COVID-19 patients share common, corticosteroid-independent features of impaired host immunity to pathogenic molds

Supplementary Methods

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Supplementary Table 1. Preparation of whole blood stimulation tubes for ex-vivo immunoassays.

	Assay	α-CD28	α-CD49d	AfuLy	AfuG	RarLy	RarG	PrS	PHA	RPMI
Concentration in ready-to-use stimulation tubes	n/a	2 µg/mL	2 µg/mL	100 µg/mL	1×10^6 AfuG/mL	100 µg/mL	1×10^6 RarG/mL	1.2 nmol	10 µg/mL	*Ad* 500 µL
Final concentration after injection of 500 µL WB	n/a	1 µg/mL	1 µg/mL	50 µg/mL	5×10^5 AfuG/mL	50 µg/mL	5×10^5 RarG/mL	0.6 nmol	5 µg/mL	n/a
Unstimulated control	AI, Cyt	X	X							X
	II									X
AfuLy stimulation	AI, Cyt	X	X	X						X
AfuG stimulation	II				X					X
RarLy stimulation	AI, Cyt	X	X			X				X
RarG stimulation	II						X			X
PrS stimulation	AI, Cyt	X	X					X		X
	II							X		X
AfuLy + PrS stimulation	AI, Cyt	X	X	X				X		X
AfuG + PrS stimulation	II				X			X		X
Positive control	AI, II, Cyt								X	X

X indicates that the compound was used for the respective condition.

Abbreviations: AfuLy = *A. fumigatus* mycelial lysate, RarLy = *R. arrhizus* mycelial lysate, CD = cluster of differentiation, n/a = not applicable, PrS = SARS-CoV-2 Protein S (Alpha variant), PHA = phytohemagglutinin, RPMI = Roswell Park Memorial Institute medium, WB = whole blood, AI = adaptive immunity, II = innate immunity, Cyt = multiplex cytokine assay.

Supplementary Table 2. Antibodies used for flow cytometric analyses.

Name	Fluorochrome	Clone	Provider	Catalogue number	Panel(s)
CD1c (BDCA-1)	PerCP-Vio700	REA694	Miltenyi Biotec	130-110-538	DC
CD3	PE-Vio615	REA613	Miltenyi Biotec	130-114-520	ID
CD4	VioGreen	REA623	Miltenyi Biotec	130-113-230	T1, T2
CD8	PerCP-Vio700	REA734	Miltenyi Biotec	130-110-682	T1, T2
CD11b	APC	REA713	Miltenyi Biotec	130-110-554	GR
CD14	APC-Vio770	TÜK4	Miltenyi Biotec	130-113-144	ID, DC
CD16	VioGreen	REA423	Miltenyi Biotec	130-113-397	ID, GR
CD19	PE	REA675	Miltenyi Biotec	130-113-646	ID
CD45RA	PE-Vio615	REA1047	Miltenyi Biotec	130-117-745	T2
CD56	FITC	REA196	Miltenyi Biotec	130-114-549	ID
CD62L	PE	REA615	Miltenyi Biotec	130-113-625	GR
CD66b	PerCP-Vio700	REA306	Miltenyi Biotec	130-119-768	ID, GR
CD69	APC-Vio770	REA824	Miltenyi Biotec	130-112-616	T1, T2
CD83	FITC/VioBright515	REA714	Miltenyi Biotec	130-110-507	DC
CD107a (LAMp-1)	APC/R667	REA792	Miltenyi Biotec	130-111-626	T2
CD154	FITC/VioBright515	REA238	Miltenyi Biotec	130-122-800	T1, T2
CD183 (CXCR3)	APC	REA232	Miltenyi Biotec	130-120-450	T1
CD194 (CCR4)	PE	REA279	Miltenyi Biotec	130-120-456	T1
CD196 (CCR6)	PE-Vio615	REA190	Miltenyi Biotec	130-120-459	T1
CD197 (CCR7)	PE	REA546	Miltenyi Biotec	130-119-583	T2
CD253 (TRAIL)	PE-Vio615	REA1113	Miltenyi Biotec	130-119-286	GR, DC
CD279 (PD-1)	PE-Vio770	REA1165	Miltenyi Biotec	130-120-385	T1, T2
CD284 (TLR4)	APC	HTA125	Miltenyi Biotec	130-096-236	DC
Dectin-1	PE	REA515	Miltenyi Biotec	130-121-993	DC
DHR 123	FITC		Sigma-Aldrich	D1054	GR
HLA-DR	VioGreen	REA805	Miltenyi Biotec	130-111-948	DC
Ki-67	AlexaFluor700	Ki-67	BioLegend	350530	T1, T2
Viobility 405/452	PB450/VioBlue		Miltenyi Biotec	130-110-205	ID, T1,T2, GR, DC

