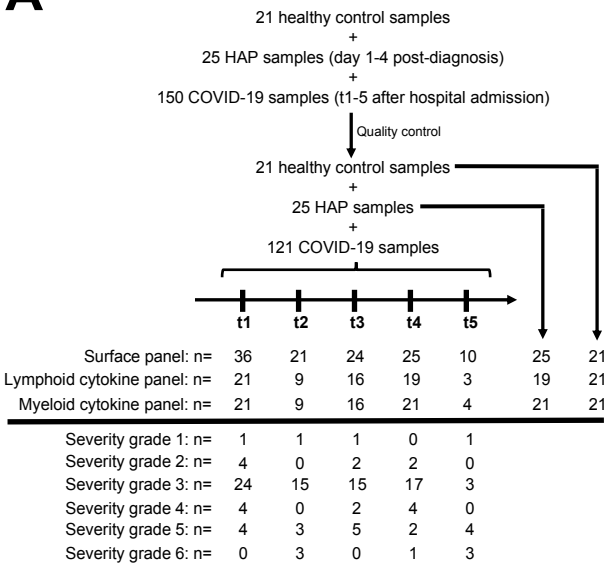
Immunity, Volume *54*

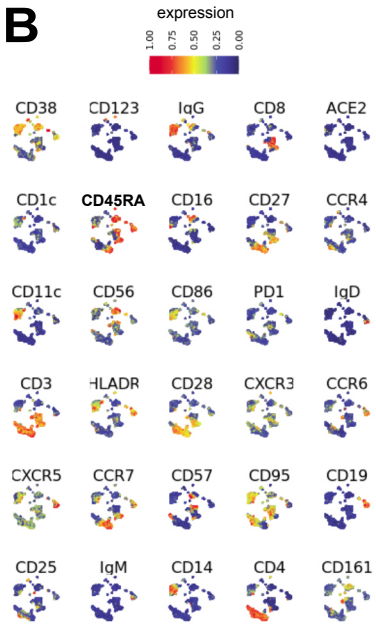
Supplemental information

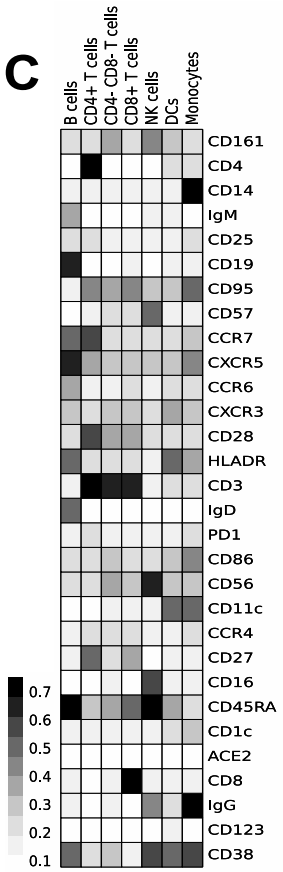
Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia

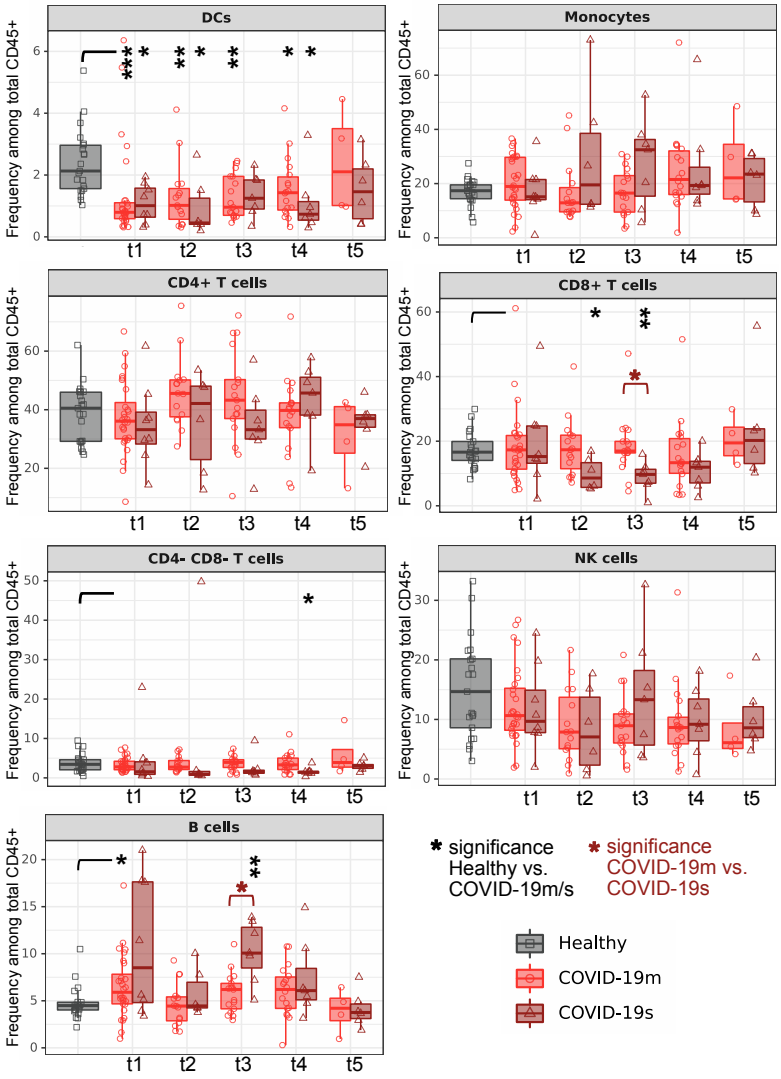
Stefanie Kreutmair, Susanne Unger, Nicolás Gonzalo Núñez, Florian Ingelfinger, Chiara Alberti, Donatella De Feo, Sinduya Krishnarajah, Manuel Kauffmann, Ekaterina Friebel, Sepideh Babaei, Benjamin Gaborit, Mirjam Lutz, Nicole Puertas Jurado, Nisar P. Malek, Siri Goepel, Peter Rosenberger, Helene A. Häberle, Ikram Ayoub, Sally Al-Hajj, Jakob Nilsson, Manfred Claassen, Roland Liblau, Guillaume Martin-Blondel, Michael Bitzer, Antoine Roquilly, and Burkhard Becher

