Online Data Supplement

Healthy versus Inflamed Lung Environments Differentially Effect MSCs

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SUPPLEMENTARY METHODS

BALF samples

BALF samples from ARDS patients without sepsis, with an APACHE II score >25, were collected prospectively as part of an unrelated clinical investigation conducted by the National Heart Lung Blood Institute (NHLBI) ARDSNET (ClinicalTrials.gov NCT0011216) [26]. ARDS BALF samples were collected approximately 48 hours after enrollment under appropriate approved IRB protocols (UCSF) at the different participating institutions. For one of the complement ELISA and one of the flow cytometry measurements, ARDS BALF obtained on study day 3 were used, for all other studies ARDS BALF obtained on study day 1 was utilized. These samples were subsequently made available for the analyses in the current studies. Control BALF samples were obtained from healthy volunteers at Dartmouth under appropriate institutional IRB protocols. The BALF samples were centrifuged to pellet cells and debris and cell-free supernatants were stored at -20°C or -70°C prior to use in the current studies. All BALF samples were de-identified and numerically coded. Only limited clinical information is available on the patient samples.

Ex vivo exposure of hMSCs

Human MSCs (hMSCs) were obtained from the NHLBI's Production Assistance for Cellular Therapies (PACT) program (University of Minnesota) and routinely cultured in MEM/EBSS media (Hyclone) supplemented with 20% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Lonza) in standard tissue culture incubators. The hMSCs utilized were obtained from a single volunteer (except for the complement ELISA and HLA the flow cytometry measurements in which bone marrow-derived hMSCs from a second donor were also utilized) and were the same as those utilized in the recent START trial of systemic MSC administration in ARDS patients [3, 4]. Cells at passage 3-5 were used for experiments. For exposures, hMSCs were seeded (200,000 cells/6-well) in normal growth media and allowed to attach overnight. Following adherence, cells were washed and synchronized for 24 hours in serum free media (MEM/EBSS). After synchronization, media was replaced with individual ARDS or healthy control BALF samples diluted into serum free media (20% BALF final concentration as delineated in prior studies [11, 13]. Serum free media with 20% PBS was added to unstimulated hMSCs, and serum free media only was added to control hMSCs. After 24 hours incubation (37°C), media was aspirated, cells washed with PBS, 1 ml serum free media added, and cells incubated for an additional 24 hours (37°C). Conditioned media was then collected for different analyses and cells lysed with TRIzol Reagent for RNA sequencing and other analyses.

BALF Assessments

Total protein was measured using Pierce BSA Protein Assay Kit (Thermo Scientific) (ARDS: n=6, healthy control: n=15). Total dsDNA content was measured using a Qubit Fluorometer dsDNA HS (10pg/ul-100ng/ul) assay (ARDS: n= 9, Healthy controls: n=15). Levels of pro- and anti-inflammatory cytokines were assessed using a Luminex assay as further described below. Some of the data from healthy control samples have also been presented in a previous

publication and are used here for comparison purposes [13]. IFN-γ was measured using ELISA MAX TM as further described below.

Cytotoxicity

Following BALF exposure, hMSC viability was assessed using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) according to manufacturer's instructions. Briefly, conditioned media samples were analyzed for LDH activity and absorbance was measured at 490 nm and 680 nm using a spectrophotometer plate reader. hMSCs exposed to serum free media only served as negative control, Triton X-100-exposed hMSCs served as positive control, and serum free media served as background control. An AMG EVOS Cell Imaging System was utilized to obtain photomicrographs of cells following exposures to assess qualitative appearance. All samples and controls were analyzed in duplicate.

Mitochondrial Respiration

For real-time analysis of the oxygen consumption rate (OCR), hMSCs pre-exposed (24 hours) to BALF samples, PBS, or serum free media were analyzed using an XF-96^{e} Extracellular Flux Analyzer (Seahorse/Agilent, Bioscience). Briefly, hMSCs were seeded into XF-96^{e} Seahorse cell culture microplates (5 x 10^4 cells/well in 100 ul normal hMSC growth media) and allowed to attach overnight. After attachment, cells were either stimulated for 24 hours with individual ARDS (n=9) or healthy control (n=8) BALF samples (20% in serum free media), 20% PBS in serum free media (n=3), or serum free media (n=3). Prior to analyses, cells were equilibrated in a non-CO_2 incubator with XF running buffer (non-buffered DMEM supplemented with 5mM glucose and 2mM L-glutamine) and XF-96^{e} probe plates were hydrated and prepared according to manufacturer's instructions. OCR rates were measured under basal conditions and in response to 1µM Oligomycin, 1.5µM fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 100nM Rotenone-Antimycin A. For the data acquisition protocol, three baseline reads were

recorded, followed by injection of Oligomycin treatment, followed by the second injection of FCCP, followed by Rotenone/Antimycin-A, followed by 27 final reads. Data were analyzed using Wave software 2.6.1 (Agilent) and all samples were analyzed in 3-4 technical replicates.

