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Supplemental information

Neutrophil profiles of pediatric COVID-19 and multisystem inflammatory syndrome in children

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SUPPLEMENTAL FIGURES

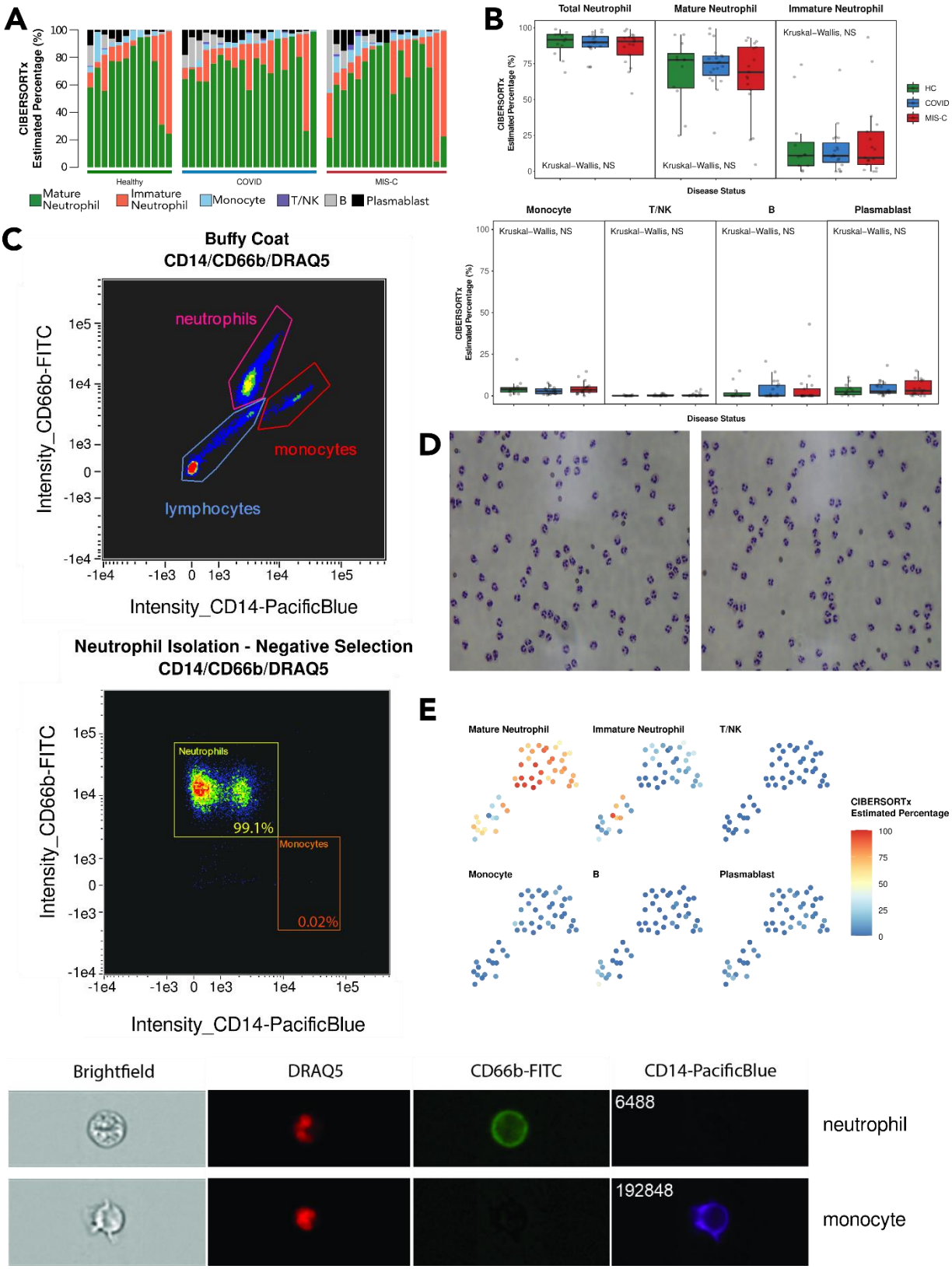

Fig. S1. Neutrophil isolation and quality control for purity, related to Fig. 1. (A) Bar plots displaying the distribution of the CIBERSORTx estimated cell type percentages for each sample. Samples are divided by disease status and ordered according to Total Neutrophil Content (sum of Mature and Immature neutrophil fractions). **(B)** Box plots showing the distribution of CIBERSORTx percentages for each cell type divided by disease status. No significant differences were found between disease grouping using the Kruskal-Wallis test. **(C)** Cells are gated on the nuclear stain, DRAQ5 (far red). Top: population of nucleated cells from the buffy coat. Bottom: population of isolated neutrophils after negative selection, confirming the high purity of neutrophil