Abbreviations: ID = immune cell distribution panel, GR = granulocyte panel, DC = dendritic cells panel, T1 = T cell panel 1, T2 = T cell panel 2. All antibodies/dyes were used at 2% v/v, except for Ki-67 (5% v/v), DHR 123 (1% v/v), and Viobility 405/452 (1% v/v).

Supplementary Table 3. Utilization of blood samples for downstream readouts.

Assay	**Flow cytometry**					**Cytokine assay**	
Panel	**ID**	**T1 & T2**	**GR & DC**	**T1 & T2**	**GR & DC**	**Multiplex**	**Multiplex**
Stimulus	**n/a**	**Afu**	**Afu**	**Rar**	**Rar**	**Afu**	**Rar**
Figures	**1C**	**1D-E** **2C-H**	**4A-C***	**S6**	**5E-F**	**3A-D** **S1** **S2**	**5A-D**
Patient 1 (Alpha)	X	X	X			X	
Patient 2 (Alpha)	X	X	X			X	
Patient 3 (Alpha)	X	X	X			X	
Patient 4 (Alpha)	X	X	X			X	
Patient 5 (Delta)	X	X	X	X		X	
Patient 6 (Delta)	X	X	X	X	X	X	X
Patient 7 (Delta)	X	X	X	X	X	X	X
Patient 8 (Delta)	X	X	X	X	X	X	X
Patient 9 (Delta)	X	X	X	X	X	X	X
Patient 10 (Delta)	X	X	X	X	X	X	X
Patient 11 (Delta)	X	X	X	X	X	X	X
Patient 12 (Delta)	X	X	X	X	X	X	X
Total number of patient samples	12	12	12	8	7	12	7
Control 1	X	X	X *	X	X	X	X
Control 2	X	X	X *	X	X	X	X
Control 3	X		X *			X	
Control 4	X		X *			X	
Control 5	X	X	X *	X	X	X	X
Control 6	X	X	X *	X	X	X	X
Control 7	X	X	X *			X	
Control 8	X	X	X *	X	X	X	X
Control 9	X	X	X	X	X		X
Total number of control samples	9	7	9 (8) *	6	6	8	6

Footnotes and abbreviations on following page.

Footnotes and abbreviations for **Supplementary Table 3**:

X indicates that the subject/sample was included in the respective analyses and figures.

* indicates that the sample was additionally used for co-stimulation experiments with *A. fumigatus* lysate and SARS-CoV-2 protein S.

The fungal killing assays (**Fig. 4D** and **Fig. 5G**), performed after the main analysis, utilized all Delta patient samples and an independent cohort of 6 additional control subjects.

Abbreviations: ID = immune cell distribution panel, T1 = T cell panel 1, T2 = T cell panel 2, GR = granulocyte panel, DC = dendritic cells panel, n/a = not applicable, Afu = *Aspergillus fumigatus*, Rar = *Rhizopus arrhizus*.

Gating strategy

Gating strategy to study global and mold antigen-reactive T-cell responses.