Cytokine Analyses

Conditioned media and BALF samples were analyzed using a human magnetic Luminex Assay kit (R&D Systems). All samples were diluted 1:2 and analyzed using Bio-Rad Bioplex Analyzer in duplicate according to manufacturer's instructions. Analytes included ADAMTS13, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CXCL8/IL-8, CD44, FAS, FAS Ligand, G-CSF, GM-CSF, HGF, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-18, IL-36β/IL-1F8, Leptin, MIF, MMP-3, Osteopontin, SP-D, and TNF-α as previously described [13]. Samples included BALF samples (ARDS patients (n=15) and healthy controls (n=15)) and conditioned media from ARDS BALF-exposed hMSCs (n=7), healthy control BALF-exposed hMSCs (n=10), and PBS-exposed hMSCs (n=7). Extrapolated values are presented as mean with SD and values out of range below were set to 1.0. The cytokine data from BALF samples of 7 of the healthy control subjects have been used as a control group in a previous publication [13]. Cytokine values detected in conditioned media were log2 normalized in order to fit on the same scale.

IFN-γ was measured using ELISA MAX TM (BioLegend) on ARDS (n=15) and healthy control (n=14) BALF samples and on conditioned media from hMSCs exposed to ARDS BALF (n=3) and healthy control BALF (n=5) BALF or PBS (n=4) according to manufacturer's instructions.

Complement Assays

The complement cascade activation product c3a des (Arg) was measured in conditioned media collected from hMSCs exposed for 24 hrs to BALF samples or PBS 24 hours using an ELISA assay (Enzo LifeSciences; ADI-900-058) following the manufacturers' instructions.

Flow Cytometry

hMSCs were harvested after exposure to BALF samples (HC and ARDS) or PBS 24 hours and used for flow-cytometry analysis. The cells were fixed with 4% PFA solution and stained for DAPI (1:2500 dilution from 5mg/ml stock solution), HLA-ABC antibody conjugated to FITC (Biolegend; #311404), and HLA-DR antibody conjugated to AlexaFluor 647 (Biolegend; #307622) at the concentration recommended by manufacturers' protocol. The samples were run on BDLSRII Flow Cytometer and analyzed in FlowJo.

Statistical Analyses

Shapiro-Wilk test was used for normality test and data was presented as mean ± SD (normal distribution) or media ± IQR (non-normal distribution). Non-parametric Mann-Whitney test was used to assess differences between two groups. Kruskal-Wallis tests (Dunn's post hoc test) or one-way ANOVA (Dunnett's post hoc test) were used to assess differences between three or more groups. Statistical analyses were performed using GraphPad Prism software. P-values ≤ 0.05 were considered as significant, except in the case of RNA sequencing data analyzed in edgeR, where a multiple hypothesis corrected false discovery rates (FDR) less than 0.05 were considered significant. Spearman correlations were calculated in base R, using the t distribution to calculate p-values in those cases that included ties in rank.

Supplementary Table 1. Delineation of each sample and how it was utilized in this study

Sample #	Status	Location	Study
1	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
2	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
3	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
4	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry, and C3a ELISA
5	HC	Dartmouth	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM
6	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, Flow cytometry, and C3a ELISA
7	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
8	HC	Dartmouth	CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
9	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
10	HC	Dartmouth	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
11	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF
12	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA, Flow cytometry, and C3a ELISA
13	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
14	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF
15	HC	Dartmouth	BALF luminex, dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry, and C3a ELISA
16	ARDS	UCSF	BALF luminex, LDH, stimulation exp., dsDNA, osmolality, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
17	ARDS	UCSF	BALF luminex, dsDNA, osmolality, IFNγ BALF
18	ARDS	Fresno	RNA seq, CM luminex, BALF luminex, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3), Flow cytometry, and C3a ELISA
19	ARDS	Fresno	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3)
20	ARDS	Fresno	BALF luminex, Seahorse, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
21	ARDS	Fresno	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, IFNγ BALF
22	ARDS	Fresno	BALF luminex, IFNγ BALF, Flow cytometry, and C3a ELISA
23	ARDS	Fresno	BALF luminex, Seahorse, IFNγ BALF
24	ARDS	OHSU	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
25	ARDS	Oregon	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF
26	ARDS	Stanford	BALF luminex, IFNγ BALF
27	ARDS	USC	BALF luminex, IFNγ BALF
28	ARDS	USC	CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF, Flow cytometry (day3), and C3a ELISA (day3)
29	ARDS	USC	RNA seq, CM luminex, BALF luminex, Seahorse, LDH, stimulation exp., dsDNA, osmolality, total protein, IFNγ BALF and CM, Flow cytometry (day3), and C3a ELISA (day3)
30	ARDS	Bstate	BALF luminex, Seahorse, IFNγ BALF
31	ARDS	Oregon	Flow cytometry (day3), and C3a ELISA (day3)
32	ARDS	Oregon	Flow cytometry, and C3a ELISA
33	ARDS	Yale	Flow cytometry, and C3a ELISA
34	ARDS	Stanford	Flow cytometry, and C3a ELISA
35	ARDS	Stanford	Flow cytometry, and C3a ELISA
36	ARDS	USC	Flow cytometry, and C3a ELISA
37	ARDS	Bstate	Flow cytometry, and C3a ELISA

