Supplemental data

Inflammasome activation in neutrophils of patients with severe COVID-19

Karen Aymonnier, Julie Ng, Laura E Fredenburgh, Katherin Zambrano-Vera, Patrick Münzer, Sarah Gutch, Shoichi Fukui, Michael Desjardins, Meera Subramaniam, Rebecca M Baron, Benjamin A Raby, Mark A Perrella, James A Lederer, Denisa D Wagner

Methods

Quantification of NETs biomarkers in Plasma

Briefly, for the measurement of DNA-MPO complexes, plasma was incubated overnight in a plate pre-

coated with an anti-MPO antibody (4 µg/mL; *Biorad, clone 4A4)*. Standard and plasma samples were
diluted in sample diluent of the incubation with anti-DNA conjugated with peroxidase (POD) from the *Cell Death Detection ELISA PLUS* Kit (Roche). Samples were incubated for 2 hours at room temperature on an orbital shaker. After washing, the DNA bound to MPO was revealed using 3,3',5,5'-tetramethylbenzidine (3,3',5,5'-TMB) substrate (Thermo Fischer Scientific). After 20 minutes of incubation in the dark, the reaction was stopped by the addition of 0.16 M sulfuric acid and absorbance was measured at 450 nm.

Plasma dsDNA and citrullinated Histone H3 were measured using the Quant-iT™ PicoGreen® kit (Invitrogen) and Citrullinated Histone H3 (Clone 11D3) ELISA Kit (Cayman) respectively according to the manufacturer protocol.

Plasma Cytokines Levels

Whole blood was collected via central venous catheter or by venipuncture into EDTA tubes. Samples were centrifuged for 15 minutes at 2000 x g, and the resulting plasma frozen in -80°C until use. 40µL of plasma was used for analysis by mouse cytokine Luminex bead assays with known standards using Luminex FlexMap 3D Assay System per the manufacturer's instructions.

Immunofluorescence staining of inflammasome sensors and ASC proteins

Fixed cells were washed once with PBS, permeabilized for 10 minutes at 4°C and incubated with blocking buffer (3% BSA, 3% donkey serum, 0.5% Tween-20 in 1x PBS) at 37°C for 1 hour. For AIM2 and NLRP1 sensors, the samples were incubated at 4°C overnight with the following primary antibodies: mouse anti-AIM2 (1:100; Santa Cruz, sc-515514) or mouse anti-NALP1 (1:50 ; Santa Cruz, sc-166368) and rabbit anti-ASC (1:400; Adipogen, clone AL177). The samples were washed in PBS and incubated with secondary antibodies: donkey anti-Mouse IgG AlexaFluor 555 (1:1500; Thermo Fisher Scientific, cat. A32787, 1:1500) or donkey anti-rabbit IgG AlexaFluor 488 (1:1500, Abcam, cat. ab150061). For NLRC4 and NLRP3 sensors, a sequential staining was performed: the samples were first incubated at 4°C overnight with the following primary antibodies: mouse anti-NLRP3 (1:200, Cell Signaling, clone D2P5E) or mouse anti-NLRC4 (1:500, Merk Millipore, cat. 06-1125). After three wash steps with PBS, the samples were incubated with the secondary antibody donkey anti-rabbit IgG (1:1500, Abcam, cat. ab150061) for 2h at RT. After five wash steps with 0.5% Tween-20, 1% BSA in 1x PBS, samples are incubated with blocking buffer during 1h at 37°C. Samples were incubated for 1.5 hours at RT with rabbit anti-ASC (1:800; Adipogen, clone AL177). Samples

were washed 3 times and incubated with second secondary antibody donkey anti-rabbit AlexaFluor 555 (1:1000 Cat # A32794) at RT for 1 hour. For sequential immunostaining, non-specific controls performed without the first primary antibody or without the second primary antibody, to ensure that second secondary antibody do not recognize the first primary antibody. All antibodies were diluted in blocking solution. After another three wash steps with PBS, the samples were mounted using mounting medium containing DAPI (4',6-diamidin-2-phenylindole). Imaging of immunostainings was carried out on a Zeiss LSM 780 confocal microscope with a 100x oil objective.

Supplemental Tables

Supplemental Table 1. Demographics and clinical characteristics of the COVID-19 cohort.

Legend: Not Available (N/A), Normal Range (NR), P_AO_2/F_IO_2 Ratio (P/F Ratio), Positive End-Expiratory Pressure (PEEP), Sequential Organ Failure Assessment (SOFA), Lung Injury Score (LIS score) and Acute Physiology And Chronic Health Evaluation II (APACHE II).

		Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
Demographics	**Age**	76	83	77	73	31	29
	Gender	Male	Male	Male	Male	Female	Female
	Race	Unknown	Unknown	White	White	White	White
	Ethnicity	Hispanic	Hispanic	Not Hispanic	Not Hispanic	Not Hispanic	Not Hispanic
Blood parameters	**Total leukocytes, 10^6/µL** NR 4.5-11	14.76	12.36	11.96	10.33	6.65	6.56
	Lymphocyte %	9.5	2.9	N/A	1	8	7
	Monocyte %	6.4	4.4	N/A	0	8	1
	Neutrophil %	79.5	90.2	N/A	94	83	90
	vWF level, % NR 50 - 160	N/A	> 500	> 500	> 500	268	235
	ADAMTS13 activity, % NR > 66	N/A	72	89	86	85	101
	Ratio vWF:Ag/ADAMTS13:Ac	N/A	> 6.9	> 5.6	> 5.8	3.2	2.3
	High-sensitivity C-reactive protein, mg/mL NR < 10	241.2	171.4	52.9	45.6	107.2	67.7
	D-dimers, ng/mL NR < 500	1274	957	432	2785	2944	1203
Ventilator parameters	**P/F ratio**	86	182.5	194.44	128.06	85.87	330
	PEEP	18	12	N/A	12	20	20
	Compliance	37	35	25.5	38	45.7	27
Severity of illness Scores	**SOFA**	10	7	5	8	7	10
	LIS	3.75	3	2.25	3	3.33	2.75
	APACHE II	36	24	20	24	24	18

Supplemental Table 2. Timing of cellular event according to MV shedding.

Time (min) of cellular events relative to MV shedding during NETosis in mouse neutrophils after 10 µg/mL of lipopolysaccharides (LPS) activation. Means ± SD.

	Speck formation (min)	MT disassembly (min)	Nuclear rounding (min)	Nuclear breakage (min)	Speck disappearance (min)	NET formation (min)
n=197	-4.345 ± 4.422	2.765 ± 4.040	23.19 ± 13.59	57.05 ± 32.44	84.24 ± 56.36	99.06 ± 35.57

Supplemental Table 3. Determination of inflammasome sensors associated to ASC speck in neutrophils from severe COVID-19 patients.

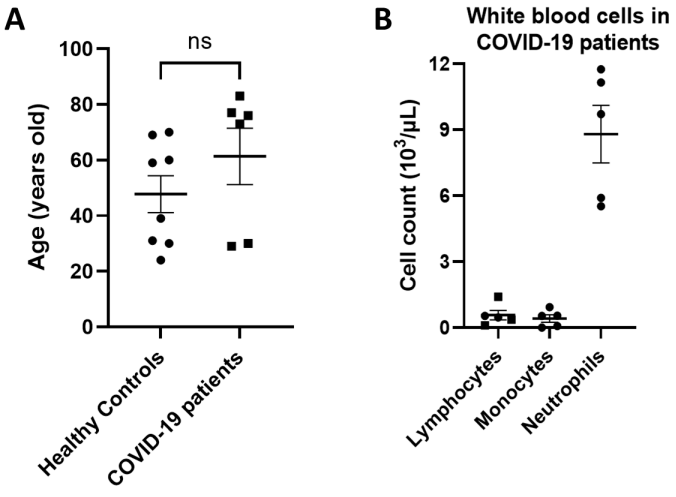
Percent of ASC specks colocalizing with AIM2, NLRP1, NLRC4 or NLRP3 in each patient. Legend: Not Available (N/A).

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Mean
AIM2-ASC	N/A	25%	N/A	0%	N/A	20%	15%
NLRP1-ASC	16.6%	0%	N/A	25%	N/A	N/A	13.9%
NLRC4-ASC	50%	38.5%	57%	44.2%	45.8%	N/A	47.1%
NLRP3-ASC	38.5%	22.2%	N/A	30.8%	33.3%	20%	28.9%

