PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

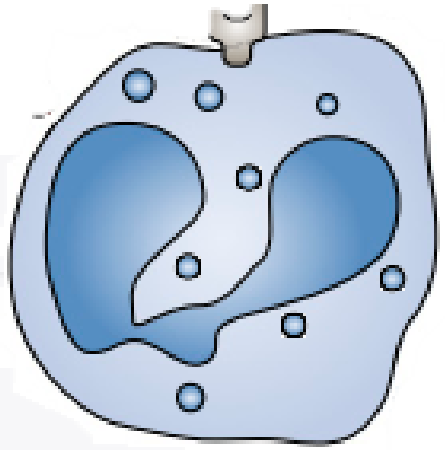
Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome

Elizabeth A. Middleton,[1,2] Xue-Yan He,[3] Frederik Denorme,[1] Robert A. Campbell,[1,2] David Ng,[3] Steven P. Salvatore,[4,5] Maria Mostyka,[4] Amelia Baxter-Stoltzfus,[4] Alain C. Borczuk,[4,5] Massimo Loda,[4,5] Mark J. Cody,[1,6] Bhanu Kanth Manne,[1] Irina Portier,[1] Estelle S. Harris,[2] Aaron C. Petrey,[1,7] Ellen J. Beswick,[2] Aleah F. Caulin,[8] Anthony Iovino,[6,8] Lisa M. Abegglen,[6,8] Andrew S. Weyrich,[1,2] Matthew T. Rondina,[1,2,9,10] Mikala Egeblad,[3] Joshua D. Schiffman,[1,6,8,*] and Christian Con Yost[1,6,*]

Outline

- Introduction
  - Neutrophils & Neutrophil extracellular traps (NETs)
- Materials & Methods
- Results
- Discussion

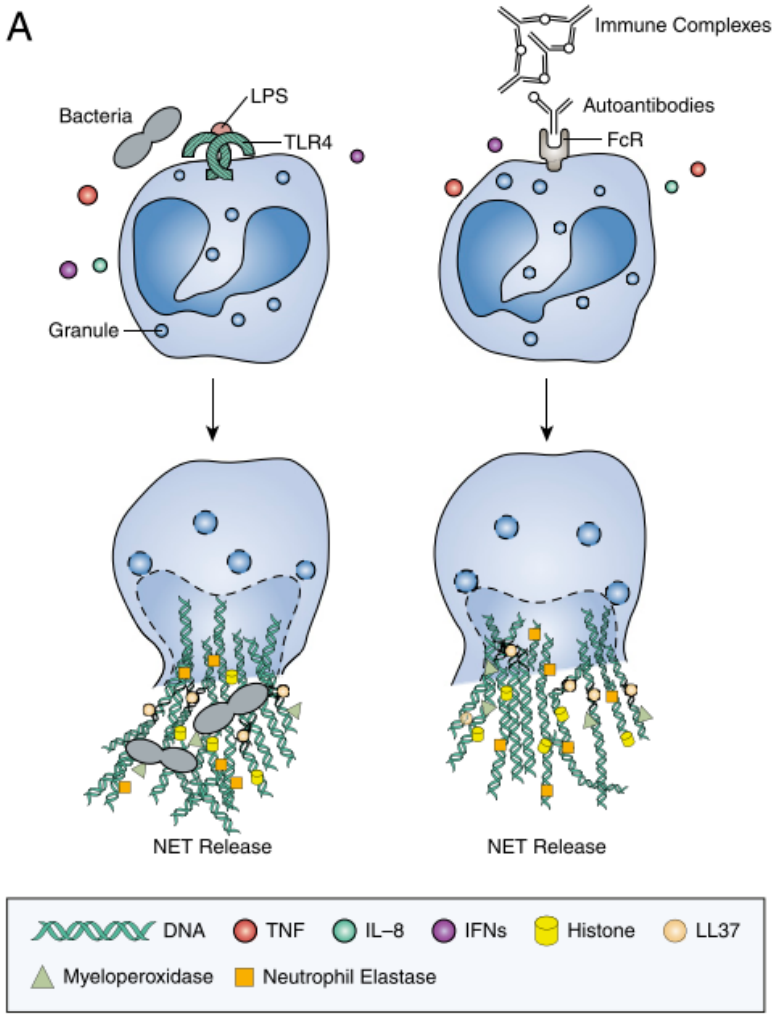
Neutrophils



- polymorphonuclear leukocyte (PMN)
- Most abundant granulocyte
- enriched cytoplasm with granules & secretory vesicles
- typically the first leukocyte recruited to inflammatory site
- capable of eliminating pathogens by multiple mechanisms

Adapted from Kaplan et al. Immunity. *J. Immunol.* **189**, 2689–2695 (2012)

Neutrophil Extracellular Traps (NETs)



Stimuli:

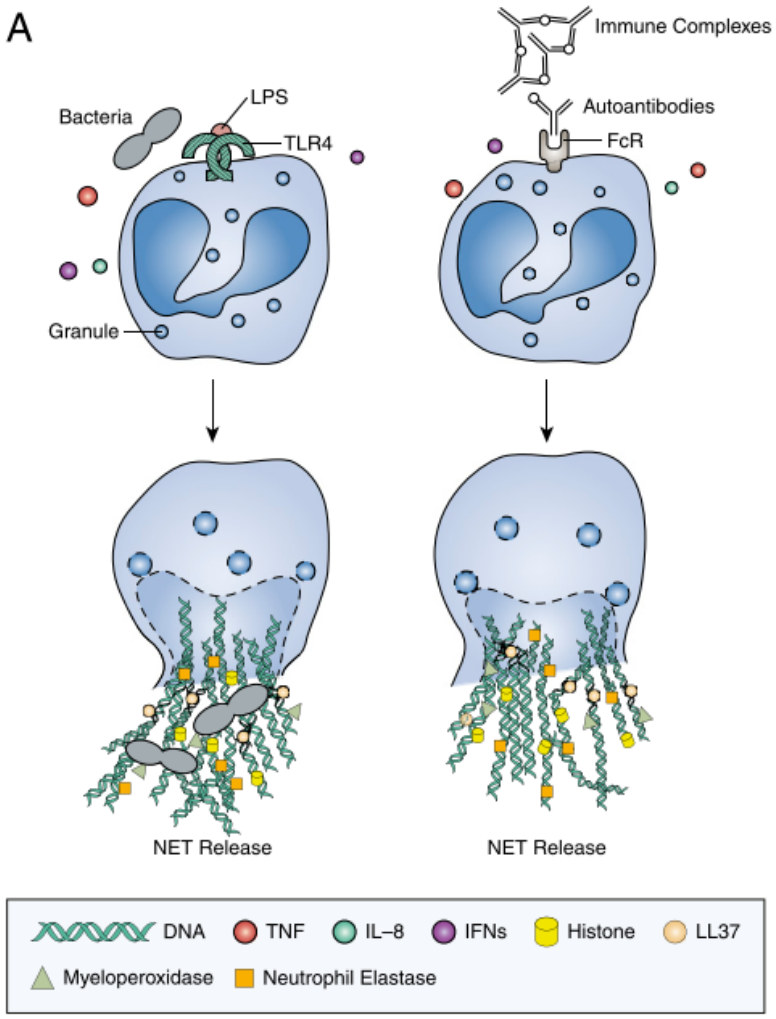
- Microbes & microbial products
- Immune complexes
- Autoantibodies
- A wide range of cytokines (e.g. IL-8, TNF, type 1 & 2 IFN)
- …