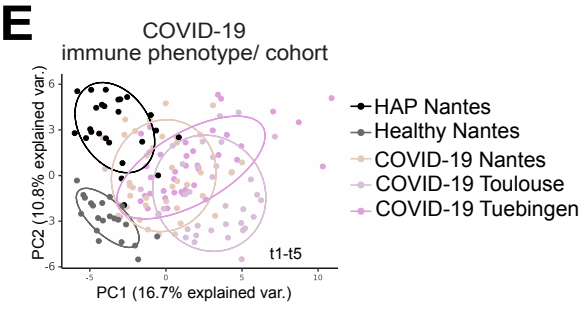
Figure S1

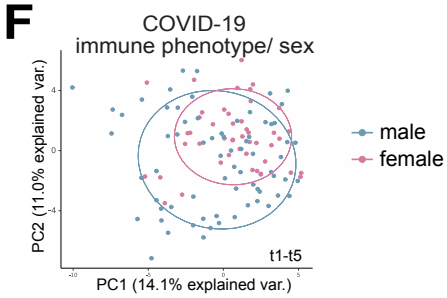

A
21 healthy control samples
+
25 HAP samples (day 1-4 post-diagnosis)
+
150 COVID-19 samples (t1-5 after hospital admission)
Quality control
21 healthy control samples
+
25 HAP samples
+
121 COVID-19 samples
t1 t2 t3 t4 t5
Surface panel: n= 36 21 24 25 10 25 21
Lymphoid cytokine panel: n= 21 9 16 19 3 19 21
Myeloid cytokine panel: n= 21 9 16 21 4 21 21
Severity grade 1: n= 1 1 1 0 1
Severity grade 2: n= 4 0 2 2 0
Severity grade 3: n= 24 15 15 17 3
Severity grade 4: n= 4 0 2 4 0
Severity grade 5: n= 4 3 5 2 4
Severity grade 6: n= 0 3 0 1 3


B
expression
1.00 0.75 0.50 0.25 0.00
CD38 CD123 IgG CD8 ACE2
CD1c CD45RA CD16 CD27 CCR4
CD11c CD56 CD86 PD1 IgD
CD3 HLADR CD28 CXCR3 CCR6
CXCR5 CCR7 CD57 CD95 CD19
CD25 IgM CD14 CD4 CD161


C
B cells
CD4+ T cells
CD4- CD8- T cells
CD8+ T cells
NK cells
DCs
Monocytes
CD161 CD4 CD14 IgM CD25 CD19 CD95 CD57 CCR7 CXCR5 CCR6 CXCR3 CD28 HLADR CD3 IgD PD1 CD86 CD56 CD11c CCR4 CD27 CD16 CD45RA CD1c ACE2 CD8 IgG CD123 CD38
0.7 0.6 0.5 0.4 0.3 0.2 0.1


D
DCs
Monocytes
CD4+ T cells
CD8+ T cells
CD4- CD8- T cells
NK cells
B cells
Frequency among total CD45+
t1 t2 t3 t4 t5
* significance Healthy vs. COVID-19m/s
* significance COVID-19m vs. COVID-19s
Healthy
COVID-19m
COVID-19s


E
COVID-19
immune phenotype/ cohort
PC2 (10.8% explained var.)
PC1 (16.7% explained var.)
t1-t5
HAP Nantes
Healthy Nantes
COVID-19 Nantes
COVID-19 Toulouse
COVID-19 Tuebingen


F
COVID-19
immune phenotype/ sex
PC2 (11.0% explained var.)
PC1 (14.1% explained var.)
t1-t5
male
female

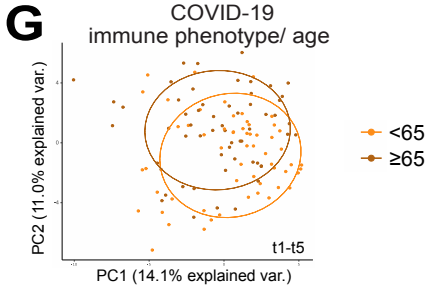

G
COVID-19
immune phenotype/ age
PC2 (11.0% explained var.)
PC1 (14.1% explained var.)
t1-t5
<65
≥65