population. Neutrophils are identified by CD66b (FITC) and monocytes by CD14 (Pacific Blue). Numbers in the top left corner of the CD-14 Pacific Blue image represent the event number of the captured cell. **(D)** Geimsa staining of isolated neutrophil sample (neutrophils stained blue/purple and eosinophils stained pink). **(E)** CIBERSORTx estimated cell type percentages.

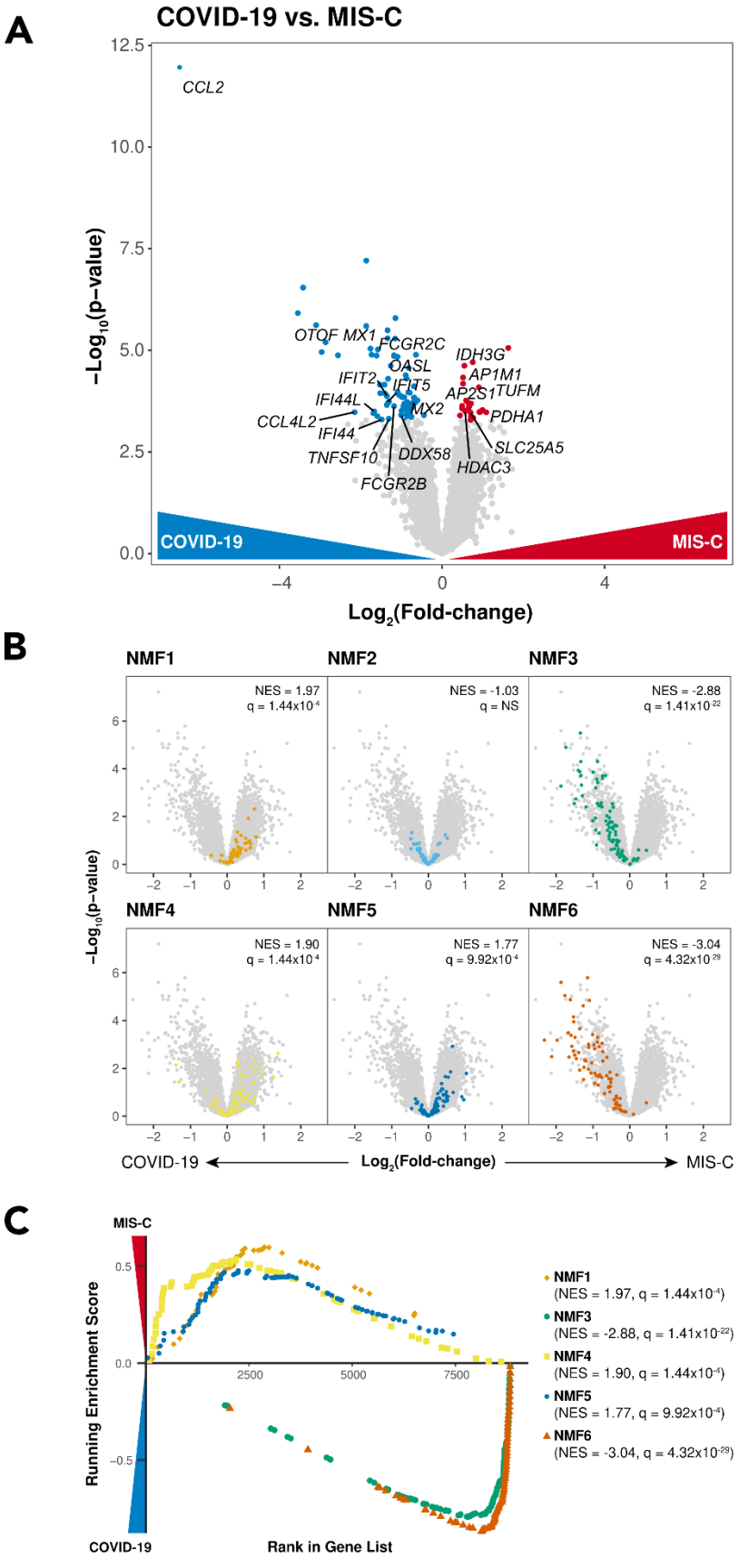
A
COVID-19 vs. MIS-C
CCL2
OTOF
MX1
FCGR2C
OASL
IFIT2
IFIT5
IFI44L
MX2
CCL4L2
IFI44
TNFSF10
DDX58
FCGR2B
IDH3G
AP1M1
AP2S1
TUFM
PDHA1
SLC25A5
HDAC3
COVID-19
MIS-C
−Log10(p−value)
Log2(Fold-change)
B
NMF1
NES = 1.97
q = 1.44x10-4
NMF2
NES = -1.03
q = NS
NMF3
NES = -2.88
q = 1.41x10-22
NMF4
NES = 1.90
q = 1.44x10-4
NMF5
NES = 1.77
q = 9.92x10-4
NMF6
NES = -3.04
q = 4.32x10-29
−Log10(p−value)
COVID-19
Log2(Fold-change)
MIS-C
C
MIS-C
COVID-19
Running Enrichment Score
Rank in Gene List
NMF1
(NES = 1.97, q = 1.44x10-4)
NMF3
(NES = -2.88, q = 1.41x10-22)
NMF4
(NES = 1.90, q = 1.44x10-4)
NMF5
(NES = 1.77, q = 9.92x10-4)
NMF6
(NES = -3.04, q = 4.32x10-29)