Binding via:

- FC receptors
- Complement receptors
- GPCRs
  - Formyl-peptide receptors
  - Chemoattractant receptors
- Adhesion receptors
  - selectins/selectin ligands
  - integrins
- Cytokine receptors
  - Type 1 & Type 2
  - IL1R family
  - TNFR family
- Innate immune receptors
  - TLRs (all except TLR3)
  - C-type lectins
  - NOD-like receptors
  - RIG-like receptors

Adapted from Kaplan et al. Immunity. *J. Immunol.* **189**, 2689–2695 (2012)

NETs



Microbe,
Inflammatory Stimulus,
Endogenous Inducer

ER → [Ca^{++}] ↑

PKC

$Gp91^{phox}$ + Rac2

NADPH Oxidase

ROS, NO ↑

Granule rupture

Nuclear envelope rupture

Mixing of nuclear, cytoplasmic, granular content

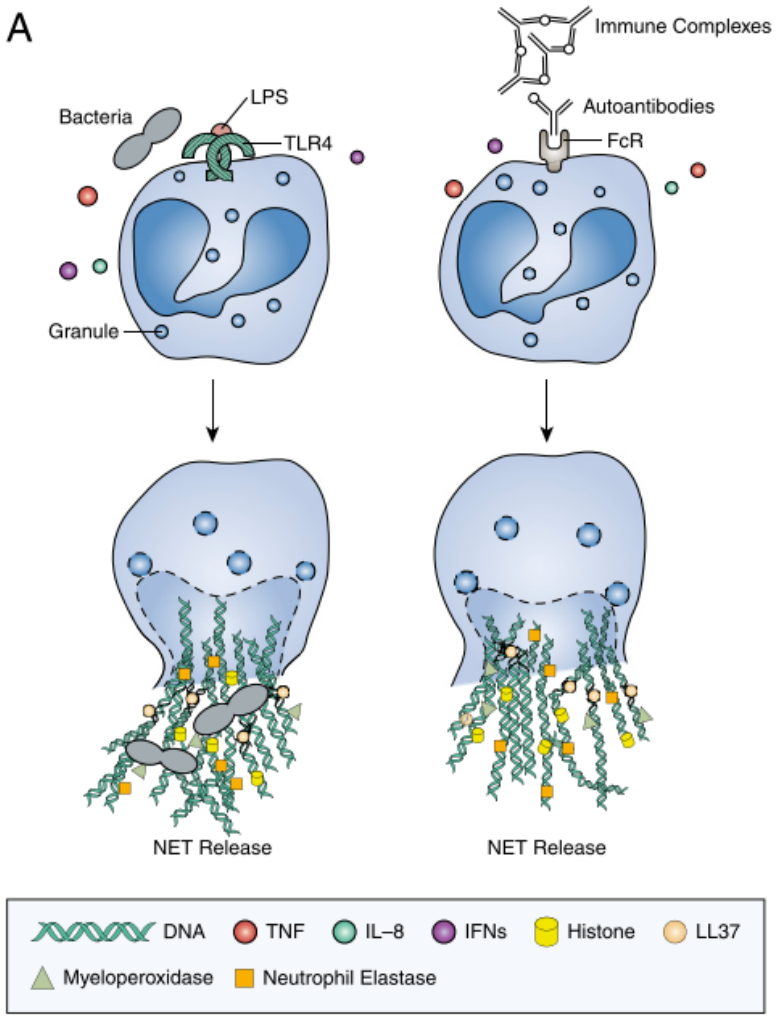
Histone deimination, Proteolytic cleavage

Chromatin decondensation

NET release

Adapted from Kaplan et al. Immunity. *J. Immunol.* **189**, 2689–2695 (2012)

NETs



Microbe,
Inflammatory Stimulus,
Endogenous Inducer

ER → [Ca^{++}] ↑

PKC

$Gp91^{phox}$ + Rac2

NADPH Oxidase

ROS, NO ↑

Granule rupture

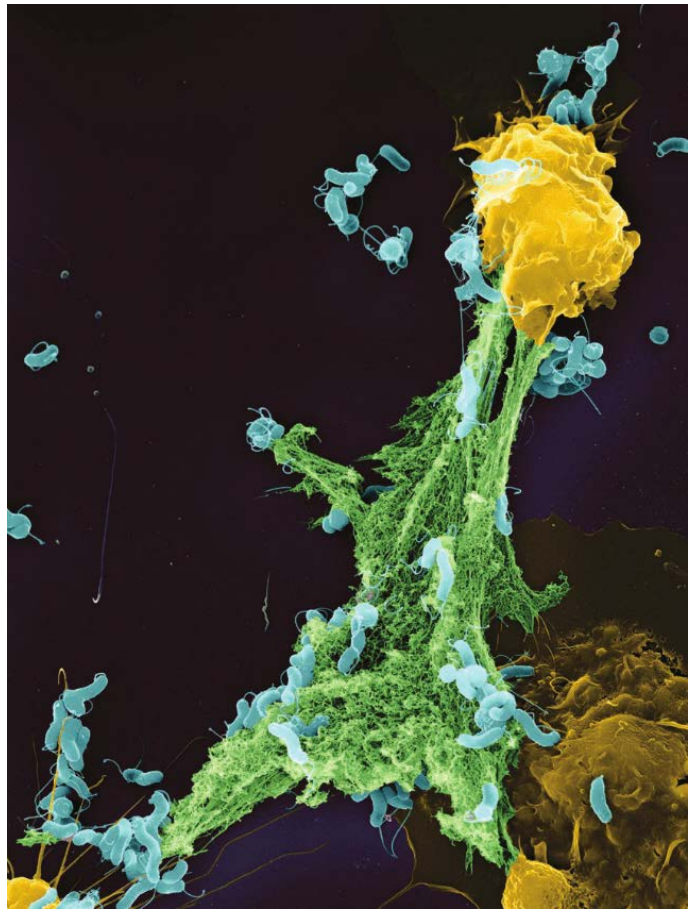
Nuclear envelope rupture

Mixing of nuclear, cytoplasmic, granular content

Histone deimination, Proteolytic cleavage

Chromatin decondensation

NET release



Adapted from Kaplan et al. Immunity. *J. Immunol.* **189**, 2689–2695 (2012)
Thålin et al. 2019, Arteriosclerosis, Thrombosis, and Vascular Biology, 39(9), 1724–1738.