Figure S2

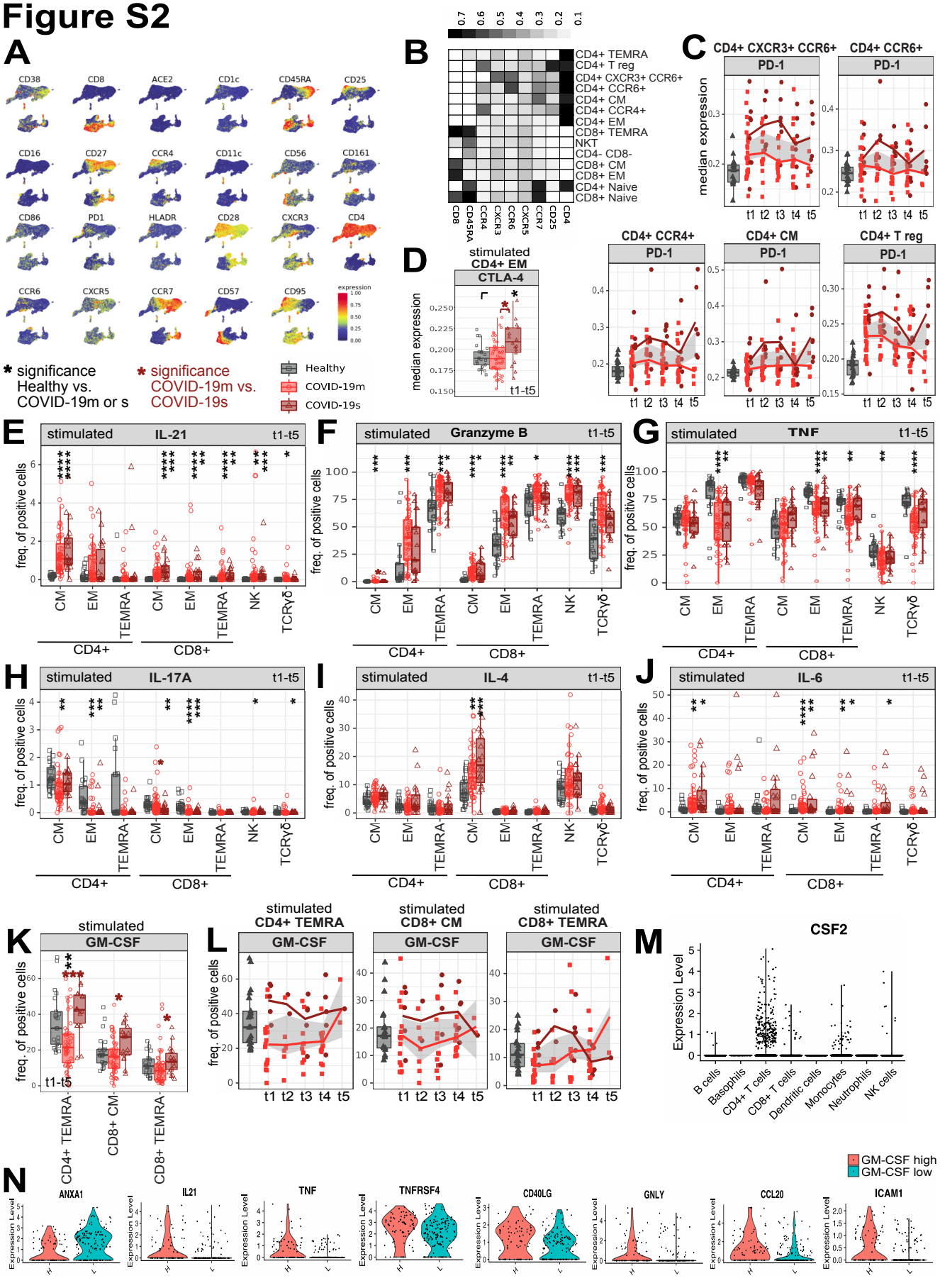

A
CD38
CD8
ACE2
CD1c
CD45RA
CD25
CD16
CD27
CCR4
CD11c
CD56
CD161
CD86
PD1
HLADR
CD28
CXCR3
CD4
CCR6
CXCR5
CCR7
CD57
CD95
expression
* significance Healthy vs. COVID-19m or s
* significance COVID-19m vs. COVID-19s
Healthy
COVID-19m
COVID-19s
B
CD4+ TEMRA
CD4+ T reg
CD4+ CXCR3+ CCR6+
CD4+ CCR6+
CD4+ CM
CD4+ CCR4+
CD4+ EM
CD8+ TEMRA
NKT
CD4- CD8-
CD8+ CM
CD8+ EM
CD4+ Naive
CD8+ Naive
C
CD4+ CXCR3+ CCR6+
CD4+ CCR6+
CD4+ CCR4+
CD4+ CM
CD4+ T reg
PD-1
median expression
D
stimulated
CD4+ EM
CTLA-4
median expression
t1-t5
E
stimulated
IL-21
t1-t5
freq. of positive cells
F
stimulated
Granzyme B
t1-t5
G
stimulated
TNF
t1-t5
H
stimulated
IL-17A
t1-t5
I
stimulated
IL-4
t1-t5
J
stimulated
IL-6
t1-t5
CM
EM
TEMRA
NK
TCRγδ
CD4+
CD8+
K
stimulated
GM-CSF
t1-t5
CD4+ TEMRA
CD8+ CM
CD8+ TEMRA
L
stimulated
CD4+ TEMRA
GM-CSF
stimulated
CD8+ CM
GM-CSF
stimulated
CD8+ TEMRA
GM-CSF
t1 t2 t3 t4 t5
M
CSF2
Expression Level
B cells
Basophils
CD4+ T cells
CD8+ T cells
Dendritic cells
Monocytes
Neutrophils
NK cells
N
GM-CSF high
GM-CSF low
ANXA1
IL21
TNF
TNFRSF4
CD40LG
GNLY
CCL20
ICAM1
Expression Level
H
L