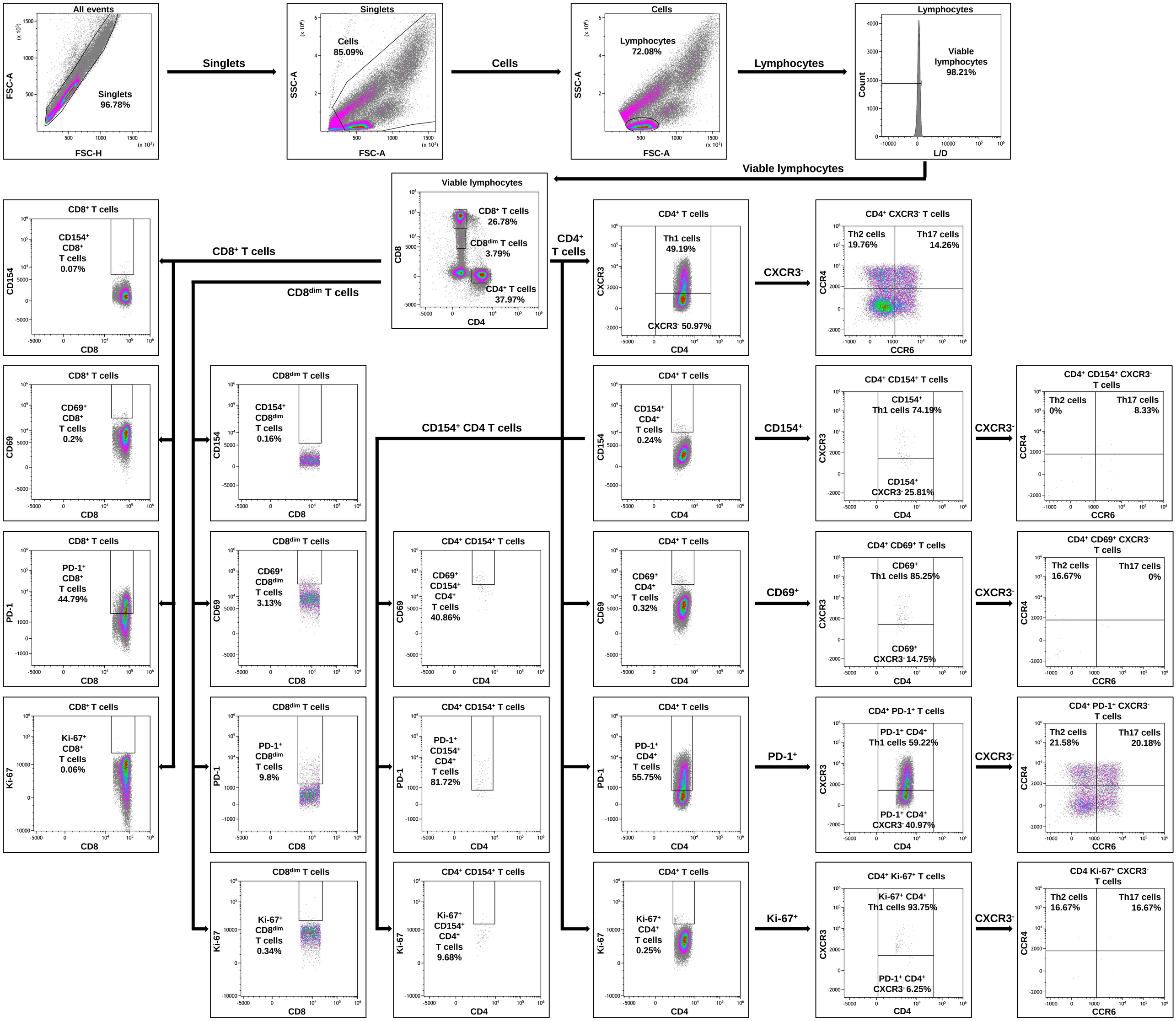
Single cells were gated based on SSC-A, FSC-A, and FSC-H properties. Lymphocytes were identified by light scatter properties and dead lymphocytes were excluded by Live/Dead staining. $CD4^+$, $CD8^{bright}$, and $CD8^{dim}$ cells were differentiated.