Neutrophils & NETs

- Platelet triggered NETosis

  → may become dysregulated

  → NET mediated tissue damage,
  hypercoagulability,
  chronic inflammatory disease

Materials & Methods

Study design

- 33 COVID-19 patients
  + 17 age- & sex-matched healthy adults
- Patients admitted to hospital with respiratory symptoms and SARS-CoV2^{+} PCR test
- Investigation of pathogensis COVID-19-related sepsis, ARDS, thrombosis → incl. NET formation & platelet-neutrophil interactions

Study Patients

- Enrolement criteria
  - >18 years
  - Respiratory symptoms (cough, shortness of breath) or fever
  - Hospital admission
  - SARS-CoV2^{+} PCR test
  - Informed consent
- 5 convalescing COVID-19 patients
  - 3 not from enrolled cohort
  - 2 from cohort
  - Blood collection 4-6 weeks post positive PCR test

Materials & Methods

Clinical characteristics of healthy donors and hospitalized patients with COVID-19

Characteristics	Healthy donors (n = 17)	Hospitalized		*P*
		Non-ICU COVID-19 infection (n = 19)	ICU COVID-19 infection (n = 14)	
Age, mean ± SD, y	50.6 ± 17.6	48.2 ± 13.6	64.5 ± 13.7	.008
Male	50	52.6	57.1	.94
Hispanic/Latino/African American	11.8	31.6	42.9	.15
BMI (kg/m^2), mean ± SD		33.9 ± 9.6	30.5 ± 9.4	.43
Diabetes		31.6	57.1	.15
Hypertension		36.8	42.9	.74
Chronic lung disease		26.3	42.9	.33
SOFA score, mean ± SD		1.6 ± 1.3	4.6 ± 1.2	<.0001
ARDS		10.5	92.9	<.0001
Mechanical ventilation		0.0	50.0	<.0001
28-d survival		100	71.4	.011
WBC (k/uL), mean ± SD		6.1 ± 2.4	8.3 ± 2.3	.02
Platelet count (k/uL), mean ± SD		245 ± 107	244 ± 56	.97

Unless otherwise noted, data are percentages (%).

BMI, body mass index; SOFA, Sequential Organ Failure Assessment; WBC, white blood cell count.

Materials & Methods

Clinical course of autopsy patients who died from COVID-19 ARDS

Case 1: Older patient with multiple preexisting medical conditions
This 64-year-old male of Hispanic decent with diabetes, end-stage renal disease on hemodialysis, heart failure, and hepatitis C on ledipasvir/sofosbuvir therapy developed fever after presenting with respiratory distress to the emergency room. SARS-CoV-2 PCR from a nasopharyngeal swab obtained prior to his demise was positive. He declined medical intervention, including intubation, and died within 5 hours of presentation. There was no clinical evidence of sepsis in this patient, premortem bacterial cultures were negative, and autopsy was conducted within 5 hours of his death. Neutrophil infiltration, but without immunofluorescence testing for NETs or platelets, of this patient's autopsy lung sample has been published.[4]
Case 2: Elderly with preexisting medical conditions and ARDS
This 73-year-old male with chronic obstructive pulmonary disease and diabetes developed ARDS with an arterial oxygen saturation of 50%. He was intubated and treated empirically with ceftriaxone, azithromycin, and doxycycline for community-acquired pneumonia with negative blood cultures. His chest radiograph showed diffuse patchy airspace opacification. SARS-CoV-2 PCR from a nasopharyngeal swab was positive. The patient required mechanical ventilation and experienced acute renal failure (creatinine increased from 2.4 to 4.1 mg/dL). His white blood cell count increased as did his absolute neutrophil count. He was lymphopenic. He remained afebrile with a temperature maximum of 37.8°C. He expired on hospital day 5 from COVID-19–related ARDS.
Case 3: Elderly with multiple preexisting medical conditions and cardiac arrest
This 71-year-old male with hypertension, hyperlipidemia, coronary artery disease, and diabetes had cough and fever for several days prior to a witnessed sudden cardiac arrest at home. His wife was a known SARS-CoV-2^{+} household exposure with minimal symptoms, similar to the patient's initial presentation. Despite attempts at cardiopulmonary resuscitation by emergency medical personal in transit and in the emergency room, he expired. A SARS-CoV-2 nasopharyngeal swab was positive in the emergency room prior to his demise.

Materials & Methods

- MPO-DNA ELISA
  - Plasma and tracheal aspirate
  - Capture: Anti-human MPO primary antibody
  - Detection: anti-DANN primary antibody
- PMN isolation
  - EasySep Direct Human Neutrophil Isolation Kit (STEMCELL)
- Neutrophil granularity quantification (n=4)
  - Flow Cytometry
  - Anti-human CD66b-V450 and SSC

Materials & Methods

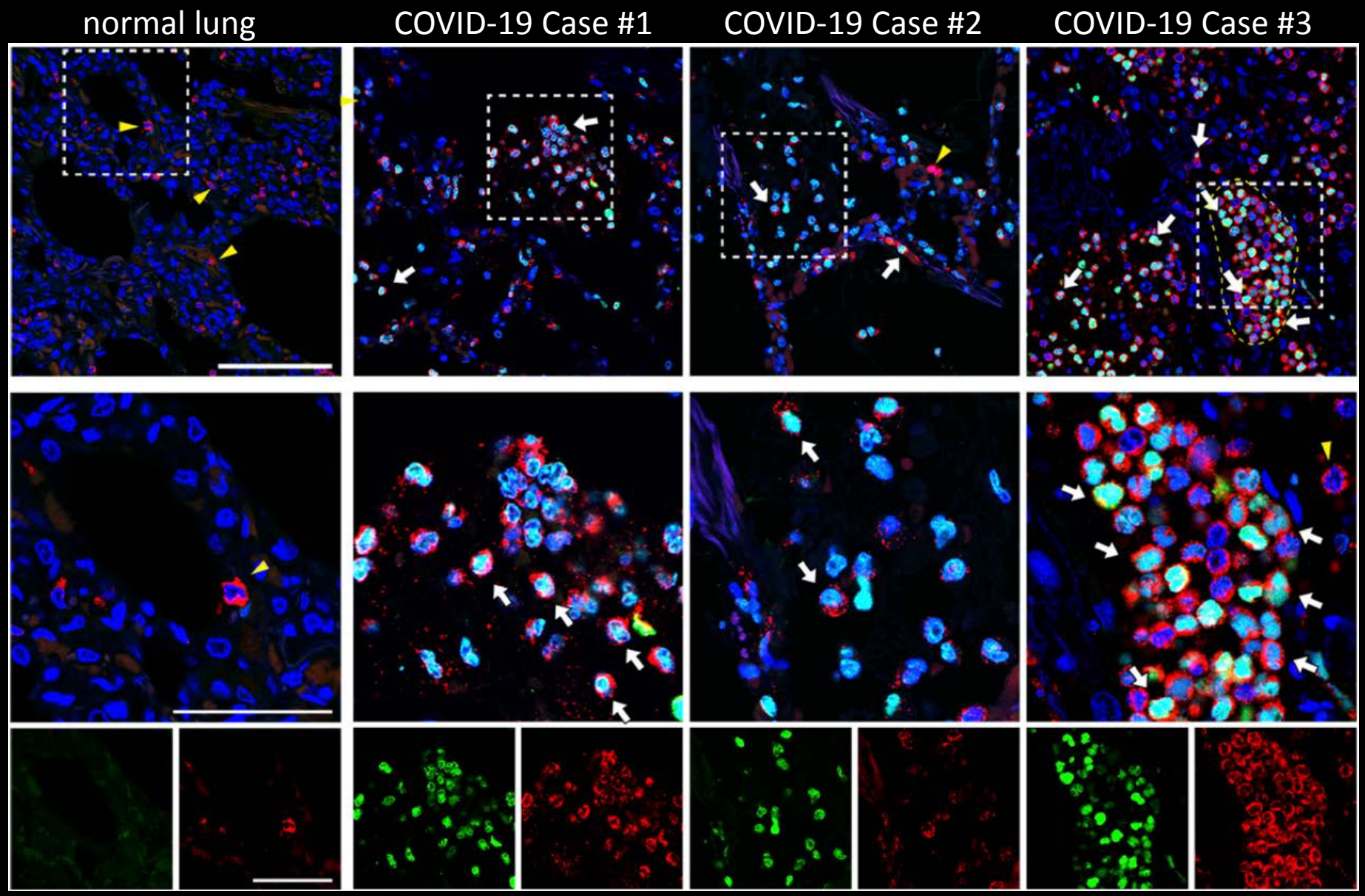
- Immunofluorescence staining for NETs and platelets
  - Paraffin-embedded autopsy lung specimens from 3 COVID-19 cases
  - Commercially available healthy tissue as control
  - Mouse-anti-human MPO
  - Rabbit-anti-human citH3
  - Mouse-anti-human PF4
- Plasma coagulation factor assays
  - ELISA: sPF4, RANTES, von Willebrand Factor (VWF) antigen, D-dimers

