Delta Ct}$ mathematical model (12).

SUPPLEMENTAL FIGURES

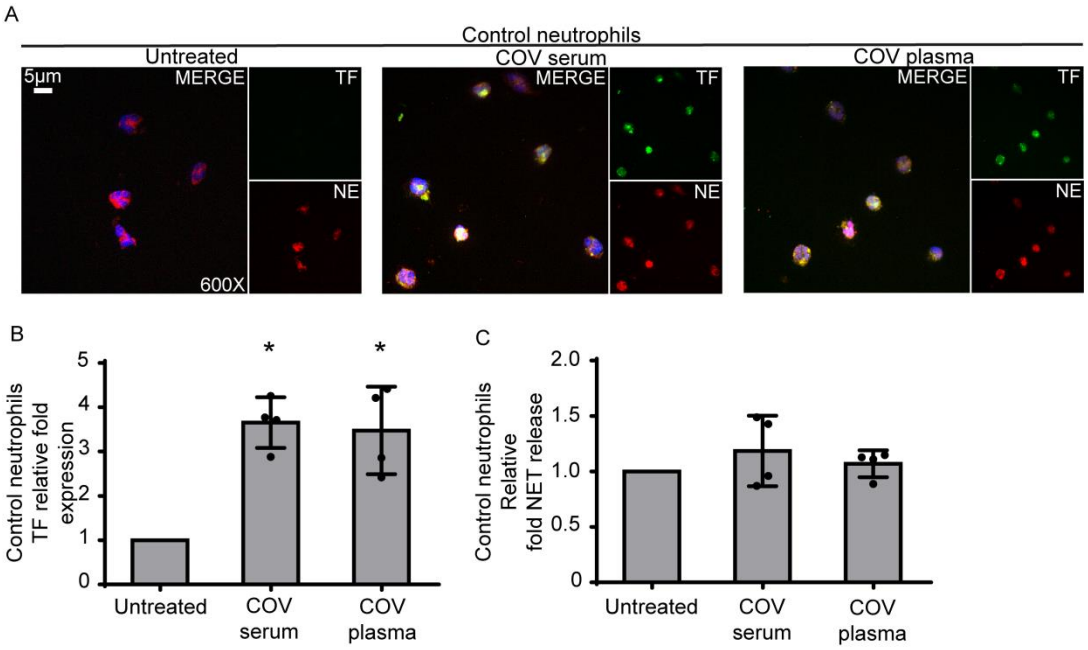

Figure S1. Tissue factor (TF) in control neutrophils treated with serum or plasma from COVID-19 patients.

A) Confocal fluorescence microscopy showing tissue factor (TF)/neutrophil elastase (NE) staining in control neutrophils treated with COVID-19-derived serum ("COV serum") or COVID-19-derived plasma ("COV plasma"). A representative example of four independent experiments is shown. Original magnification: x600, scale bar: 5 µm. Blue: DAPI, green: TF, red: NE. **B)** Relative fold expression of TF mRNA in control neutrophils treated with COV serum or COV plasma. Data are from four independent experiments (mean ± SD). **C)** Myeloperoxidase (MPO)-DNA complex levels in NET structures isolated from control neutrophils treated with COV serum or COV plasma. Data are from four independent experiments (mean ± SD). All conditions were compared to control/untreated, and statistical significance at $p < 0.05$ is indicated by *. B, C: Friedman's test. Supplemental Table S2 summarizes these in vitro experiments.

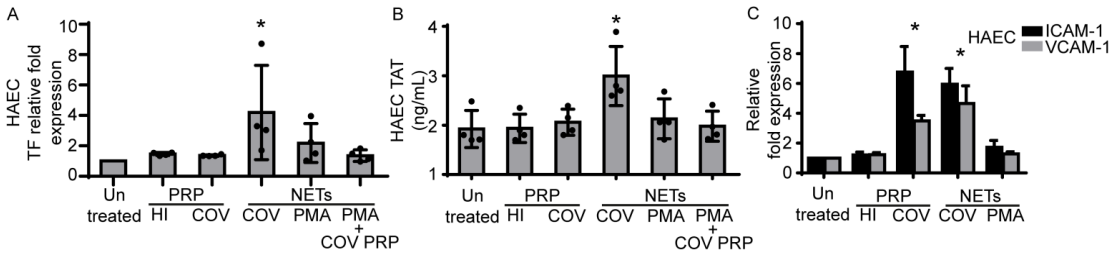

Figure S2: COVID-19 thromboinflammation in human aortic endothelial cell (HAEC) cultures.

A) Relative fold expression of tissue factor (TF) mRNA in HAEC treated with: PRP from healthy individuals (HI), PRP from COVID-19-suffered patients (COV PRP), NET structures from control neutrophils after stimulation with COV PRP, or NET structures from control neutrophils after stimulation with PMA (PMA-induced NETs) or PMA-induced NETs mixed with COV PRP. Data are from four independent experiments (mean ± SD). **B)** Thrombin-antithrombin (TAT) complex levels in control plasma treated with HAEC supernatants stimulated as described in (A). Data are from four independent experiments (mean ± SD). **C)** Relative fold expression of mRNA for intercellular adhesion molecule-1 (ICAM-1)/vascular cell adhesion molecule-1 (VCAM-1) in HAEC stimulated as described in (A). Data are from four independent experiments (mean ± SD). All conditions were compared to control/untreated, and statistical significance at $p < 0.05$ is indicated by *. A-C: Friedman's test. These in vitro experiments are summarized in Supplemental Table S2.