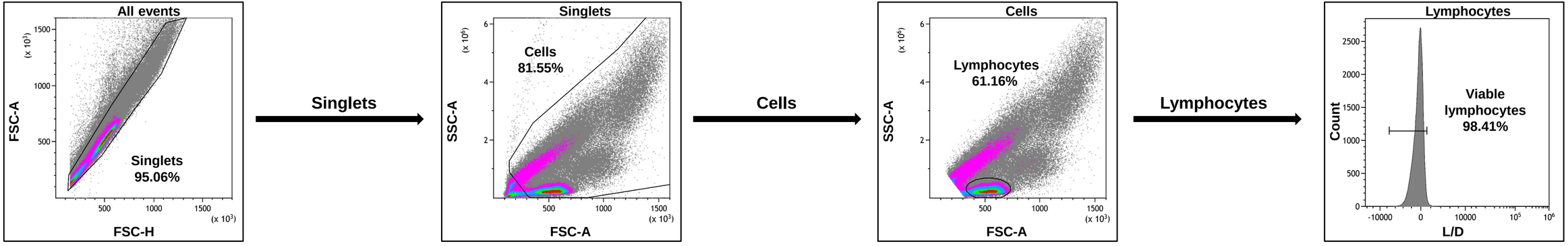
In panel T1 (**1st figure**), positive cells for activation markers CD154 and CD69, exhaustion marker PD-1, and proliferation marker Ki-67 were gated within each T-cell subpopulation. $CD4^+$ T-helper (Th) cells were then subdivided into Th1 ($CXCR3^+$), Th2 ($CXCR3^-$ $CCR6^-$ $CCR4^+$), and Th17 ($CXCR3^-$ $CCR6^+$ $CCR4^+$) cells. Th-cell polarization was also determined for $CD154^+$, $CD69^+$, $PD\text{-}1^+$ and $Ki\text{-}67^+$ Th cells. Mean fluorescence intensity was determined for all markers.

In panel T2 (**2nd figure**), positive cells for activation markers CD154, CD69, and CD107a, exhaustion marker PD-1, and proliferation marker Ki-67 were gated within each T-cell subpopulation. Effector memory T cells (T_{EM}, $CD45RA^-$ $CCR7^-$), central memory T cells (T_{CM}, $CD45RA^-$ $CCR7^+$), effector T cells (T_{EMRA}, $CD45RA^+$ $CCR7^-$) and naïve T cells (T_N, $CD45RA^+$ $CCR7^+$) were differentiated. Mean fluorescence intensity was determined for all markers.