Materials & Methods

- Platelet-neutrophil aggregates
  - FACs
  - Platelets: CD41-APC
  - PMNs: CD66b-V450
- Cytokine array
  - Multiplex bead array for IL-8 & IL-6
- nNIF peptide synthesis
  - neonatal NET-inhibitory factor in umbilical cord blood
  - inhibits key terminal events in NET formation, including (PAD4) activity, nuclear histone citrullination, & nuclear decondensation

Increased plasma NETs correlate with increased COVID-19 severity

- Robust PMN infiltration in lung based on MPO staining
- Numerous citH3+ MPO+ PMNs
- Rare latticees of extracellular DNA



B

- Sign. Increase in plasma NET levels in
  - Non-intubated
  - (endotracheally) intubated
- Upon recovery: plasma NET levels decreased to similar levels as healthy donors

C

- Plasma NET levels sign. higher in non-survivors compared

D

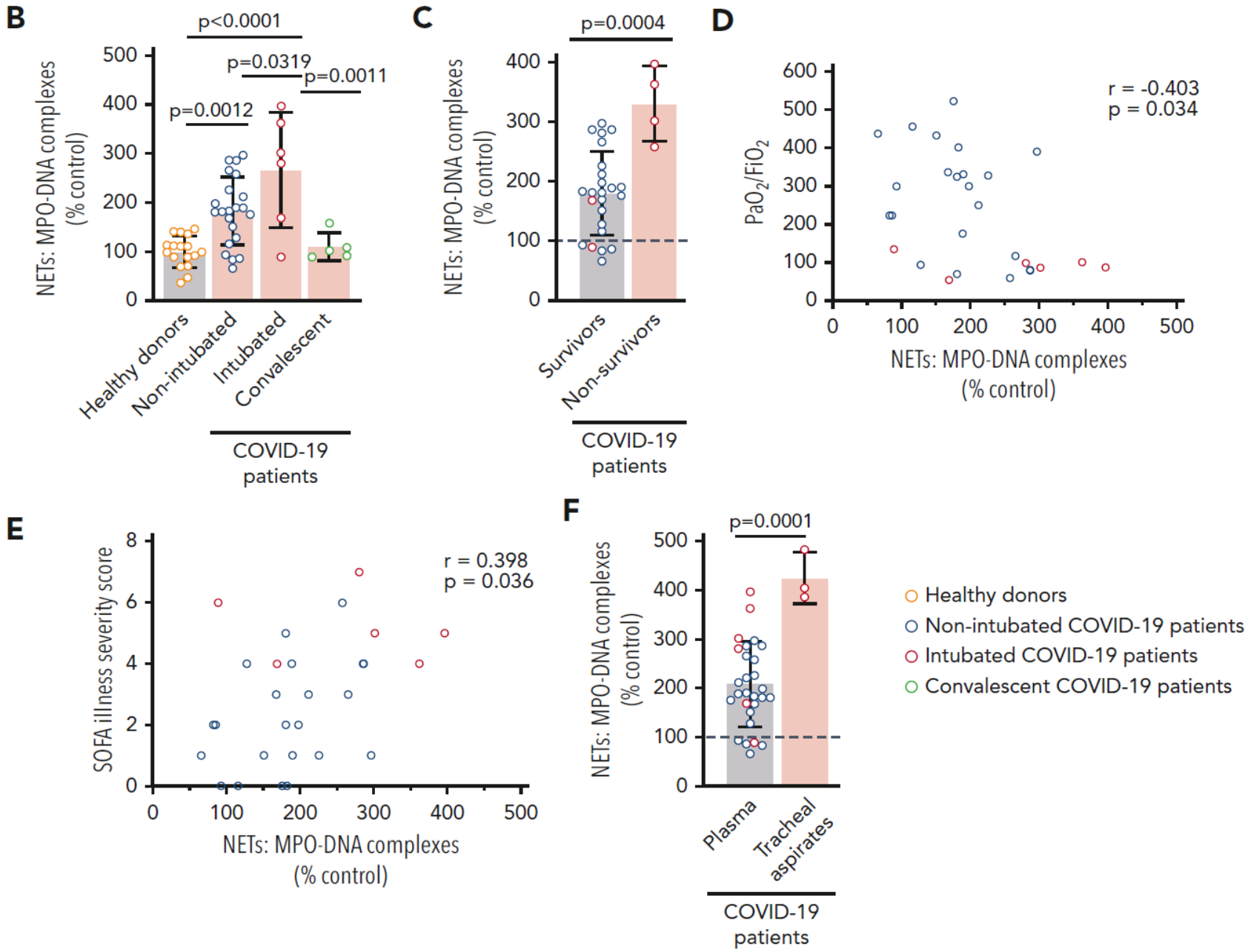
- PaO2/FiO2 varies inversely with plasma NETs