Fig. S2. Direct comparison of acute pediatric COVID-19 and MIS-C, Related to Fig. 3. (A) Volcano plot showing differentially expressed genes between MIS-C samples and acute pediatric COVID-19 samples. Color-coded points indicate genes that pass FDR correction with $q < 0.05$. **(B)** Color-coded points in the volcano plot of differentially expressed genes between acute pediatric COVID-19 and MIS-C by whether the gene is a marker for one of the NMF subtypes with axis limits set to +/- 3 Log2(fold-change). **(C)** GSEA enrichment plots for the NMF1 (Pro-Neu), NMF3 (PD-L1+ ISG+), NMF4 (Immature), NMF5 (G-MDSC), and NMF6 (ISG+) signatures, all of which passed FDR correction as indicated.

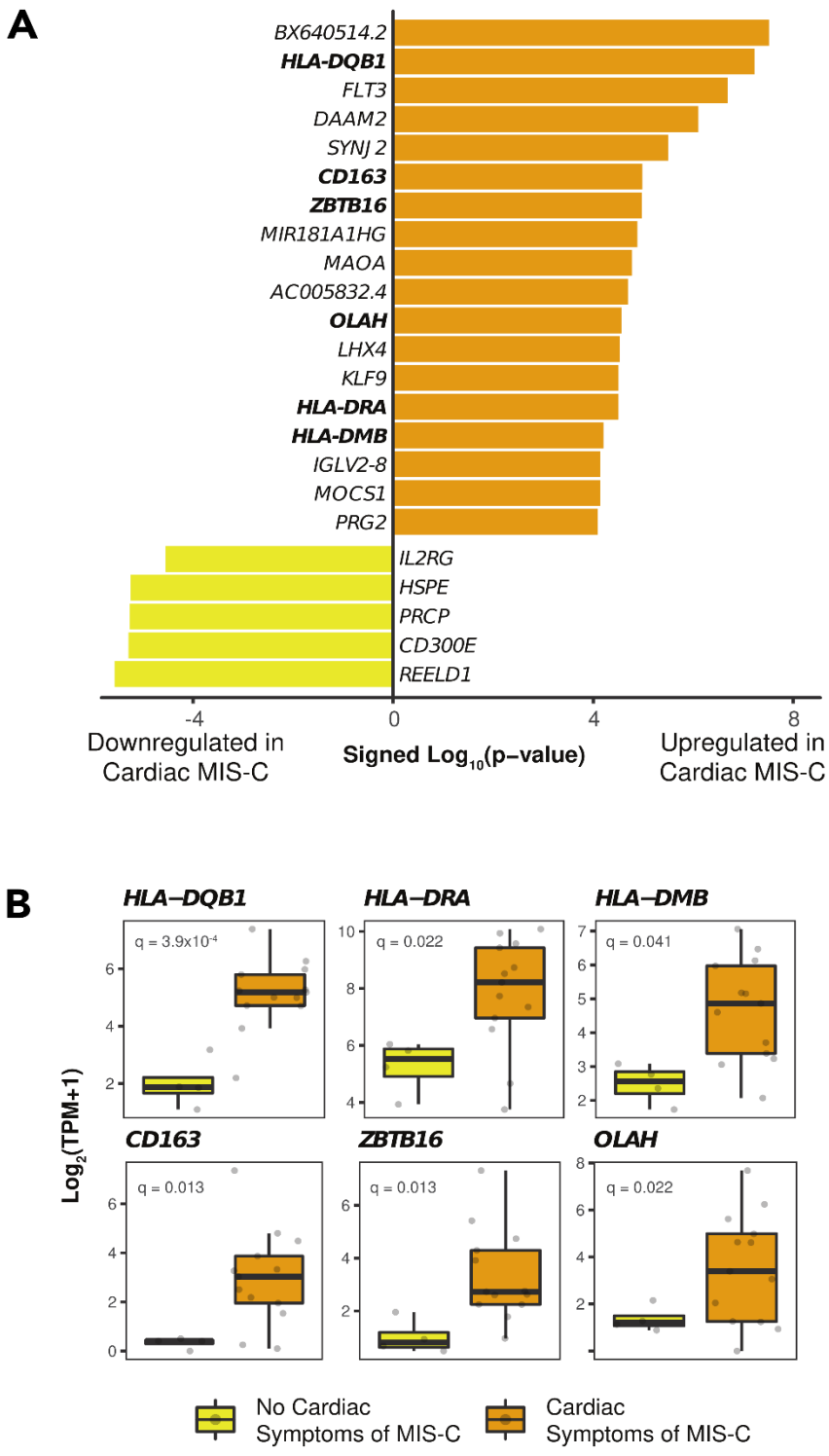

Fig. S3. Comparison of MIS-C, acute pediatric COVID-19, and healthy children Related to Fig. 3. (A) Bar plots displaying the top differentially expressed genes between samples from MIS-C patients with and without cardiac involvement of disease. Bar length corresponds to the signed $\log_{10}$(p-value) for the differential expression analysis. Bolded genes are displayed individually in **(B)**. **(B)** Box plots of $\log_2$(TPM+1) values for selected genes differentially expressed in **(A)**. FDR q-values are from DESeq2.