Figure S3

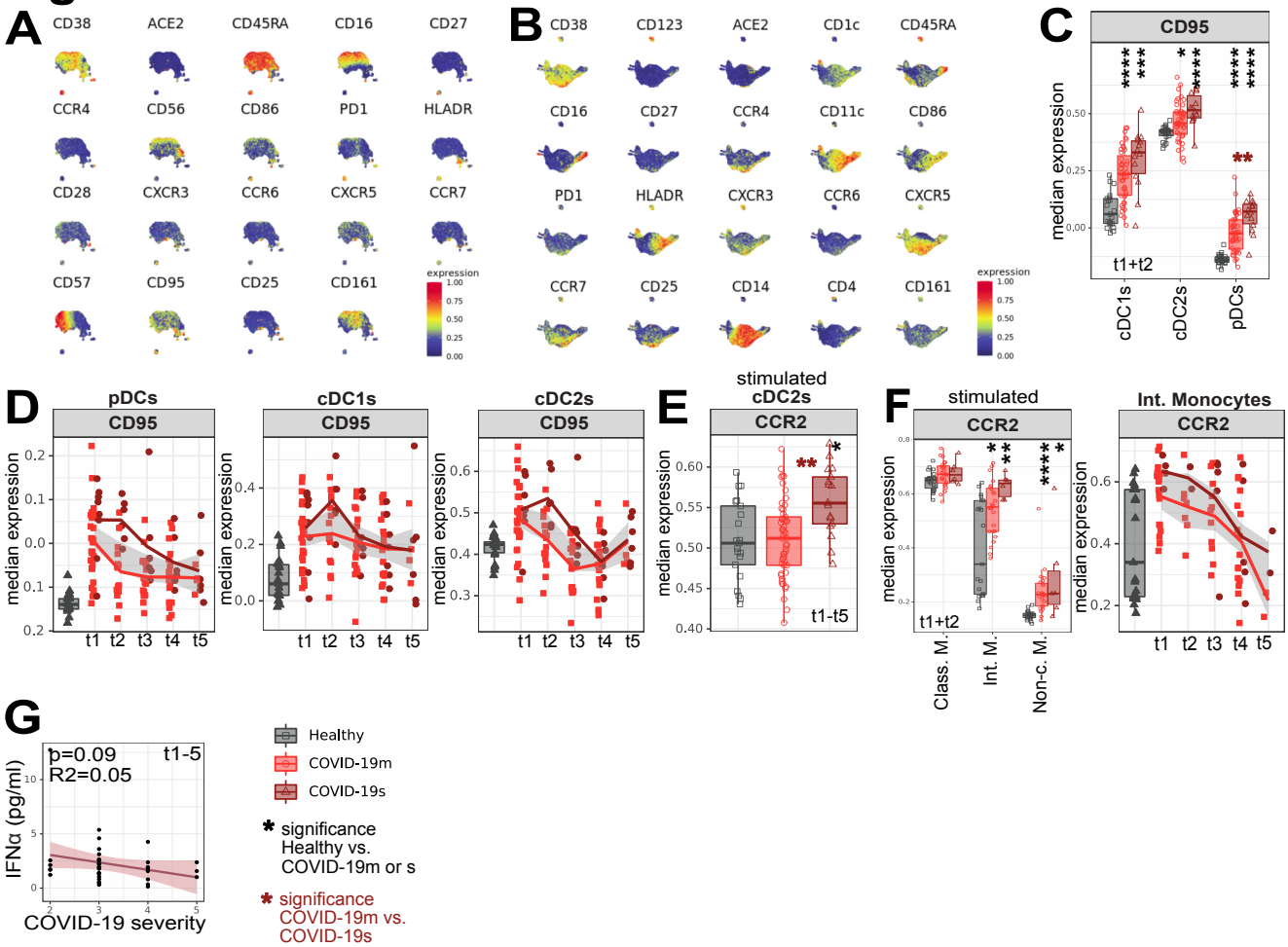
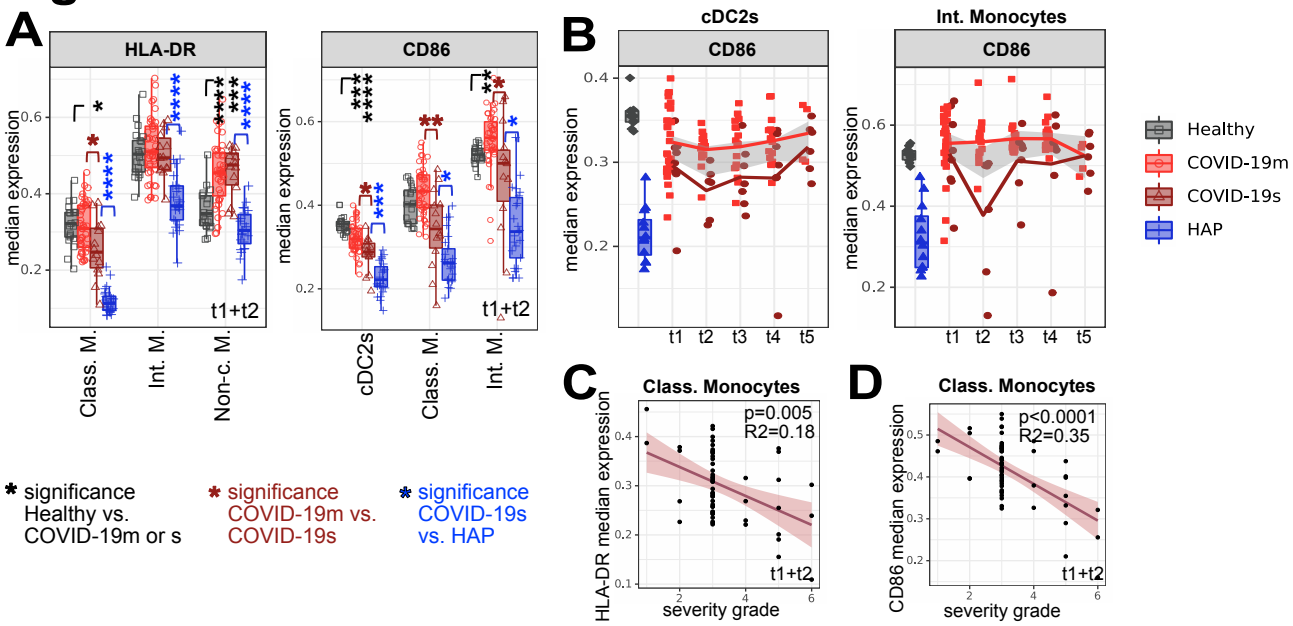

A
CD38
ACE2
CD45RA
CD16
CD27
CCR4
CD56
CD86
PD1
HLADR
CD28
CXCR3
CCR6
CXCR5
CCR7
CD57
CD95
CD25
CD161
expression
B
CD38
CD123
ACE2
CD1c
CD45RA
CD16
CD27
CCR4
CD11c
CD86
PD1
HLADR
CXCR3
CCR6
CXCR5
CCR7
CD25
CD14
CD4
CD161
expression
C
CD95
median expression
t1+t2
cDC1s
cDC2s
pDCs
D
pDCs
CD95
cDC1s
CD95
cDC2s
CD95
median expression
t1 t2 t3 t4 t5
E
stimulated cDC2s
CCR2
median expression
t1-t5
F
stimulated
CCR2
median expression
t1+t2
Class. M.
Int. M.
Non-c. M.
Int. Monocytes
CCR2
median expression
t1 t2 t3 t4 t5
G
p=0.09
R2=0.05
t1-5
IFNα (pg/ml)
COVID-19 severity
Healthy
COVID-19m
COVID-19s
* significance Healthy vs. COVID-19m or s
* significance COVID-19m vs. COVID-19s