E

- Plasma NETs correlate directly with SOFA score

F

- Sign. Increased NET levels in tracheal aspirates



COVID-19 PMNs demonstrate increased activation and NETs at baseline

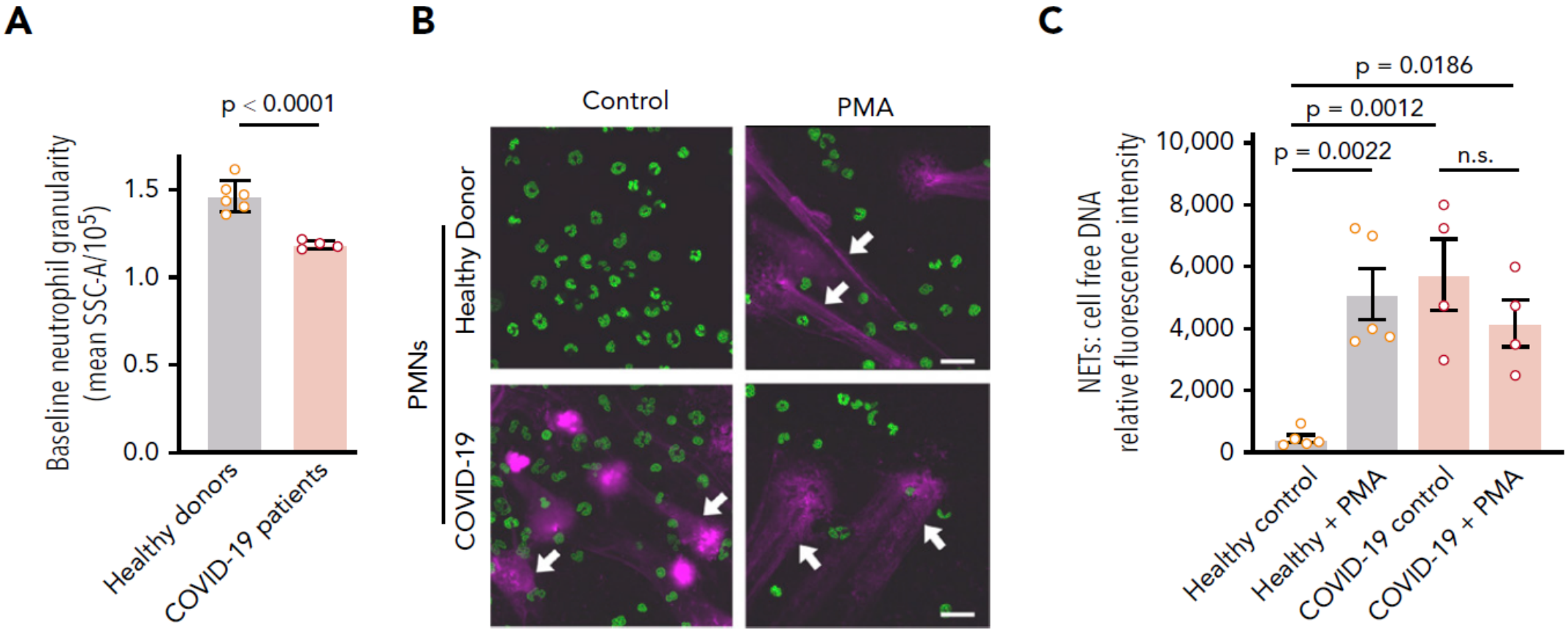
COVID-19 PMNs additionally fail to respond to NET-inducing stimuli with increased NET formation

A

- Sign. Decrease in PMN granularity in COVID-19 PMNs

B & C

- PMA failed to further increase NET formation



NETs associate with microthrombi formation & platelet deposition

A

- NETs localized in structures consistent with blood vessels
- Co-localization of citH3^{+} neutrophils with PF4^{+} platelets

B

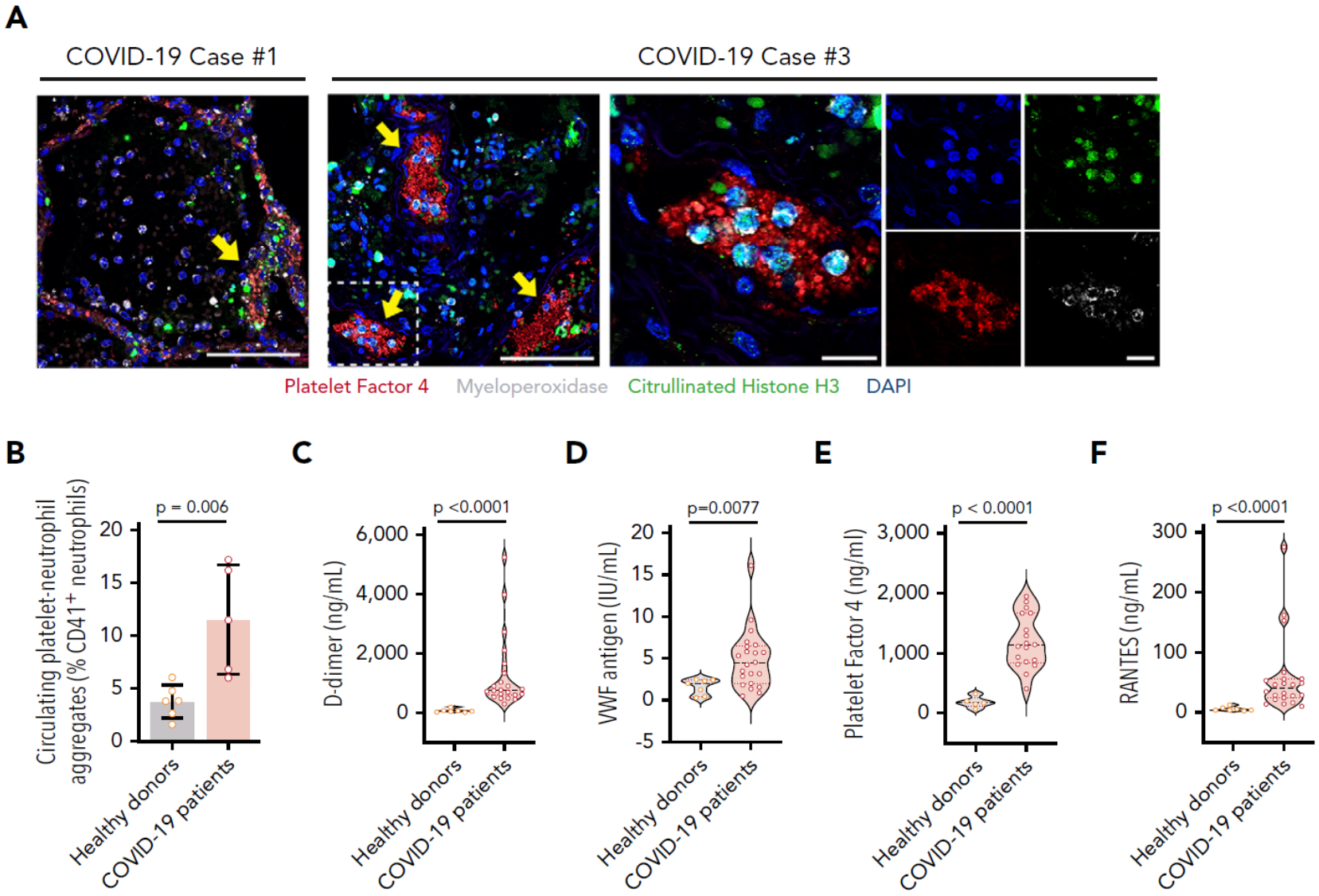
- Sign. Higher levels of circulating platelet-neutrophil aggregates in COVID-19 patients