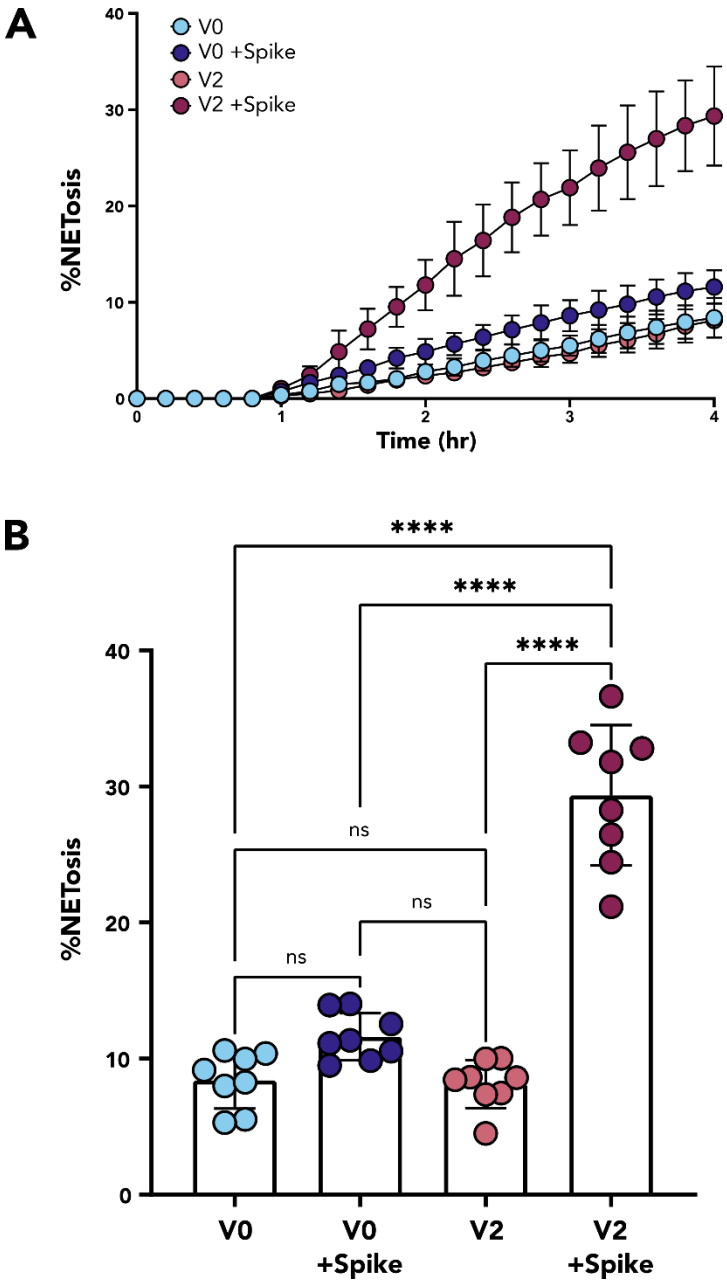

Fig. S4. Quantification of NETosis in neutrophils stimulated with matched pre- and post-vaccine plasma, related to Fig. 5. Paired plasma samples were collected from children 12-15 years of age prior to vaccination (V0) and 2-4 weeks after the 2nd dose (V2) with the Pfizer BNT162b2 vaccine series. None of the children had prior COVID-19. **(A)** Temporal dynamics of NET release in neutrophils (n = 8) stimulated with V0 plasma, V0-plasma-coated Spike beads, V2 plasma, and V2-plasma-coated Spike beads. **(B)** End-point percentage of NET release in neutrophils (n = 8) stimulated with conditions as described in **(A)**.