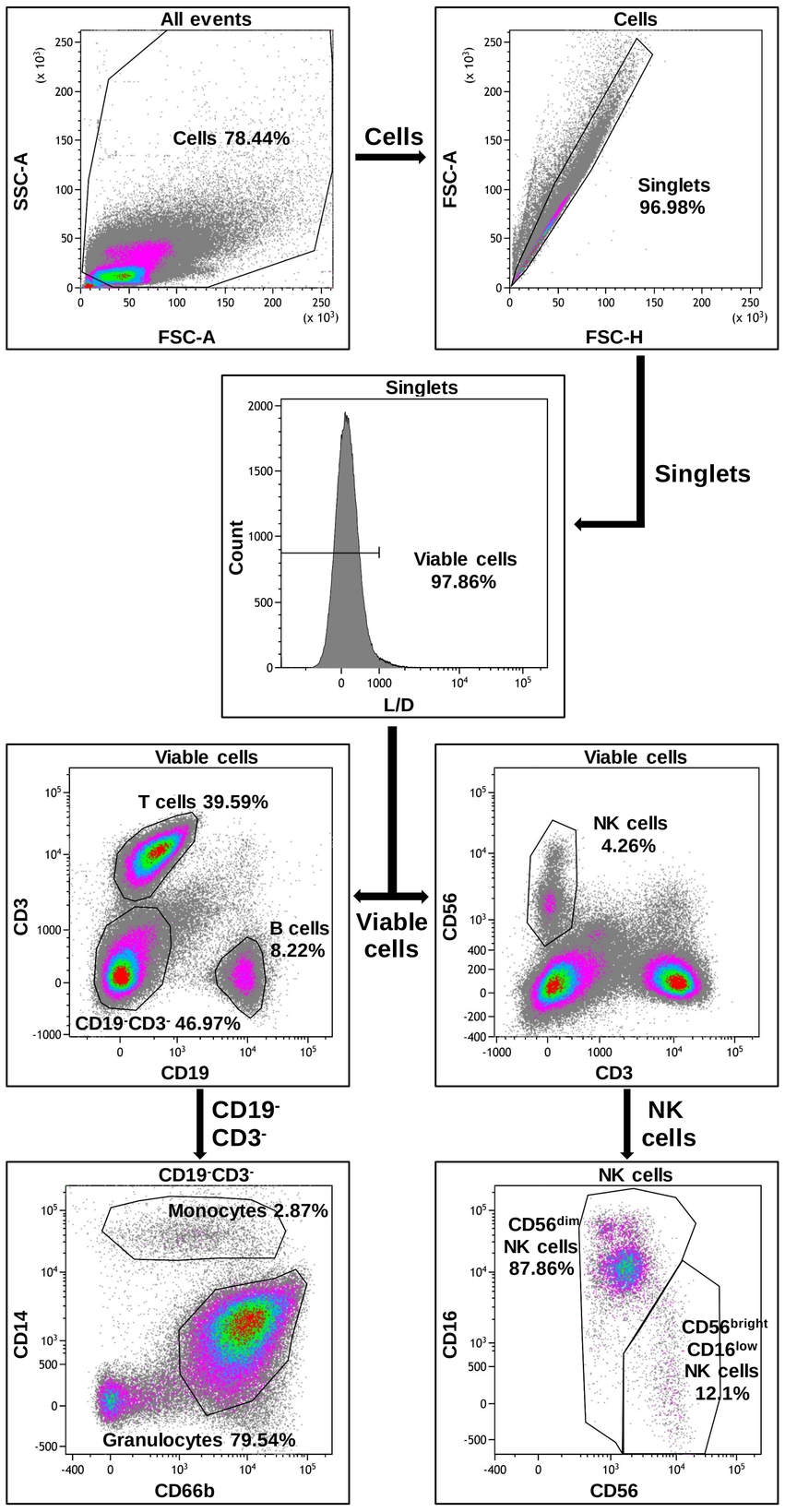
Gating strategy to determine baseline leukocyte distributions and to study mold antigen-induced activation of dendritic cells and granulocytes (3rd figure).

Debris was excluded by light scatter properties and dead cells were excluded by Live/Dead staining. For determination of leukocyte distributions (performed on unstimulated samples), T cells ($CD19^-$ $CD3^+$) and B cells ($CD19^+$ $CD3^-$) were gated. $CD19^-$ $CD3^-$ cells were further subdivided into monocytes ($CD14^+$) and granulocytes ($CD66b^+$). Natural killer (NK) cells were identified as $CD3^-$ $CD56^+$ and subdivided into $CD56^{dim}$ $CD16^+$ NK cells and $CD56^{bright}$ $CD16^{low}$ NK cells. Granulocytes were identified by CD66 expression and analyzed for ROS, CD253, CD16, CD11b, and CD62L expression. Dendritic cells, identified as $CD1c^+$ cells, were analyzed for Dectin-1, CD253, HLA-DR, CD83, CD284 and CD14 expression. Mean fluorescence intensity was determined for all markers.