Figure S4



* significance Healthy vs. COVID-19m or s

* significance COVID-19m vs. COVID-19s

* significance COVID-19s vs. HAP

Figure S5

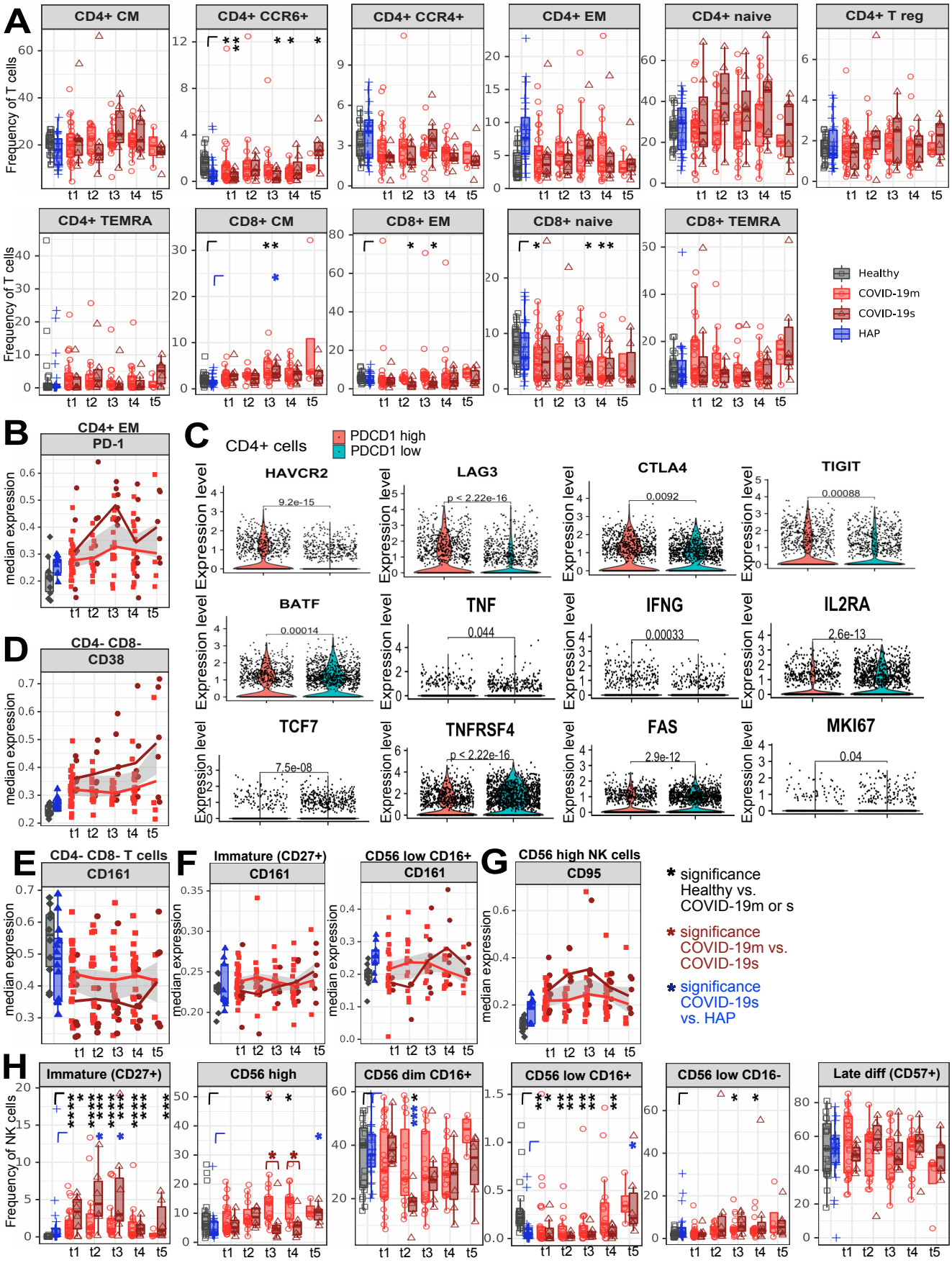
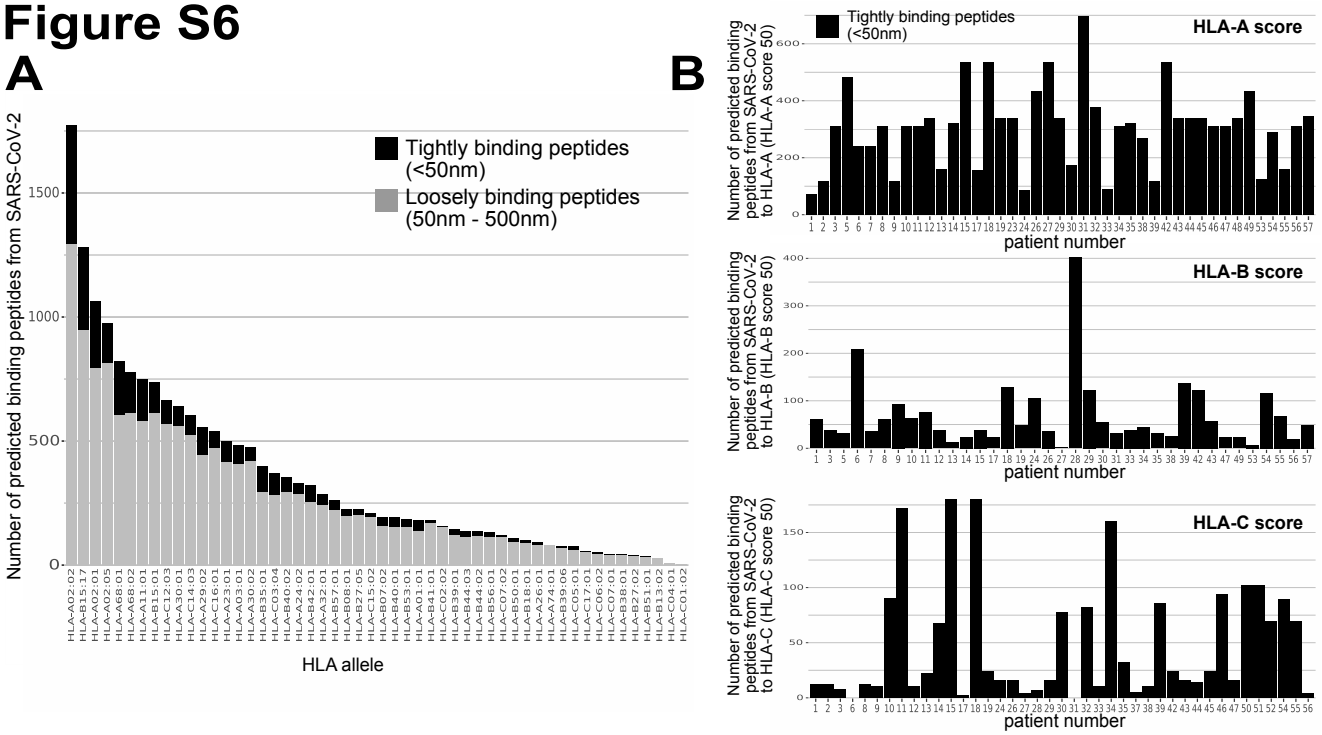
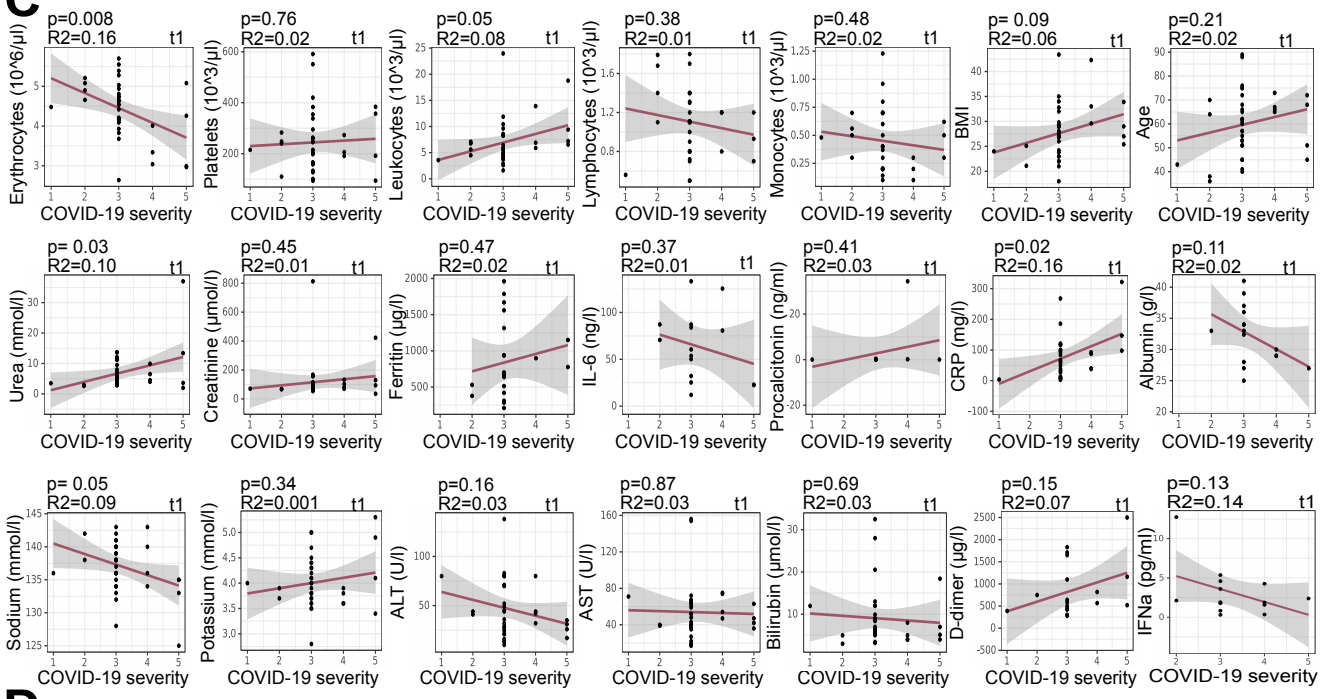