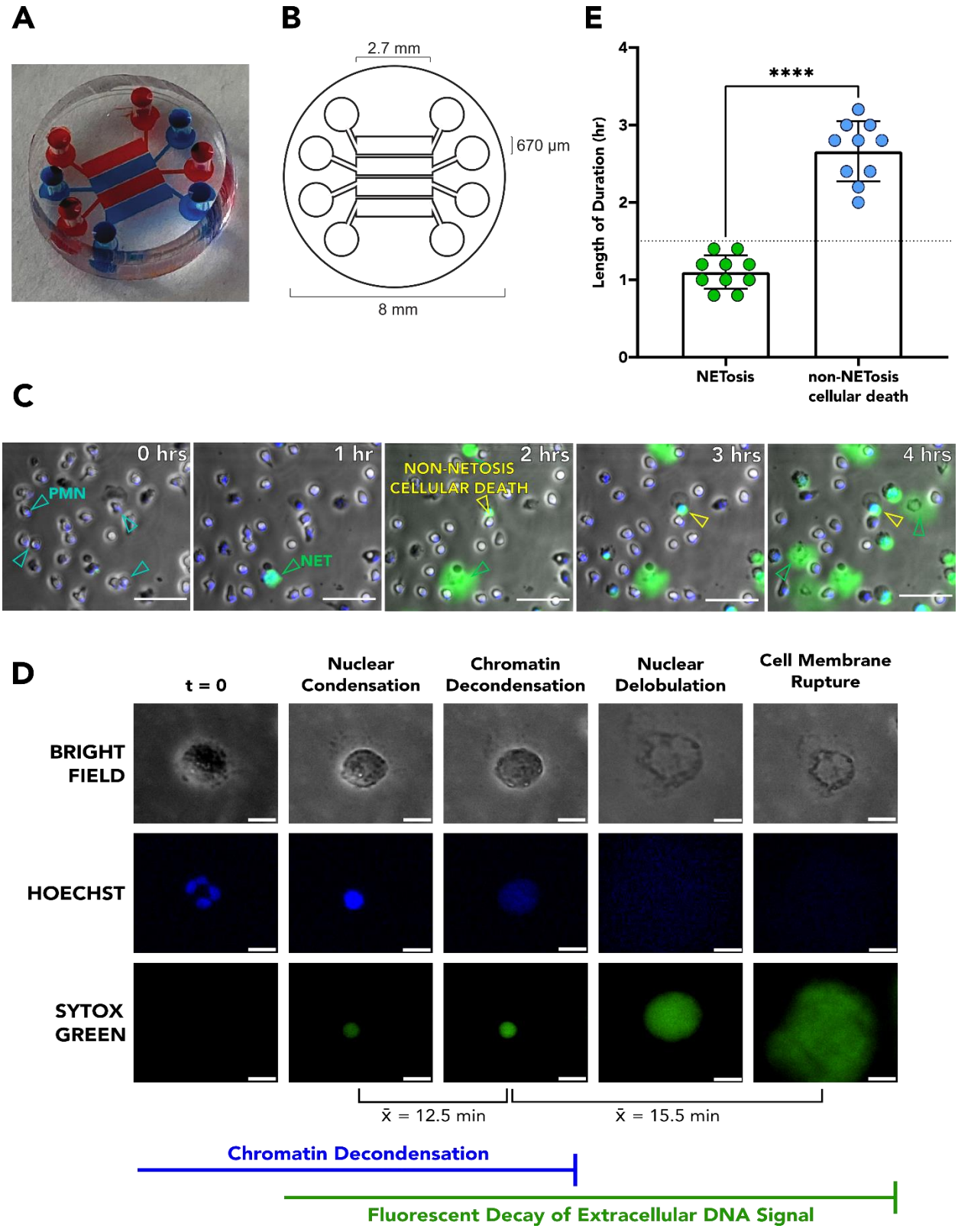
A
B
2.7 mm
670 µm
8 mm
E
****
Length of Duration (hr)
0
1
2
3
4
NETosis
non-NETosis
cellular death
C
0 hrs
PMN
1 hr
NET
2 hrs
NON-NETOSIS
CELLULAR DEATH
3 hrs
4 hrs
D
t = 0
Nuclear Condensation
Chromatin Decondensation
Nuclear Delobulation
Cell Membrane Rupture
BRIGHT FIELD
HOECHST
SYTOX GREEN
x̄ = 12.5 min
x̄ = 15.5 min
Chromatin Decondensation
Fluorescent Decay of Extracellular DNA Signal

Fig. S5. Design and schematic of the employed microfluidic device, related to Fig. 4 and 5, methodology and validation of quantification of NETosis. (A) Microfluidic device used to visualize NET release over time. Dye showing four separated channels which allow for four simultaneous measurements. **(B)** Dimension of the microfluidic device used to visualize NET release over time. **(C)** Time-lapse images of NET formation and non-NOTosis cellular death within the microfluidic devices. Neutrophil (PMN) nucleus was stained with Hoechst stain (DAPI), and extracellular DNA was stained with SYTOX green (FITC). Diffused SYTOX green staining and disrupted membrane seen in the brightfield channel over time was defined to be NETosis. Circular, stable SYTOX green staining and circular neutrophils were seen over time was defined as a cell undergoing non-NETosis cellular death. Scale bar = 50 μM. **(D)** Visualization of a neutrophil undergoing NETosis through nuclear condensation, chromatin decondensation, nuclear delobulation, cell membrane rupture, and extracellular staining where there is, on average (n = 10), 3 minutes between the changes in the nucleus and staining of the extracellular DNA. Scale bar = 10 μM **(E)** Validation of quantification of NETosis. Neutrophils were manually tracked to determine the length of SYTOX green duration in NET release (n = 10) and non-NETosis cellular death (n = 10). NETs were determined to be SYTOX green staining detected for ≤ 1.5 hrs, and non-NETosis cellular death was defined as tracked cells with a duration > 1.5 hrs. Significance was determined by 1-way ANOVA with multiple comparisons in GraphPad Prism v9. Mean values and standard deviation are presented. Statistical significance is defined as **** $P < 0.0001$.