HC, healthy control subjects; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; RNA Seq, RNA sequencing; CM, conditioned media; LDH, lactate dehydrogenase; dsDNA, double stranded DNA; IFNγ, Interferon gamma; Dartmouth, Dartmouth Hitchcock Medical Centre; UCSF, University of California, San Francisco; Fresno, University of California Fresno; USC, Univ. of Southern California; OHSU, Oregon Health & Science University; Stanford, Stanford University; Bstate, Bay State Medical Center, Day3, samples collected on study day 3.

Supplementary Table 2. Mediators detected in BALF from ARDS patients and healthy controls

Cytokine	Cytokines in BALF (pg/ml)		Mann-Whitney
	HC BALF	ARDS BALF	
	Mean (SD)	*Mean (SD)*	*P-value*
ADAMTS13	606 (2342)	40454 (49330)	<0.0001 (****)
CXCL8/IL-8	85 (69)	3540 (2487)	<0.0001 (****)
Fas Ligand	3.1 (2.3)	20 (30)	0.0143 (*)
GM-CSF	4.4 (3.5)	55 (157)	0.0100 (*)
IL-10	3.4 (5.2)	2.0 (2.3)	0.5545
IL-13	25 (93)	843 (2140)	0.2241
IL-2	1.0 (0.0)	34 (34)	0.0002 (***)
IL-4	34 (15)	50 (40)	0.3295
Leptin	1.0 (0.0)	736 (1908)	0.2241
MIF	20415 (11140)	100999 (150610)	0.0408 (*)
CCL4	210 (167)	50575 (192241)	0.0006 (***)
Osteopontin	5926 (860)	47322 (129511)	<0.0001 (****)
TNF-α	2.0 (1.3)	42 (125)	0.0017 (**)
CD44	356 (215)	1099 (1557)	0.0408 (*)
Fas	99 (111)	2509 (3626)	<0.0001 (****)
G-CSF	50 (48)	505 (569)	0.0497 (*)
HGF	18 (9.9)	1081 (1605)	<0.0001 (****)
IL-1β	7.7 (8.6)	151 (377)	0.0068 (**)
IL-12 p70	43 (42)	86 (70)	0.0825
IL-18	34 (31)	67 (77)	0.2854
IL-36β	7.5 (3.9)	8.1 (5.7)	0.9589
IL-6	3.6 (5.8)	1268 (2599)	<0.0001 (****)
CCL2	68 (41)	4667 (5018)	<0.0001 (****)
CCL3	171 (98)	617 (981)	0.0168 (*)
MMP-3	16 (22)	988 (798)	<0.0001 (****)
SP-D	89435 (36372)	38750 (50805)	0.0017 (**)
IFN-γ #	4.6 (7.7)	25 (40)	0.1751

Other mediators	HC BALF	ARDS BALF	Unpaired t-test
	Mean (SD)	*Mean (SD)*	*P-value*
dsDNA (ng/ul)	0.3 (0.1)	10.2 (9.9)	0.0007 (***)
Total Protein (ug/ml)	110 (73.6)	1894 (2007)	0.0020 (**)

Cytokines detected in clinical ARDS BALF samples (n=15) and healthy control subjects (n=15) using 27-plex Luminex assay. Samples were analyzed on two luminex plates. Mean with SD of extrapolated values are presented and statistical analysis was performed by Mann-Whitney test. Values out of range below were set to 1.0. IFN-γ was measured on a separate ELISA on BALF samples from ARDS patients (n=15) and healthy control subjects (n=14) and presented as mean with SD and statistical analysis was performed by Mann-Whitney test. To further assess what inflammatory mediators might be present in ARDS BALF samples, dsDNA content (HC: n=15 and ARDS: n=9) and total protein (HC: n=15 and ARDS: n=6), were assessed and compared to those found in BALF obtained from normal healthy controls. Data are presented as mean with SD and statistical analysis was performed by unpaired t-test. BALF: bronchoalveolar lavage fluid; ARDS: acute respiratory distress syndrome; HC: healthy control subjects; # IFN-γ was measured on a separate ELISA; SD: standard deviation; * $p < 0.05$; **; $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Supplementary Table 3. Correlations of IL-1β in BALF with cytokines detected in conditioned media

Cytokines in CM	Pearson-correlating ranks	Number	
	P-value	HC	ARDS
IL-6	0.0173 (*)	10	6
IL-36	0.0334 (*)	8	5
IL-2	0.0340 (*)	8	5
MMP-3	0.0034 (**)	8	5
FAS	0.0427 (*)	9	5
IL-8	0.0346 (*)	10	6

IL-1β levels (ranked) detected in BALF samples were Pearson correlated with IL-6, IL-36, IL-2, MMP-3, FAS, and CXCL8/IL-8 levels (ranked) detected in conditioned media from BALF-exposed hMSCs. CM: conditioned media, HC: healthy control, ARDS: acute respiratory distress syndrome.