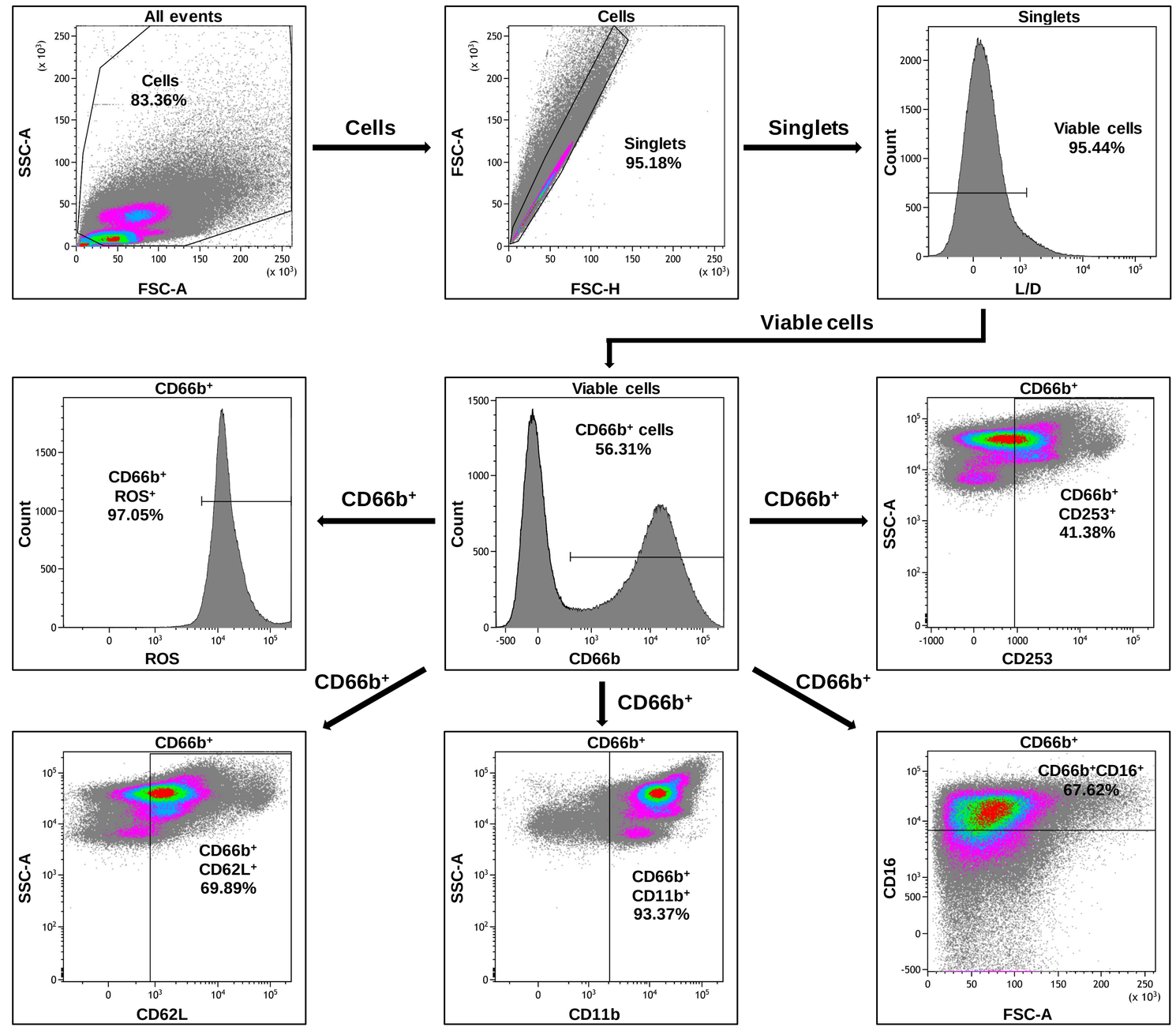

All events
FSC-A
FSC-H
Singlets
96.78%
Singlets
SSC-A
FSC-A
Cells
85.09%
Cells
SSC-A
FSC-A
Lymphocytes
72.08%
Lymphocytes
Count
L/D
Viable lymphocytes
98.21%
Viable lymphocytes
CD8
CD4
CD8+ T cells
26.78%
CD8dim T cells
3.79%
CD4+ T cells
37.97%
CD8+ T cells
CD154
CD8
CD154+ CD8+ T cells
0.07%
CD8dim T cells
CD154
CD8
CD154+ CD8dim T cells
0.16%
CD4+ T cells
CXCR3
CD4
Th1 cells
49.19%
CXCR3- 50.97%
CXCR3-
CD4+ CXCR3- T cells
CCR4
CCR6
Th2 cells
19.76%
Th17 cells
14.26%
CD4+ T cells
CD154
CD4
CD154+ CD4+ T cells
0.24%
CD154+
CD4+ CD154+ T cells
CXCR3
CD4
CD154+ Th1 cells 74.19%
CD154+ CXCR3- 25.81%
CXCR3-
CD4+ CD154+ CXCR3- T cells
CCR4
CCR6
Th2 cells
0%
Th17 cells
8.33%
CD8+ T cells
CD69
CD8
CD69+ CD8+ T cells
0.2%
CD8dim T cells
CD69
CD8
CD69+ CD8dim T cells
3.13%
CD4+ CD154+ T cells
CD69
CD4
CD69+ CD154+ CD4+ T cells
40.86%
CD4+ T cells
CD69
CD4
CD69+ CD4+ T cells