SUPPLEMENTAL TABLES

Table S1. Demographics and clinical characteristics of pediatric acute COVID-19 patients included in the study, Related to Fig. 1, Tab. 1.

Patients with acute COVID-19 infection	Age (years)	Gender	SARS-CoV-2 RT-PCR On Admission	Hospitalized	Highest Level of Care	Treatment Required	Treated with Steroids	Respiratory Support Needed	O_2 Needed	Patient Intubated
1	10	M	(+)	yes	ward	no	no	no	no	no
2	14	M	(+)	yes	ward	no	no	no	no	no
3	15	F	(+)	yes	ward	yes	no	yes	yes	no
4	0.05	M	(+)	yes	ward	yes	no	yes	yes	no
5	16	F	(+)	yes	ward	yes	no	yes	yes	no
6	2.5	M	(+)	yes	PICU	yes	no	yes	yes	no
7	12.6	M	(+)	yes	ward	yes	no	yes	yes	yes
8	13.81	M	(+)	yes	PICU	yes	yes	yes	yes	no
9	17.15	M	(+)	yes	ward	no	no	no	no	no
10	15.71	M	(+)	yes	PICU	yes	no	yes	yes	no
11	22.16	M	(+)	yes	ward	yes	no	yes	no	no
12	13	F	(+)	yes	PICU	no	no	no	no	no
13	20.35	M	(+)	yes	ward	yes	yes	no	no	no
14	17.59	F	(+)	yes	PICU	yes	yes	yes	yes	no
15	21.99	F	(+)	yes	ward	yes	yes	yes	yes	no
16	21.86	M	(+)	yes	ward	yes	yes	yes	yes	no
17	19.15	M	(+)	yes	ward	yes	yes	yes	yes	no
18	1.55	M	(+)	yes	ward	yes	no	yes	yes	no
19	3.74	M	(+)	yes	PICU	yes	yes	yes	yes	no
20	16.33	M	(+)	yes	PICU	no	no	yes	yes	no
21	0.92	M	(+)	yes	outpatient	no	no	no	no	no
22	18	F	(+)	no	outpatient	no	no	no	no	no
23	20	F	(+)	no	outpatient	no	no	no	no	no

Table S1. Demographics and clinical characteristics of pediatric acute COVID-19 patients included in the study, Related to Fig. 1, Tab. 1 (continued).

Patients with acute COVID-19 infection	Age (years)	Gender	SARS-CoV-2 RT-PCR On Admission	Hospitalized	Highest Level of Care	Treatment Required	Treated with Steroids	Respiratory Support Needed	O_2 Needed	Patient Intubated
24	20	M	(+)	no	outpatient	no	no	no	no	no
25	16	F	(+)	no	outpatient	no	no	no	no	no
26	13	M	(+)	no	outpatient	no	no	no	no	no
27	13	M	(+)	no	outpatient	no	no	no	no	no
28	19	M	(+)	no	outpatient	no	no	no	no	no
29	17	F	(+)	no	outpatient	no	no	no	no	no
30	15	F	(+)	no	outpatient	no	no	no	no	no
31	16	M	(+)	no	outpatient	no	no	no	no	no
32	10	M	(+)	no	outpatient	no	no	no	no	no
33	19	F	(+)	no	outpatient	no	no	no	no	no
34	2	F	(+)	no	outpatient	no	no	no	no	no
35	17	M	(+)	no	outpatient	no	no	no	no	no
36	10	F	(+)	no	outpatient	no	no	no	no	no
37	21.4	M	(+)	no	outpatient	no	no	no	no	no
38	11.3	M	(+)	no	outpatient	no	no	no	no	no
39	18	M	(+)	no	outpatient	no	no	no	no	no
40	15.2	F	(+)	no	outpatient	no	no	no	no	no
41	16	M	(+)	no	outpatient	no	no	no	no	no
42	17.3	M	(+)	no	outpatient	yes	yes	yes	yes	no
43	12	M	(+)	no	outpatient	no	no	no	no	no

Table S2. Demographics and clinical characteristics of pediatric MIS-C patients included in the study, Related to Fig. 1, Tab. 1.