A
CD4+ CM
CD4+ CCR6+
CD4+ CCR4+
CD4+ EM
CD4+ naive
CD4+ T reg
CD4+ TEMRA
CD8+ CM
CD8+ EM
CD8+ naive
CD8+ TEMRA
Frequency of T cells
t1 t2 t3 t4 t5
Healthy
COVID-19m
COVID-19s
HAP
B
CD4+ EM
PD-1
median expression
C
CD4+ cells
PDCD1 high
PDCD1 low
HAVCR2
9.2e-15
LAG3
p < 2.22e-16
CTLA4
0.0092
TIGIT
0.00088
BATF
0.00014
TNF
0.044
IFNG
0.00033
IL2RA
2.6e-13
TCF7
7.5e-08
TNFRSF4
p < 2.22e-16
FAS
2.9e-12
MKI67
0.04
Expression level
D
CD4- CD8-
CD38
median expression
E
CD4- CD8- T cells
CD161
median expression
F
Immature (CD27+)
CD161
CD56 low CD16+
CD161
median expression
G
CD56 high NK cells
CD95
median expression
* significance
Healthy vs.
COVID-19m or s
* significance
COVID-19m vs.
COVID-19s
* significance
COVID-19s
vs. HAP
H
Immature (CD27+)
CD56 high
CD56 dim CD16+
CD56 low CD16+
CD56 low CD16-
Late diff (CD57+)
Frequency of NK cells
t1 t2 t3 t4 t5

Figure S6


A
Tightly binding peptides (<50nm)
Loosely binding peptides (50nm - 500nm)
Number of predicted binding peptides from SARS-CoV-2
HLA allele
B
Tightly binding peptides (<50nm)
HLA-A score
HLA-B score
HLA-C score
Number of predicted binding peptides from SARS-CoV-2 to HLA-A (HLA-A score 50)
Number of predicted binding peptides from SARS-CoV-2 to HLA-B (HLA-B score 50)
Number of predicted binding peptides from SARS-CoV-2 to HLA-C (HLA-C score 50)
patient number


C
Erythrocytes (10^6/µl)
Platelets (10^3/µl)
Leukocytes (10^3/µl)
Lymphocytes (10^3/µl)
Monocytes (10^3/µl)
BMI
Age
Urea (mmol/l)
Creatinine (µmol/l)
Ferritin (µg/l)
IL-6 (ng/l)
Procalcitonin (ng/ml)
CRP (mg/l)
Albumin (g/l)
Sodium (mmol/l)
Potassium (mmol/l)
ALT (U/l)
AST (U/l)
Bilirubin (µmol/l)
D-dimer (µg/l)
IFNa (pg/ml)
COVID-19 severity