C & D

- Soluble markers of thrombosis (D-dimer and VWF) sing. Elevated

E & F

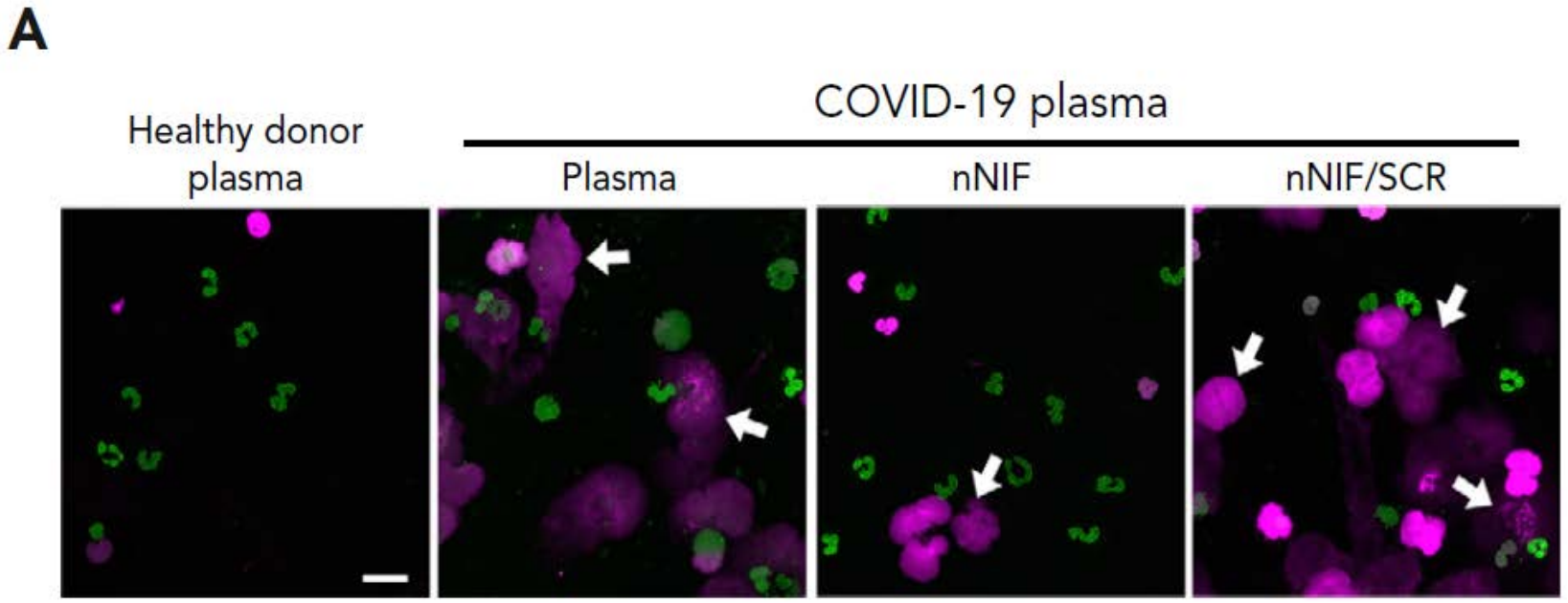
- Sign. Elevated levels of soluble platelet-derived factors (triggering NETosis); PF4, RANTES (CCL5)

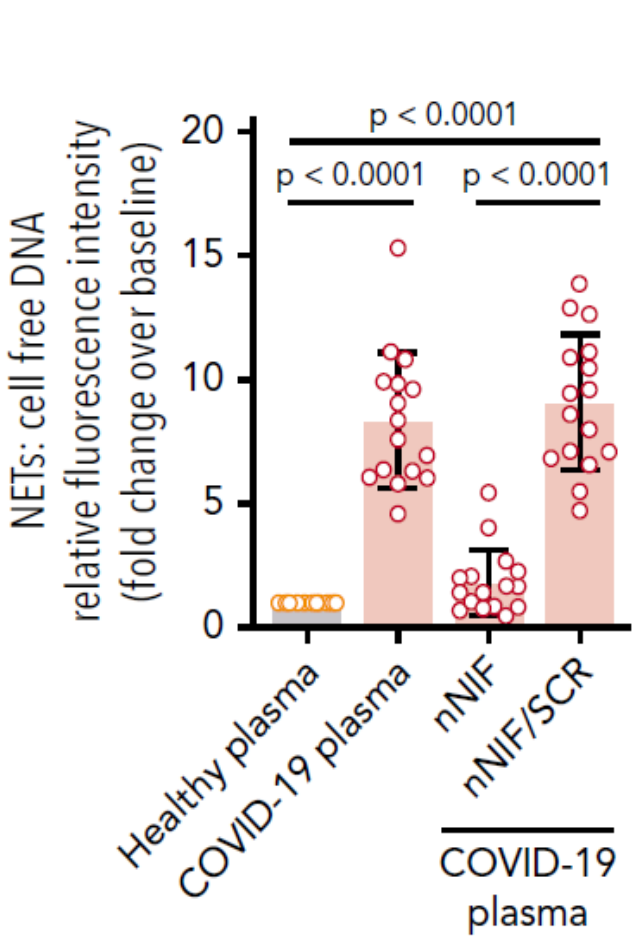


COVID-19 plasma induces NET formation in healthy PMNs

nNIF blocks NETs induced by soluble factors in plasma from COVID-19 patients

- NET formation of healthy PMNs upon incubation with COVID-19 plasma
- nNIF inhibits COVID-19 plasma-induced NET formation