Patients with MIS-C	Age (years)	Gender	SARS-CoV-2 RT-PCR On Admission	SARS-CoV-2 Antibody	COVID-19 Exposure	Highest Level of Care	Cardiovascular Involvement	Treatment Required	Treated with IVIG	Treated with Steroids	Treated with Anikinra
1	2.9	M	n/a	n/a	(+)	ward	no	no	no	no	no
2	1.2	M	neg	neg	(+)	ward	no	no	no	no	no
3	1	F	neg	(+)	(+)	ward	no	no	no	no	no
4	1.5	M	neg	(+)	(+)	ward	no	yes	yes	no	no
5	17	F	neg	(+)	(+)	ward	no	yes	no	yes	no
6	2	F	neg	(+)	(+)	ward	no	yes	yes	yes	no
7	18	F	neg	(+)	(+)	ward	no	yes	no	tes	no
8	9	M	neg	neg	(+)	ward	no	no	no	no	no
9	0.17	M	(+)	n/a	(+)	ward	no	no	no	no	no
10	0.17	M	neg	neg	(+)	ward	no	no	no	no	no
11	0.17	F	(+)	n/a	(+)	ward	no	no	no	no	no
12	1.34	M	neg	(+)	(+)	PICU	no	yes	yes	yes	yes
13	14.79	M	neg	(+)	(+)	ward	myocarditis	no	no	no	no
14	7.6	F	n/a	(+)	(+)	PICU	ventricular dysfunction (EF 48%), vasopressor support (epinephrine)	yes	yes	yes	yes
15	2.6	M	neg	neg	(+)	ward	atypical Kawasaki's, lack of coronary tapering	yes	yes	no	no
16	13.6	M	neg	(+)	(+)	PICU	myocarditis	yes	yes	yes	yes
17	12.5	M	neg	(+)	(+)	PICU	myocarditis, elevated NT-proBNP	yes	yes	yes	yes
18	3.5	M	neg	(+)	(+)	ward	coronary arterial aneurysm	yes	yes	yes	no
19	7.11	M	(+)	n/a	(+)	PICU	coronary aneurysm, elevated troponin, ventricular dyfunction (EF 48)	yes	yes	yes	no
20	21	M	n/a	(+)	(+)	PICU	myocarditis, ventricular dysfunction (EF 21%), vasopressor support	yes	yes	yes	no
21	19.4	F	n/a	(+)	(+)	PICU	extracorporeal membrane oxygenation	yes	yes	yes	no
22	10.3	M	(+)	(+)	(+)	PICU	ventricular dysfunction, vasopressor suppport, Mobitz type I and II	yes	yes	yes	yes
23	9.63	M	n/a	(+)	(+)	PICU	extracorporeal membrane oxygenation	yes	yes	yes	no
24	8.27	M	neg	(+)	(+)	PICU	ventricular dysfunction (EF 40)	yes	yes	yes	no

Table S2. Demographics and clinical characteristics of pediatric MIS-C patients included in the study, Related to Fig. 1, Tab. 1 (continued).

Patients with MIS-C	Age (years)	Gender	SARS-CoV-2 RT-PCR On Admission	SARS-CoV-2 Antibody	COVID-19 Exposure	Highest Level of Care	Cardiovascular Involvement	Treatment Required	Treated with IVIG	Treated with Steroids	Treated with Anikinra
25	5.61	F	neg	(+)	(+)	ward	ventricular dysfunction (EF 52%); conduction defect (1st degree AV block)	yes	yes	yes	no
26	9	M	neg	(+)	(+)	ward	elevated BNP, coronary dilation or aneurysm	yes	yes	yes	yes
27	3	F	(+)	(+)	(+)	PICU	bradycardia	yes	no	yes	no
28	9	M	neg	(+)	(+)	PICU	elevated troponin and pro-BNP	yes	yes	no	no
29	7	M	neg	(+)	(+)	PICU	dilated left ventricle	yes	yes	yes	yes
30	4	M	(+)	(+)	(+)	ward	corornary dilation or aneurysm	yes	yes	yes	no
31	8	M	neg	(+)	(+)	PICU	hypotension, ventricular failure, extracorporeal membrane oxygenation, arrhythmia, elevated troponin, elevated bnp, coronary dilation or aneurysm	yes	yes	yes	yes