Supplementary Table 4. ACE2 and TMPRSS2 expression in BALF- and PBS-exposed hMSCs

Gene	CPM values (log2)		
	PBS	HC BALF	ARDS BALF
	Mean (SD)	*Mean (SD)*	*Mean (SD)*
ACE2	-3.16 (0.359)	-3.17 (0.423)	-3.37 (0.219)
TMPRSS2	-2.01 (1.07)	-1.58 (1.32)	-1.90 (0.469)

ACE2 and TMPRSS2 gene expression exposed by PBS-exposed (n=3), healthy control BALF-exposed, and ARDS BALF-exposed (n=) hMSCs. CPM, counts per million; PBS, Phosphate-Buffered Saline; HC, Healthy control, ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; ACE2, Angiotensin-converting enzyme 2; TMPRSS2, Transmembrane protease, serine 2.

SUPPLEMENTARY FIGURE LEGENDS

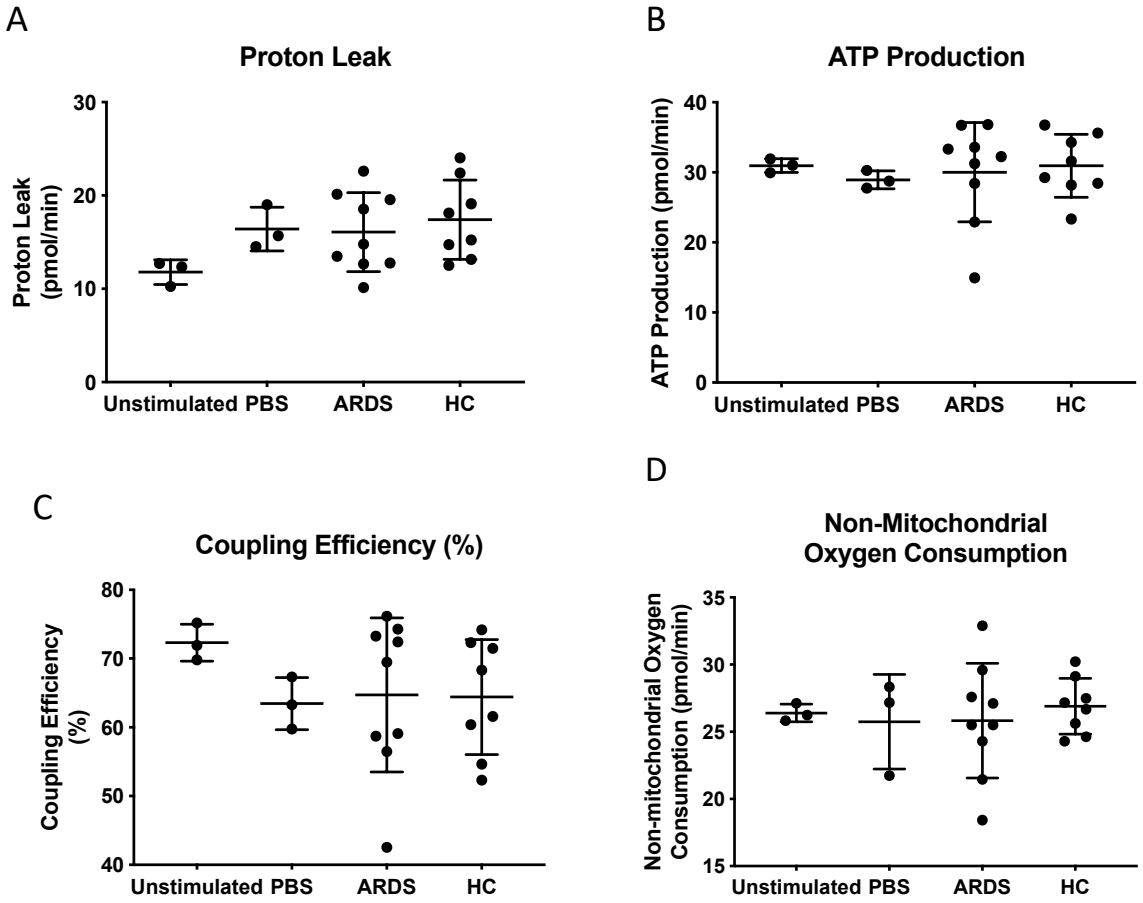


Figure S1. No differences in mitochondrial function. To assess the impact of ARDS and healthy control BALF samples on hMSC mitochondrial function, proton leak (**A**), ATP production (**B**), coupling efficiency (%) (**C**), and non-mitochondrial oxygen consumption (**D**) was measured in hMSCs pre-exposed (24 hours) utilizing XF-96e Extracellular Flux Analyzer. Data are presented as means ± SD and statistical analysis was performed by Shapiro-Wilk test, followed by a one-way ANOVA with Dunnett's post hoc test. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; uns, unstimulated (serum free media only).