Discussion

Discussion

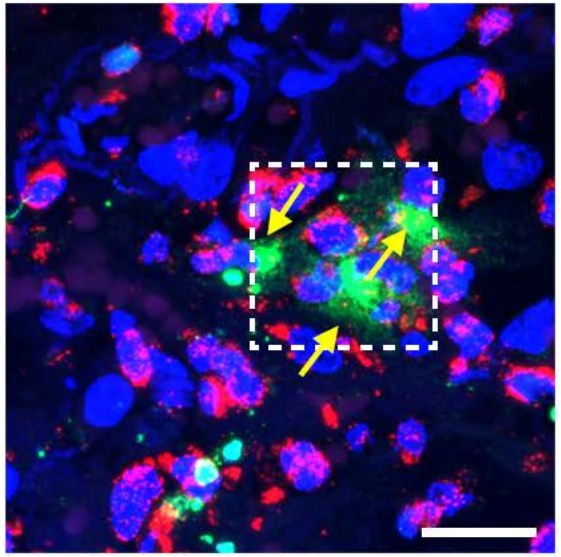
- Highest levels of circulating NETs in COVID-19 patients with endotracheal intubation
- Infiltration of platelet colocalization with citH3+ neutrophils in pulmonary microthrombi (patients died from COVID-19)
- 50-fold increase in NET release in COVID-19 PMNs

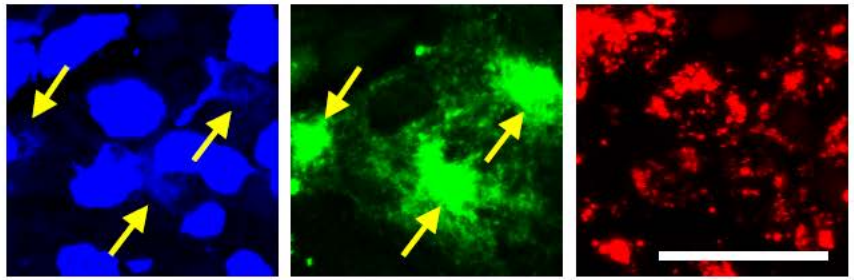
Discussion

- Robust NET induction in healthy PMNs by COVID-19 plasma
- NET levels may return to normal in convalescent patients
- Elevated levels of PF4 & platelet-neutrophil aggregates in COVID-19 patients
- High levels of thrombosis associated markers