0.32%
CD69+
CD4+ CD69+ T cells
CXCR3
CD4
CD69+ Th1 cells 85.25%
CD69+ CXCR3- 14.75%
CXCR3-
CD4+ CD69+ CXCR3- T cells
CCR4
CCR6
Th2 cells
16.67%
Th17 cells
0%
CD8+ T cells
PD-1
CD8
PD-1+ CD8+ T cells
44.79%
CD8dim T cells
PD-1
CD8
PD-1+ CD8dim T cells
9.8%
CD4+ CD154+ T cells
PD-1
CD4
PD-1+ CD154+ CD4+ T cells
81.72%
CD4+ T cells
PD-1
CD4
PD-1+ CD4+ T cells
55.75%
PD-1+
CD4+ PD-1+ T cells
CXCR3
CD4
PD-1+ Th1 cells 59.22%
PD-1+ CD4+ CXCR3- 40.97%
CXCR3-
CD4+ PD-1+ CXCR3- T cells
CCR4
CCR6
Th2 cells
21.58%
Th17 cells
20.18%
CD8+ T cells
Ki-67
CD8
Ki-67+ CD8+ T cells
0.06%
CD8dim T cells
Ki-67
CD8
Ki-67+ CD8dim T cells
0.34%
CD4+ CD154+ T cells
Ki-67
CD4
Ki-67+ CD154+ CD4+ T cells
9.68%
CD4+ T cells
Ki-67
CD4
Ki-67+ CD4+ T cells
0.25%
Ki-67+
CD4+ Ki-67+ T cells
CXCR3
CD4
Ki-67+ Th1 cells 93.75%
PD-1+ CD4+ CXCR3- 6.25%
CXCR3-
CD4 Ki-67+ CXCR3- T cells
CCR4
CCR6
Th2 cells
16.67%
Th17 cells
16.67%