Correlation Between BALCytokines and Conditioned Media Cytokines
ADAMTS13
CCL2.MCP.1
CCL3.MIP.1.alpha
CCL4.MIP.1.beta
CD44
CXCL8.IL.8
Fas
Fas.Ligand
G.CSF
GM.CSF
HGF
IL.1.beta
IL.10
IL.12.p70
IL.13
IL.18
IL.2
IL.36.beta.IL.1F8
IL.4
IL.6
Leptin
MIF
MMP.3
Osteopontin
SP.D
TNF.alpha
SP−D
Osteopontin
MMP−3
MIF
IL−6
IL−4
IL−36 beta/IL−1F8
IL−2
IL−18
IL−12 p70
IL−10
IL−1 beta
HGF
GM−CSF
G−CSF
Fas Ligand
Fas
CXCL8/IL−8
CD44
CCL4/MIP−1 beta
CCL3 MIP−1 alpha
CCL2/MCP−1
−0.5
0.0
0.5
Spearman Correlation
P < .05
FALSE
TRUE

Figure S2. Correlation between BALF cytokines and hMSC cytokines detected in conditioned media. Cytokines measured in BALF samples (top) were correlated with cytokines detected in conditioned media (left) from ARDS (n=6) and HC (n=10) BALF-exposed hMSC cultures. Red color indicates no significant difference ($p<0.05$) and blue color indicates significant difference ($p<0.05$).

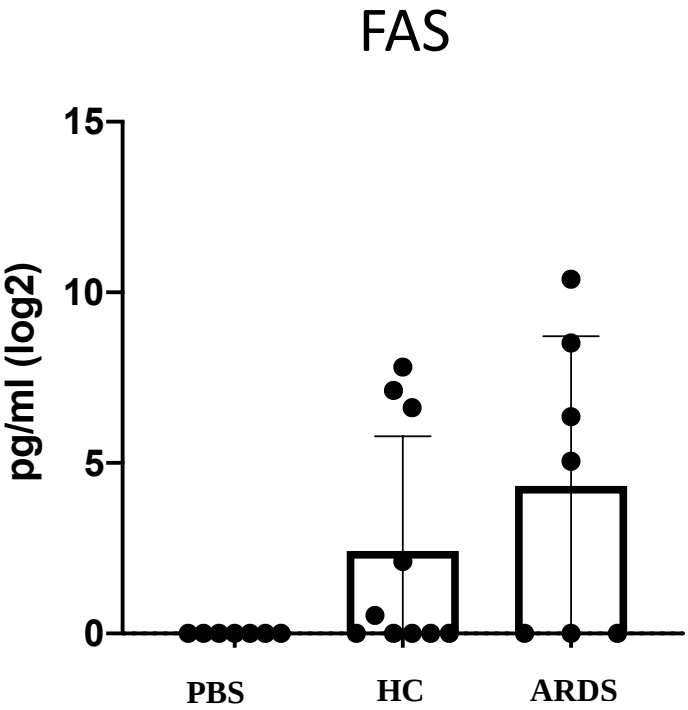
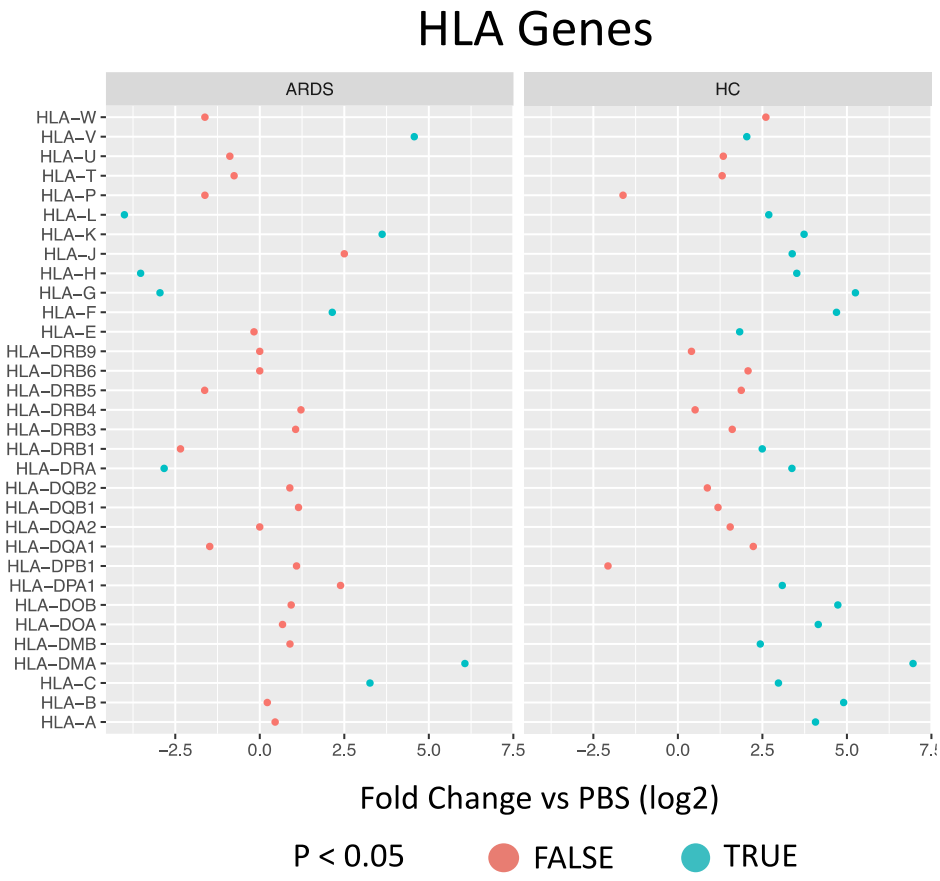