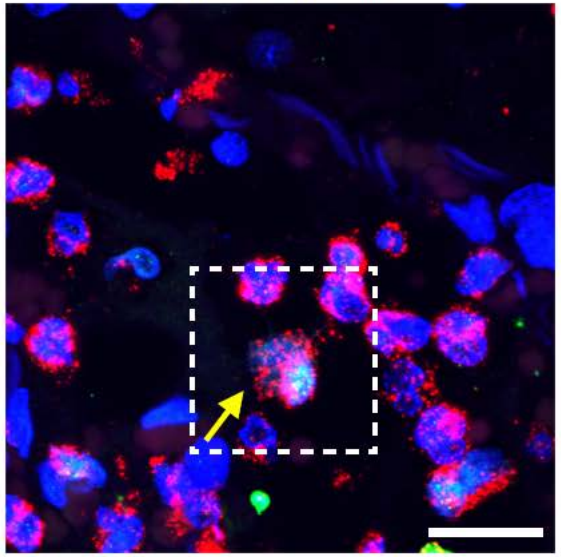
supplementary

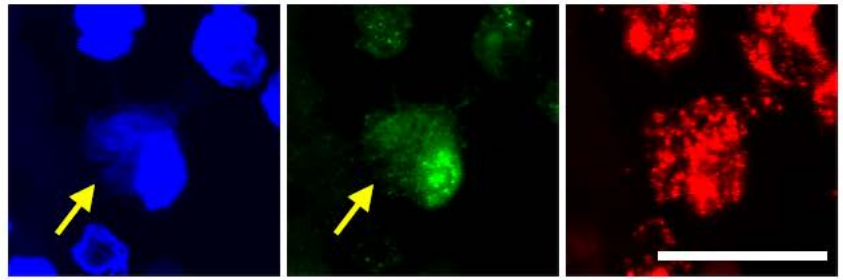
COVID-19 Case #3



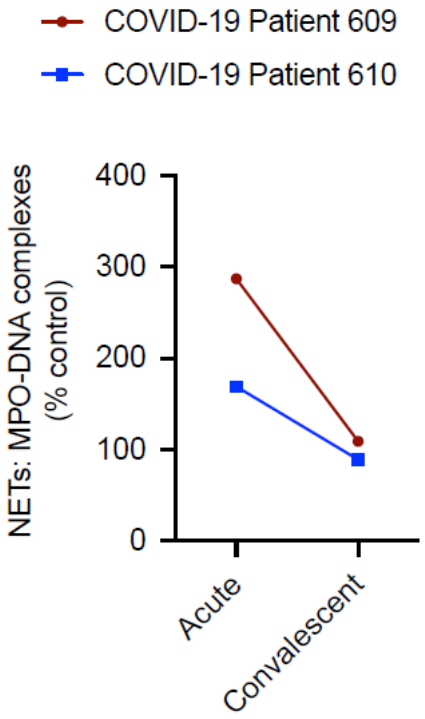


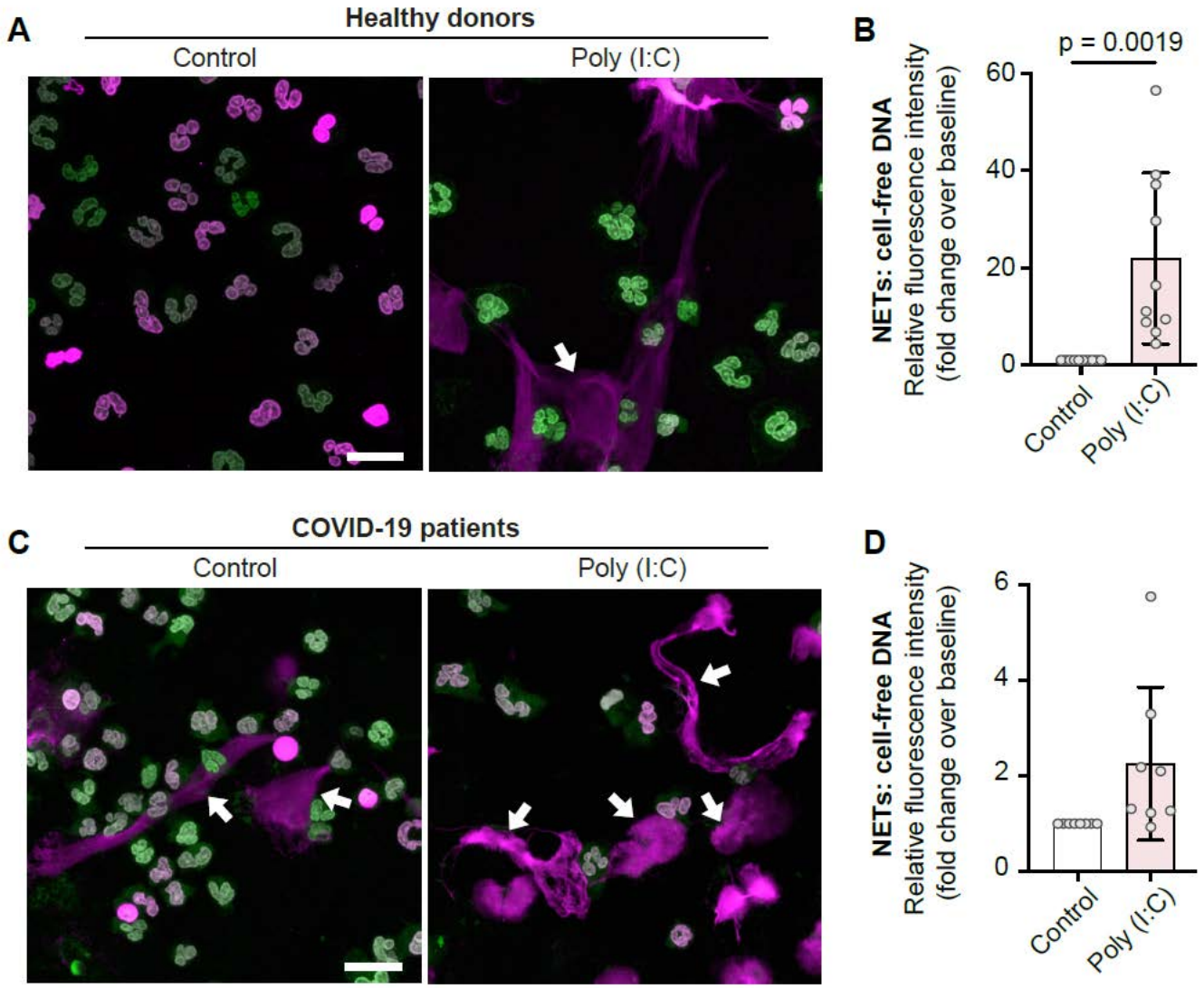
COVID-19 Case #3

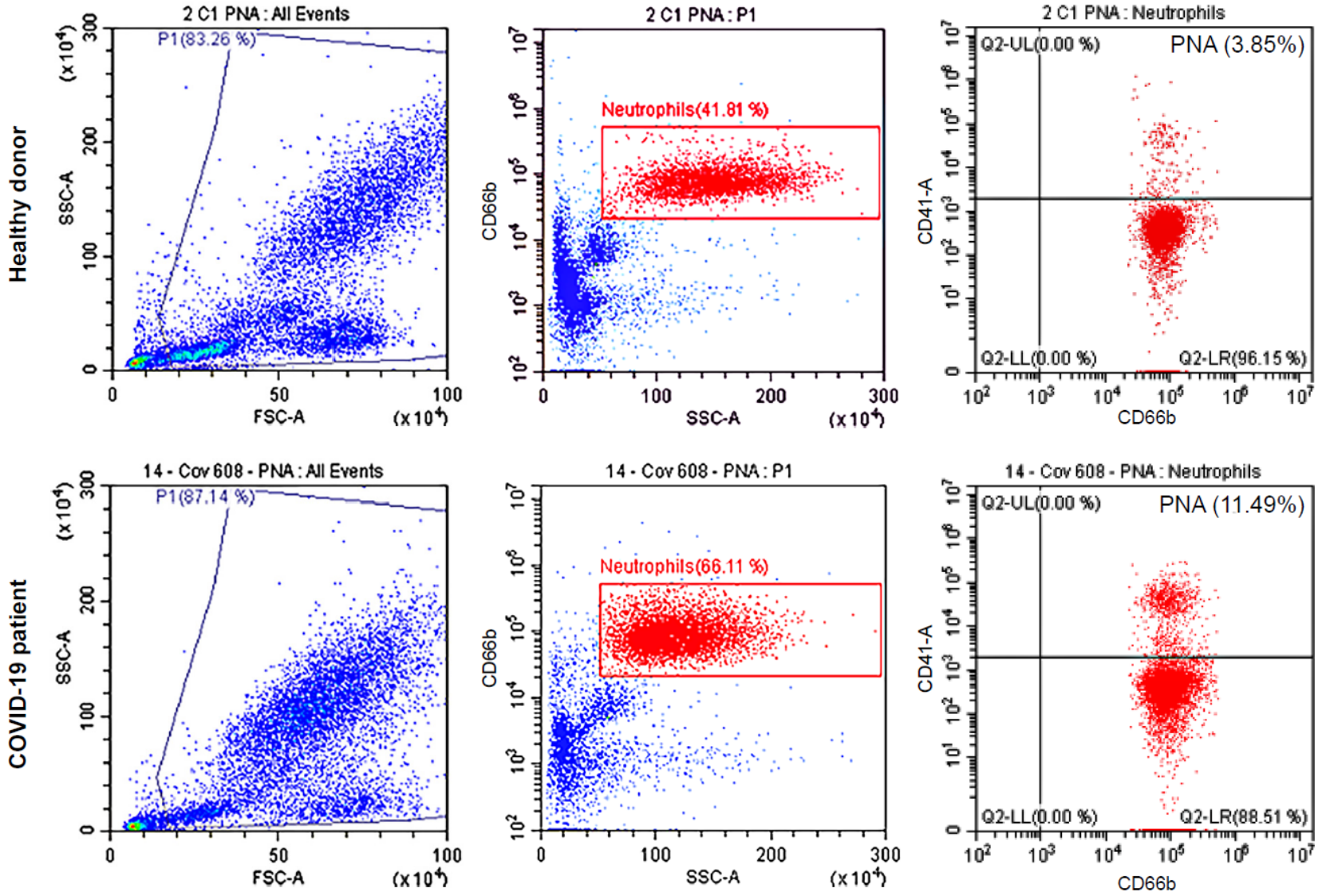




Myeloid peroxidase Citrullinated histone H3 DNA


COVID-19 Patient 609
COVID-19 Patient 610
NETs: MPO-DNA complexes
(% control)
400
300
200
100
0
Acute
Convalescent


A
Healthy donors
Control
Poly (I:C)
B
p = 0.0019
NETs: cell-free DNA
Relative fluorescence intensity
(fold change over baseline)
60
40
20
0
Control
Poly (I:C)
C
COVID-19 patients
Control
Poly (I:C)
D
NETs: cell-free DNA
Relative fluorescence intensity
(fold change over baseline)
6
4
2
0
Control
Poly (I:C)


Healthy donor
2 C1 PNA : All Events
P1(83.26 %)
SSC-A
FSC-A
2 C1 PNA : P1
Neutrophils(41.81 %)
CD66b
SSC-A
2 C1 PNA : Neutrophils
Q2-UL(0.00 %)
PNA (3.85%)
Q2-LL(0.00 %)
Q2-LR(96.15 %)
CD41-A
CD66b
COVID-19 patient
14 - Cov 608 - PNA : All Events
P1(87.14 %)
SSC-A
FSC-A
14 - Cov 608 - PNA : P1
Neutrophils(66.11 %)
CD66b
SSC-A
14 - Cov 608 - PNA : Neutrophils
Q2-UL(0.00 %)
PNA (11.49%)
Q2-LL(0.00 %)
Q2-LR(88.51 %)
CD41-A
CD66b