All events
FSC-A
FSC-H
Singlets
95.06%
Singlets
Singlets
SSC-A
FSC-A
Cells
81.55%
Cells
Cells
Lymphocytes
61.16%
Lymphocytes
Lymphocytes
Count
L/D
Viable lymphocytes
98.41%
Viable lymphocytes
Viable lymphocytes
CD8
CD4
CD8+ T cells
27.58%
CD8dim T cells
3.76%
CD4+ T cells
38.63%
CD8+ T cells
CD4+ T cells
CD8dim T cells
CD8+ T cells
CCR7
CD45RA
CM 2.64%
N 2.02%
EM 61.87%
EMRA
33.47%
CD8dim T cells
CCR7
CD45RA
CM 2%
N 3.55%
EM 15.05%
EMRA
79.4%
CD4+ T cells
CCR7
CD45RA
CM 29.17%
N 11.54%
EM 57.47%
EMRA
1.81%
CD4+ T cells
CD154
CD4
CD154+
CD4+
T cells
0.23%
CD4+ CD154+ T cells
CCR7
CD45RA
CM 55%
N 13.33%
EM 30.83%
EMRA
0.83%
CD8+ CD69+ T cells
CCR7
CD45RA
CM 15.28%
N 11.11%
EM 52.78%
EMRA
20.83%
CD8+ T cells
CD69
CD8
CD69+
CD8+
T cells
0.19%
CD8dim T cells
CD69
CD8
CD69+
CD8dim
T cells
3.23%
CD8dim CD69+ T cells
CCR7
CD45RA
CM 7.36%
N 10.43%
EM 7.98%
EMRA
74.23%
CD4+ T cells
CD69
CD4
CD69+
CD4+
T cells
0.31%
CD4+ CD69+ T cells
CCR7
CD45RA
CM 44.44%
N 6.17%
EM 48.77%
EMRA
0.62%
CD8+ PD-1+ T cells
CCR7
CD45RA
CM 1.76%
N 1.78%
EM 62.29%
EMRA
34.17%
CD8+ T cells
PD-1
CD8
PD-1+
CD8+
T cells
44.88%
CD8dim T cells
PD-1
CD8
PD-1+
CD8dim
T cells
9.77%
CD8dim PD-1+ T cells
CCR7
CD45RA
CM 7.91%
N 23.94%
EM 42.6%
EMRA
25.56%
CD4+ T cells
PD-1
CD4
PD-1+
CD4+
T cells
55.41%
CD4+ PD-1+ T cells
CCR7
CD45RA
CM 21.26%
N 1.6%
EM 75.62%
EMRA
1.52%
CD8+ Ki-67+ T cells
CCR7
CD45RA
CM 12.5%
N 4.17%
EM 75%
EMRA
8.33%
CD8+ T cells
Ki-67
CD8
Ki-67+
CD8+
T cells
0.06%
CD8dim T cells
Ki-67
CD8
Ki-67+
CD8dim
T cells
0.34%
CD8dim Ki-67+ T cells
CCR7
CD45RA
CM 17.65%
N 11.76%
EM 52.94%
EMRA
17.65%
CD4+ T cells
CD154
CD4
Ki-67+
CD4+
T cells
0.25%
CD4+ Ki-67+ T cells
CCR7
CD45RA
CM 33.33%
N 12.4%
EM 50.39%
EMRA
3.88%
CD8+ CD107a+ T cells
CCR7
CD45RA
CM
0.76%
N 1.24%
EM 46.32%
EMRA
51.68%
CD8+ T cells
CD107a
CD8
CD107a+
CD8+
T cells
60.77%
CD8dim T cells
CD107a
CD8
CD107a+
CD8dim
T cells
84.46%
CD8dim CD107a+ T cells
CCR7
CD45RA
CM 0.99%
N 2.72%
EM 9.23%
EMRA
87.07%
CD4+ T cells
CD107a
CD4
CD107a+
CD4+
T cells
2.09%
CD4+ CD107a+ T cells
CCR7
CD45RA
CM 4.43%
N 1.01%
EM 86.53%
EMRA
8.03%

Immune cell distribution (Flow panel ID)



Granulocytes (Flow panel GR)



Dendritic cells (Flow panel DC)