Figure S3. BALF-exposed hMSCs express FAS protein.
To assess if the hMSCs exposed to ARDS BALF (n=7) samples differed from healthy control BALF-exposed hMSCs (n=10) and PBS-exposed hMSCs (n=7) expressed FAS protein, conditioned media after BALF or PBS exposure was assessed for FAS. Data are presented as median with interquartile range of log2 normalized values, and statistical analysis was performed by Mann-Whitney test. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline.

A


HLA Genes
ARDS
HC
HLA–W
HLA–V
HLA–U
HLA–T
HLA–P
HLA–L
HLA–K
HLA–J
HLA–H
HLA–G
HLA–F
HLA–E
HLA–DRB9
HLA–DRB6
HLA–DRB5
HLA–DRB4
HLA–DRB3
HLA–DRB1
HLA–DRA
HLA–DQB2
HLA–DQB1
HLA–DQA2
HLA–DQA1
HLA–DPB1
HLA–DPA1
HLA–DOB
HLA–DOA
HLA–DMB
HLA–DMA
HLA–C
HLA–B
HLA–A
−2.5
0.0
2.5
5.0
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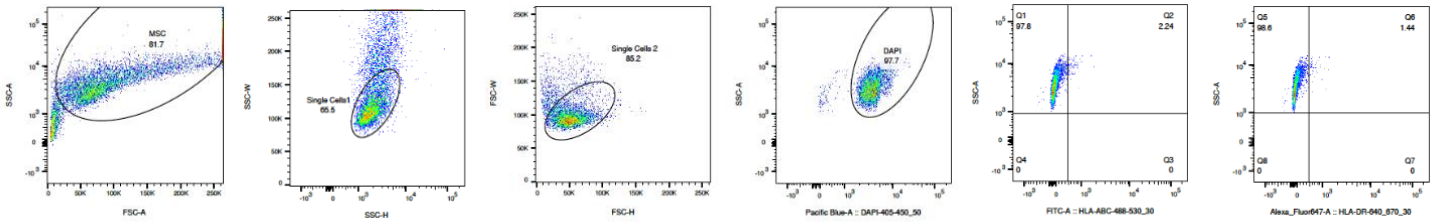
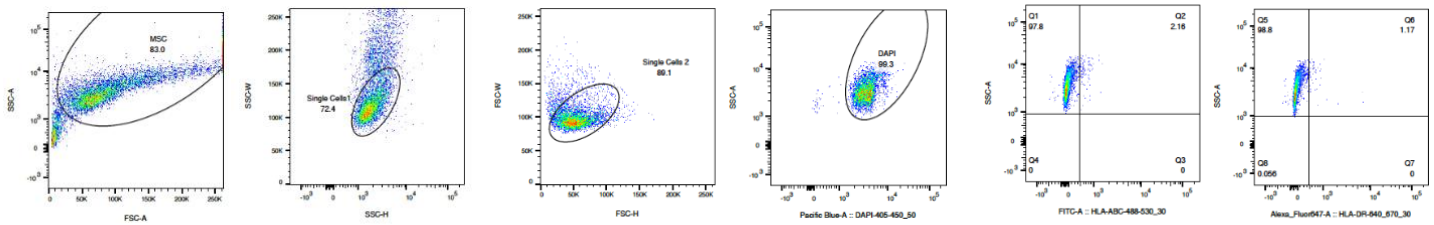
Fold Change vs PBS (log2)
P < 0.05
FALSE
TRUE