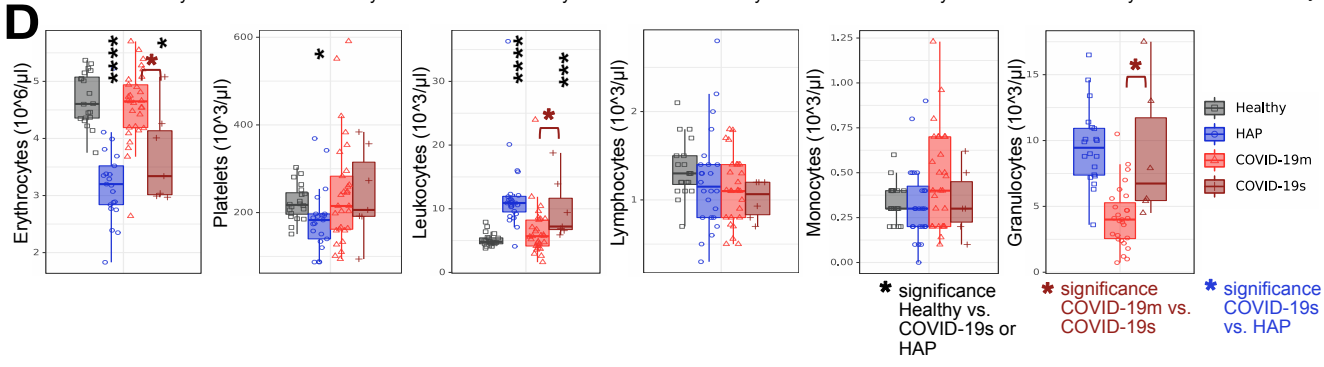
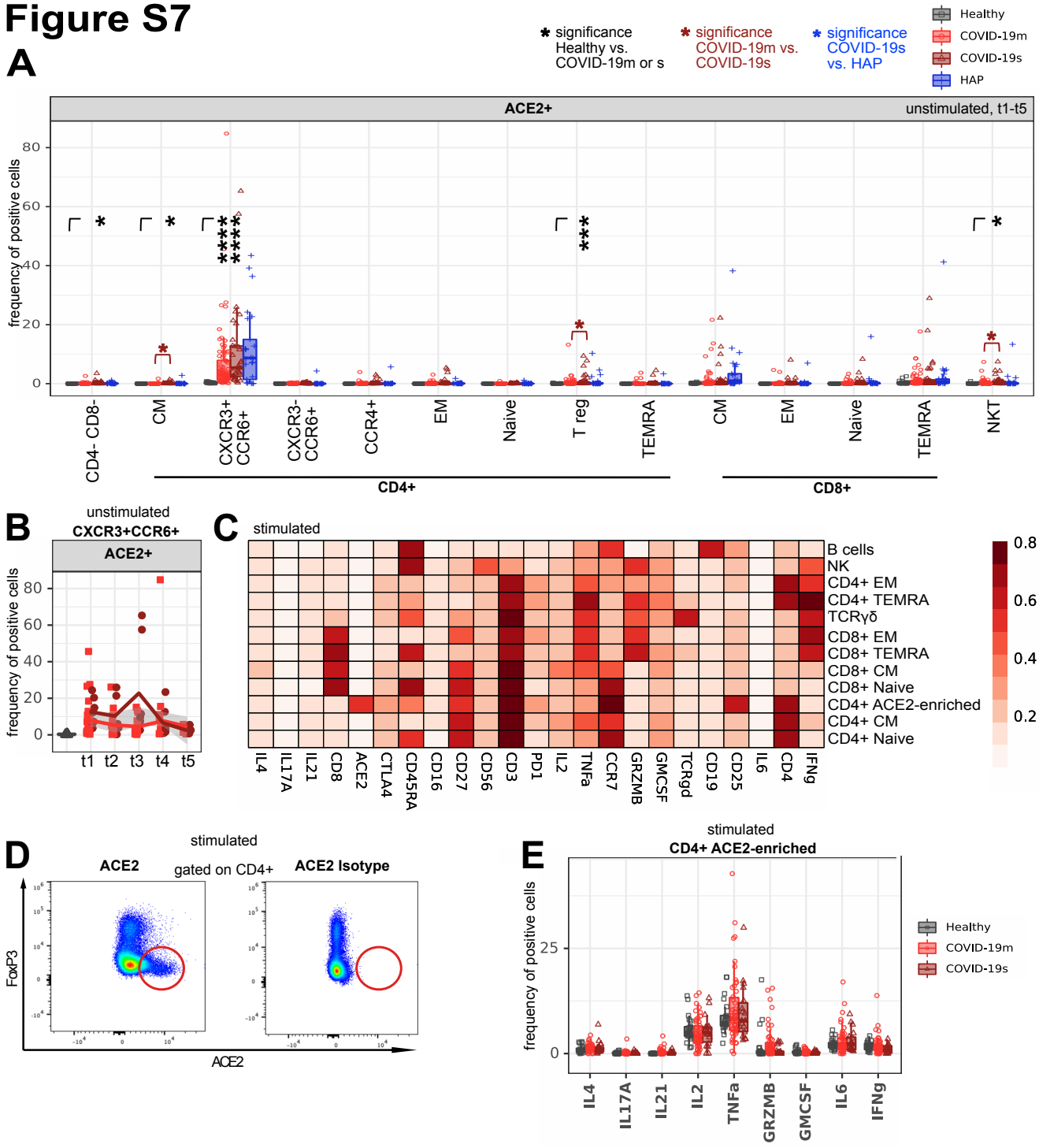

D
Erythrocytes (10^6/µl)
Platelets (10^3/µl)
Leukocytes (10^3/µl)
Lymphocytes (10^3/µl)
Monocytes (10^3/µl)
Granulocytes (10^3/µl)
Healthy
HAP
COVID-19m
COVID-19s
* significance Healthy vs. COVID-19s or HAP
* significance COVID-19m vs. COVID-19s
* significance COVID-19s vs. HAP

Figure S7


A
* significance Healthy vs. COVID-19m or s
* significance COVID-19m vs. COVID-19s
* significance COVID-19s vs. HAP
Healthy
COVID-19m
COVID-19s
HAP
ACE2+
unstimulated, t1-t5
frequency of positive cells
CD4- CD8-
CM
CXCR3+ CCR6+
CXCR3- CCR6+
CCR4+
EM
Naive
T reg
TEMRA
CM
EM
Naive
TEMRA
NKT
CD4+
CD8+
B
unstimulated
CXCR3+CCR6+
ACE2+
t1 t2 t3 t4 t5
C
stimulated
B cells
NK
CD4+ EM
CD4+ TEMRA
TCRγδ
CD8+ EM
CD8+ TEMRA
CD8+ CM
CD8+ Naive
CD4+ ACE2-enriched
CD4+ CM
CD4+ Naive
IL4 IL17A IL21 CD8 ACE2 CTLA4 CD45RA CD16 CD27 CD56 CD3 PD1 IL2 TNFa CCR7 GRZMB GMCSF TCRgd CD19 CD25 IL6 CD4 IFNg
D
stimulated
gated on CD4+
ACE2
ACE2 Isotype
FoxP3
ACE2
E
stimulated
CD4+ ACE2-enriched
frequency of positive cells
IL4 IL17A IL21 IL2 TNFa GRZMB GMCSF IL6 IFNg
Healthy
COVID-19m
COVID-19s

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (referring to Figure 1)

A: Schematic of experimental approach. Stated are the sample numbers from each cohort (COVID-19, HAP and HCs) before and after quality control, for every cytometry panel and in case of COVID-19 for every severity grade per TP.

B: UMAPs showing the total $CD45^{pos}$ compartment of combined samples. Individual plots are overlaid with the expression of included markers. 1000 cells were subsetted from every sample from each cohort.

C: Heatmap depicting median expression of various markers in FlowSOM-derived main population clusters.

D: Box plots depicting median frequencies and 25th and 75th percentile of FlowSOM-generated immune cell cluster. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$, Mann-Whitney test, BH correction).

E: Principle Component (PC) analysis of the total immune compartment based on the markers used in the surface panel and stratified by the sample origin.

F: Principle Component (PC) analysis of the total immune compartment based on the markers used in the surface panel and stratified by sex.

G: Principle Component (PC) analysis of the total immune compartment based on the markers used in the surface panel and stratified by age.

Figure S2 (referring to Figure 2)

A: UMAPs showing the total T cell compartment of combined samples. Individual plots are overlaid with the expression of included markers. 1000 cells were subsetted from every sample from each cohort.

B: Heatmap depicting median expression of relevant markers in FlowSOM-derived T cell clusters.