Supplemental Figures

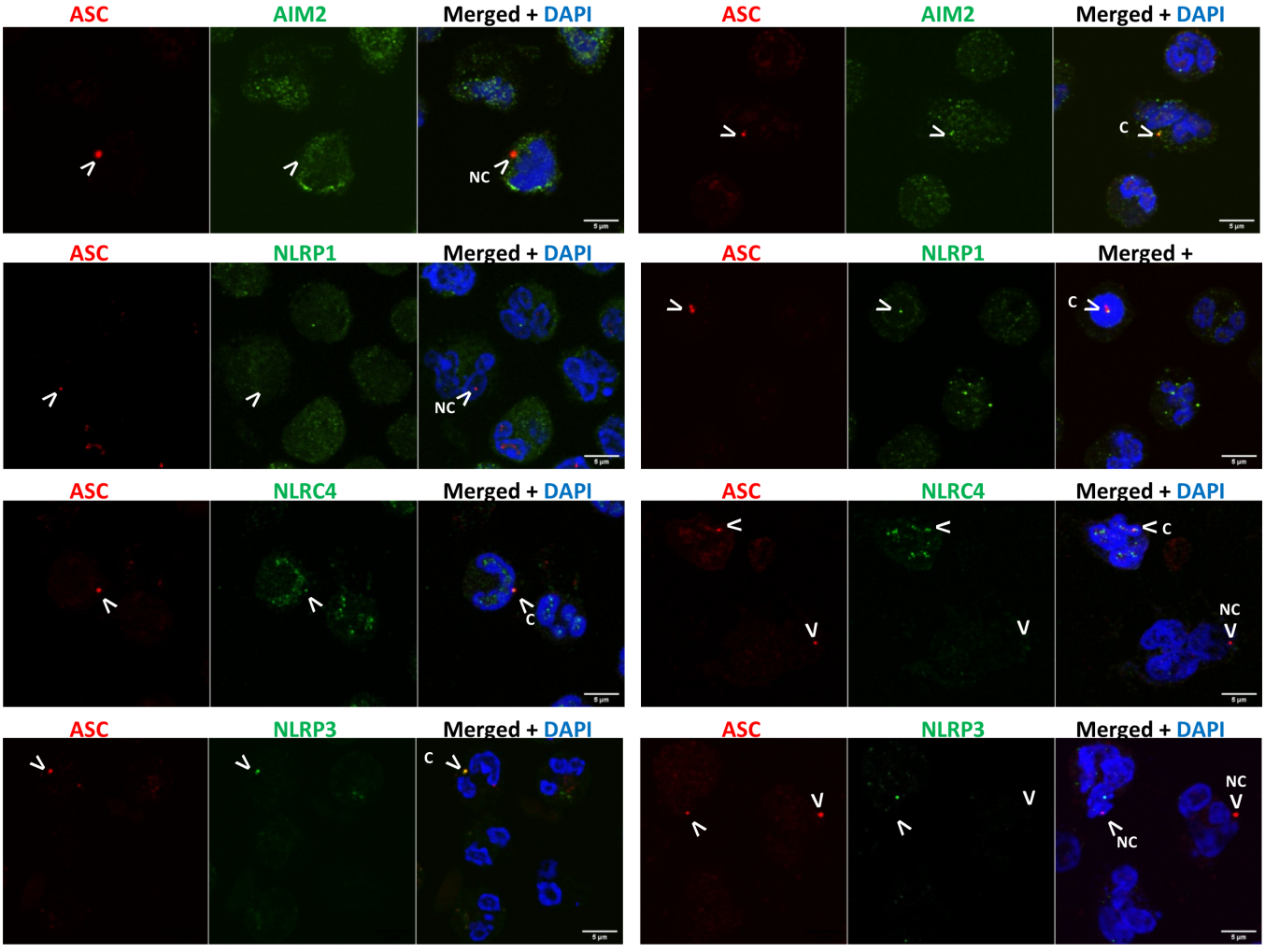
Supplemental Figure 1. Age and white blood cells count in patients with severe COVID-19.

(A) Ages of COVID-19 patients (n=6) and healthy donors (n=8). (B) White blood cells count in COVID-19 patients was evaluated. Values are means ± SEM. Data were analyzed using Mann-Whitney test. Legend: non-significant (ns).



Supplemental Figure 2. Determination of inflammasome sensors associated to ASC speck in neutrophils from severe COVID-19 patients.

Neutrophils freshly isolated from COVID-19 patients (n=3-6) were stained with DAPI (blue), an anti-ASC antibody (red) and an anti-inflammasome sensor antibody that is AIM2 or NLRP1 or NLRC4 or NLRP3 (green). Representative images from confocal microscopy are shown. White arrows indicate the presence of the ASC speck. Legend: Not Colocalized (NC), Colocalized (C).



Video Legends

Video 1. Time-lapse visualization of ASC speck formation during NETosis.

Representative time-lapse differential interference contrast (DIC, grayscale) and spinning-disk confocal microscopy movie of NETosis over 4 hours by murine neutrophils expressing fluorescent ASC. Blue, DNA (siR-DNA); green, ASC-citrine. Scale bar equals 10 µm.

Video 2. Time-lapse visualization of ASC speck formation and microtubules during NETosis.

Representative time-lapse differential interference contrast (DIC, grayscale) and spinning-disk confocal microscopy movie of NETosis over 4 hours by murine neutrophils. Red, microtubules (siR-Tubulin); green, ASC-citrine. Scale bar equals 10 µm.