B

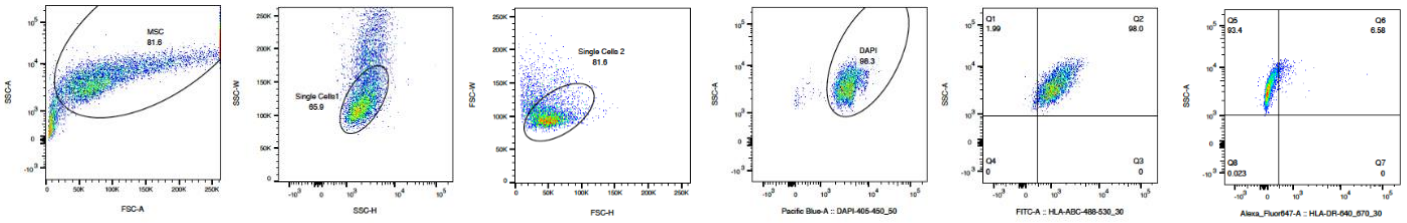
DAPI only sample #1



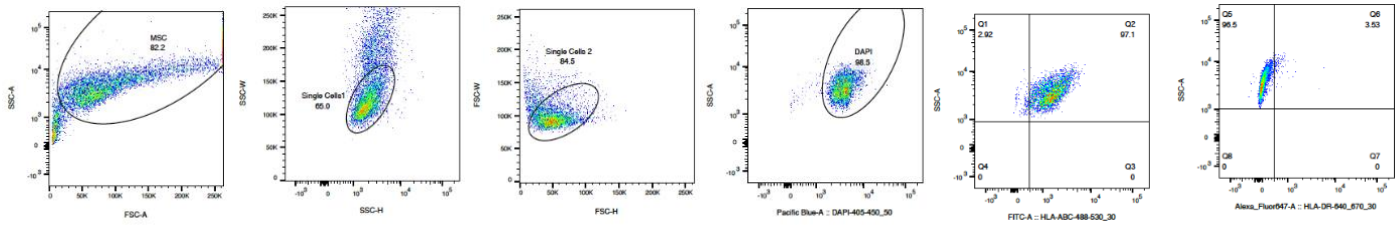
DAPI only sample #2



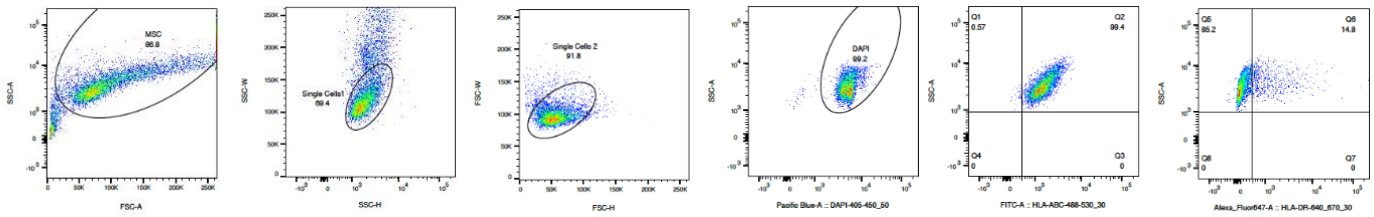
PBS-exposed sample #1



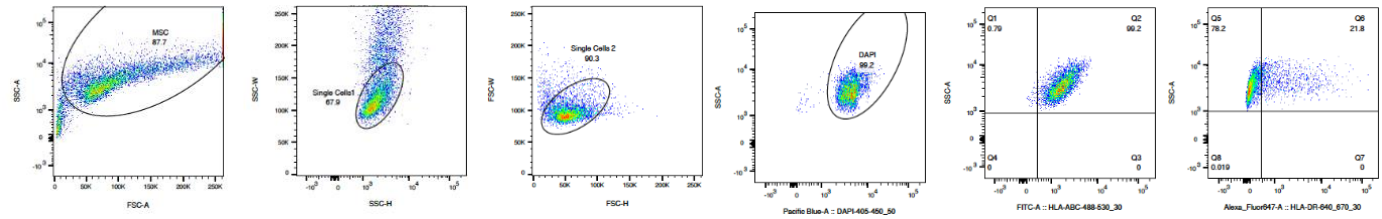
PBS-exposed sample #2



HC sample #10



HC sample #6



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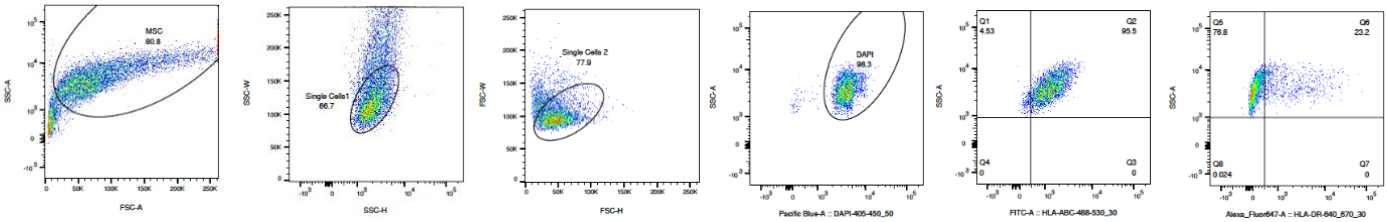