C: Median expression of PD-1 within indicated FlowSOM-generated T cell subsets of HCs shown in grey and of mild and severe COVID-19 patients across TPs 1-5 shown in red.

D: Box plots depicting median frequency and 25th and 75th percentile of CTLA-4 positive $CD4^{+}$ EM T cells of HCs, COVID-19m and COVID-19s patients. Data from all TPs were

pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, Mann-Whitney test, BH correction).

E-J: Box plots depicting median frequency and 25th and 75th percentile of cytokine positive cells in indicated FlowSOM-generated T and NK cell subsets of HCs, COVID-19m and COVID-19s patients. Data from all TPs were pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

K: Box plots depicting frequency and 25th and 75th percentile of GM-CSF positive T cell subsets of HCs, COVID-19m and COVID-19s patients. Data from all TPs were pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$, Mann-Whitney test, BH correction).

L: Median frequency of GM-CSF positive cells within indicated FlowSOM-generated T cell subsets of HCs shown in grey and of COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

M: Expression levels of *CSF2* (GM-CSF) in indicated cell subsets of COVID-19 patients measured by scRNA-seq.

N: Expression levels of indicated genes in *CSF2* (GM-CSF) high and low $CD4^+$ T cells of COVID-19 patients measured by scRNA-seq.

Figure S3 (referring to Figure 3)

A: UMAPs showing the total NK cell compartment of combined samples. Individual plots are overlaid with the expression of included markers. 1000 cells were subsetted from every sample from each cohort.

B: UMAPs showing the total monocyte and DC compartment of combined samples. Individual plots are overlaid with the expression of included markers. 1000 cells were subsetted from every sample from each cohort.

C: Box plots depicting median expression and 25th and 75th percentile of CD95 in indicated FlowSOM-generated DC subsets. Data from TP 1 and 2 were pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

D: Median expression of CD95 within indicated cell subsets of HCs shown in grey and of COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

E: Box plots depicting median expression and 25th and 75th percentile of CCR2 in FlowSOM-generated cDC2 subset after R848 restimulation. Data from all TPs were pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$ and ** = $p < 0.01$, Mann-Whitney test, BH correction).

F: Box plots depicting median expression and 25th and 75th percentile of CCR2 in FlowSOM-generated monocyte subsets, combined for TP 1 and 2 (left panel) or displayed for every individual TP (right panel). Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

G: Correlation between IFN-α serum protein level against the severity grade of COVID-19 patients. All TPs were pooled.

Figure S4 (referring to Figure 4)

A: Box plots depicting median expression and 25th and 75th percentile of HLA-DR (left panel) and CD86 (right panel) in indicated FlowSOM-generated monocyte or DC subsets. Data from TP 1 and 2 were pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

B: Median expression of CD86 within cDC2s (left panel) or intermediate monocytes (right panel). Boxplot of HCs shown in grey, HAP in blue and COVID-19m and COVID-19s patients shown in red across TPs 1-5.

C: Correlation between median expression of HLA-DR in classical monocytes (TP 1 and 2 pooled) against the severity grade of COVID-19 patients.

D: Correlation between median expression of CD86 in classical monocytes (TP 1 and 2 pooled) against the severity grade of COVID-19 patients.

Figure S5 (referring to Figure 5)

A: Box plots depicting median frequency and 25th and 75th percentile of FlowSOM-generated T cell subsets. Significant p values are depicted using an asterisk (* = $p < 0.05$ and ** = $p < 0.01$, Mann-Whitney test, BH correction).

B: Median expression of PD-1 within FlowSOM-generated $CD4^+$ EM T cell subset. Boxplot of HCs shown in grey, HAP patients in blue and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

C: Expression levels of indicated genes in *PDCD1* (PD-1) high and low $CD4^+$ T cells of COVID-19 patients measured by scRNA-seq.

D: Median expression of CD38 within FlowSOM-generated $CD4^-$ $CD8^-$ (TCR$\gamma\delta$ enriched) T cell subset. Boxplot of HCs shown in grey, HAP patients in blue and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

E: Median expression of CD161 within FlowSOM-generated $CD4^-$ $CD8^-$ (TCR$\gamma\delta$ enriched) T cell subset. Boxplot of HCs shown in grey, HAP patients in blue and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

F: Median expression of CD161 within indicated FlowSOM-generated NK cell subsets. Boxplot of HCs shown in grey, HAP patients in blue and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

G: Median expression of CD95 within indicated FlowSOM-generated NK cell subsets. Boxplot of HCs shown in grey, HAP patients in blue and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

H: Box plots depicting median frequency and 25th and 75th percentile of FlowSOM-generated NK cell subsets. Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure S6 (referring to Figure 6)

A: Bar graph demonstrates the predicted number of binding peptides from SARS-CoV-2 per indicated HLA class I allele which occurred in our study population.

B: Bar graphs show the number of predicted tightly binding peptides from SARS-CoV-2 per indicated HLA class I gene (summarized from both alleles) calculated for each individual study patient. The resulting values are further called HLA-A, HLA-B or HLA-C score 50.

C: Correlation between indicated blood values (TP 1 only) as well as age and BMI against the severity grade of COVID-19 patients.

D: Box plots depicting median counts and 25th and 75th percentile of erythrocytes, platelets, leukocytes, lymphocytes, monocytes and granulocytes at TP 1. Significant p values are depicted using an asterisk (* = $p < 0.05$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure S7 (referring to Figure 7)

A: Box plots depicting the median frequency and 25th and 75th percentile of ACE2 positive cells in indicated FlowSOM-generated T cell subsets. All TPs have been pooled. Significant p values are depicted using an asterisk (* = $p < 0.05$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

B: Median frequency of ACE2 positive cells within FlowSOM-generated CXCR3$^+$ CCR6$^+$ (Th1 Th17-enriched) CD4$^+$ T cell subset. Boxplot of HCs shown in grey and COVID-19m and COVID-19s patients across TPs 1-5 shown in red.

C: Complete heatmap depicting the median expression of various markers in FlowSOM-derived clusters of PMA and ionomycin stimulated (5h) samples.

D: Representative flow cytometry plot showing ACE2 and isotype staining (x axis) against FoxP3 staining (y axis) within the CD4$^+$ T cell compartment of PMA and ionomycin stimulated (5h) HC samples.

E: Box plots depicting the median frequency and 25th and 75th percentile of cytokine positive cells in FlowSOM-generated CD4$^+$ ACE2 enriched cluster after PMA and ionomycin stimulation (5h). All TPs have been pooled.