SUPPLEMENTAL TABLES

Supplemental Table S1. Patient characteristics

Patient No	Sex	Age range (decade)	Disease status
1*	F	63	Severe
2	M	84	Moderate
3	F	55	Moderate
4	M	78	Critical
5	M	72	Critical
6	F	81	Moderate
7*	M	49	Severe
8	M	53	Moderate
9	F	53	Moderate
10	M	55	Moderate
11	F	45	Moderate
12	F	58	Moderate
13	M	63	Moderate
14	F	74	Moderate
15	F	53	Moderate
16	M	74	Critical
17	F	62	Moderate
18	F	86	Severe
19	M	63	Moderate
20	F	61	Moderate
21	M	55	Severe
22	F	75	Moderate
23*	F	50	Severe
24	M	26	Severe
25*	M	65	Severe

M: male, F: female
Moderate cases: clinical symptoms associated with dyspnea and radiological findings of pneumonia on thoracic X-ray or/and CT scan, and requiring a maximum of 3 L/min of oxygen.
Severe cases: respiratory distress requiring more than 3 L/min of oxygen, with no other organ failure.
Critical cases: respiratory failure requiring mechanical ventilation, with shock and/or other organ failure requiring an intensive care unit (ICU) admission.
The disease status of COVID-19 patients was classified based on the adaptation of the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance suggested by Hadjadj et al. (13).
All patients were analyzed 8 to 15 days after disease onset. At the time of sampling, none of the patients were undergoing chemotherapy, radiotherapy, or an immunomodulatory treatment such as tocilizumab, anakinra or corticosteroids.
*Patient whose biological material was used for ex vivo confocal immunofluorescence microscopy experiments and in vitro stimulation studies

Supplemental Table S2. Summary of the in vitro experiments

	Cell type	Treatment with	Outcome
Figure 2	Control neutrophils	None	TF (nsa) NETs (nsa) TAT (nsa)
		COVID PRP	TF (↑) NETs (↑) TAT (↑)
		COVID PRP plus dabigatran	TF (nsa) NETs (nsa) TAT (nsa)
		COVID PRP plus FLLRN	TF (nsa) NETs (nsa)
		COVID PRP plus HCQ	TF (↑) NETs (nsa) TAT (nsa)
		COVID PRP plus C5aRa	TF (nsa) NETs (nsa) TAT (nsa)
Figure 3	Control neutrophils	Healthy serum	TF (nsa)
		Healthy serum mixed with COVID serum	TF (↑)
		Healthy serum treated with Cp40 and mixed with COVID serum	TF (nsa)
		COVID serum	TF (↑)
		COVID serum treated with Cp40	TF (↑)
Figure S1	Control neutrophils	None	TF (nsa) NETs (nsa)
		COVID serum	TF (↑) NETs (nsa)
		COVID plasma	TF (↑) NETs (nsa)
Figure S2	HAEC	None	TF (nsa) TAT (nsa) ICAM-1 (nsa) VCAM-1 (nsa)
		Healthy PRP	TF (nsa) TAT (nsa) ICAM-1 (nsa) VCAM-1 (nsa)
		COVID PRP	TF (nsa) TAT (nsa) ICAM-1 (↑) VCAM-1 (↑)
		COVID PRP-induced NETs	TF (↑) TAT (↑) ICAM-1 (↑) VCAM-1 (↑)
		PMA-induced NETs	TF (nsa) TAT (nsa) ICAM-1 (nsa) VCAM-1 (nsa)
		PMA-induced NETs mixed with COVID PRP	TF (nsa) TAT (nsa)

nsa: no significant alteration; ↑: significant upregulation/increase

Supplemental Table S3.

Sequence of Primers[1] and real-time RT-PCR conditions[2]

GENE	PRIMER	SEQUENCE OF PRIMERS	RT-PCR CONDITIONS
V-CAM	FRD:	5′ GACTCCGTCTCATTGACTTGC 3′	1. 52^{O}C for 5 min 2. 95^{O}C for 2 min 3. 35 cycles of: ➢ 95^{O}C for 15 sec ➢ 56 ^{O}C for 40 sec 4. 52 ^{O}C for 5 min 5. Melting curve analysis
	REV:	5′ CATTCGTCACCTTCCCATTCAG 3′	
I-CAM	FRD:	5′ AACCTTCCTCACCGTGTACTG 3′	
	REV:	5′ CTCCACCTGGCAGCGTAG 3′	
RPL13AF	FRD:	5′ GCCCTACGACAAGAAAAAGCG 3′	
	REV:	5′ TACTTCCAGCCAACCTCGTGA 3′	
TF	FRD:	5′ TTCQGTGTTCAAGCAGTGATTCC 3′	
	REV:	5′ ATGATGACCACAAATACCACAGC 3′	
GAPDH	FRD:	5′ GGGAAGCTTGTCATCAATGG 3′	
	REV:	5′ CATCGCCCCACTTGATTTTG 3′	

[1]Oligonucleotide primers were designed by Beacon Designer™ ver. 4.0

[2]Real-time PCR was performed using SYBR Green qPCR Master Mix (2x) gene expression master mix (Fermentas, St. Leon-Rot, Germany) on a Chromo4^{TM}Real-Time Detector (Bio-Rad, CA, USA).

SUPPLEMENTAL REFERENCES

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