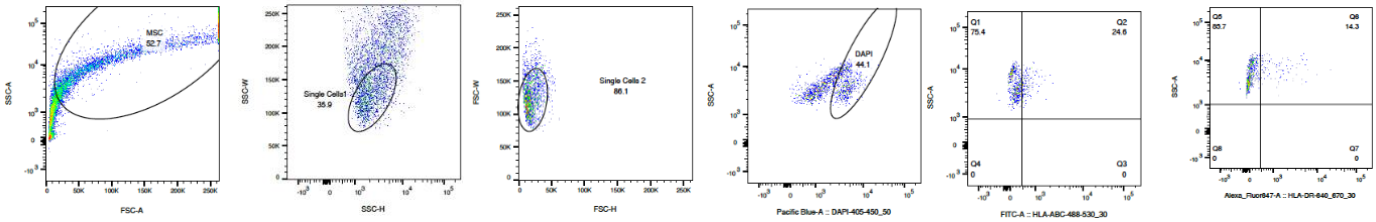
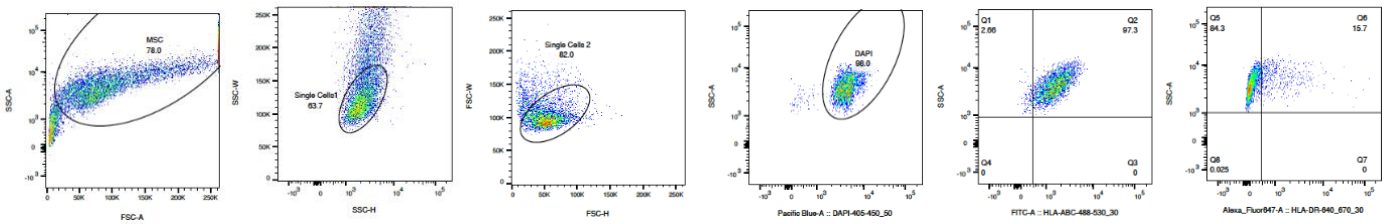


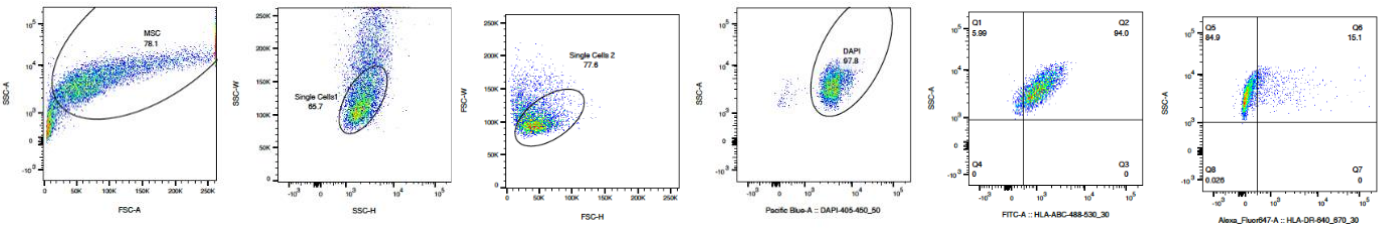
HC sample #8



HC sample #15



HC sample #9



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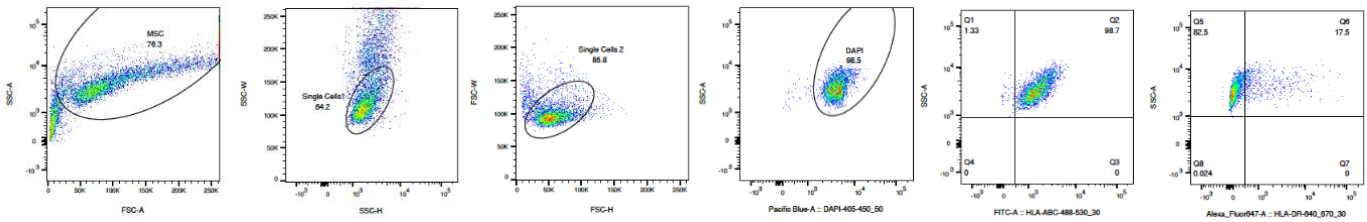

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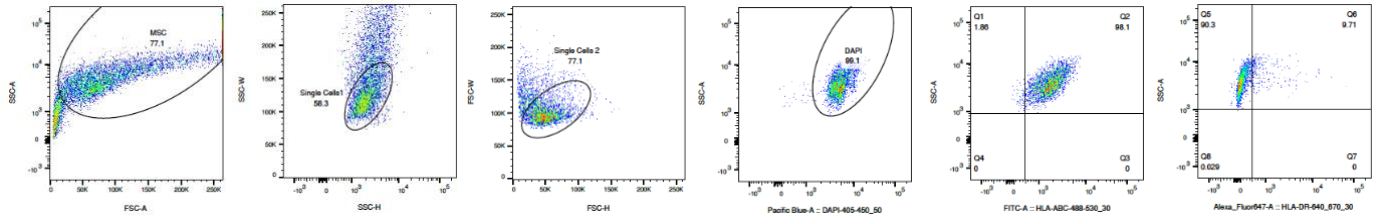

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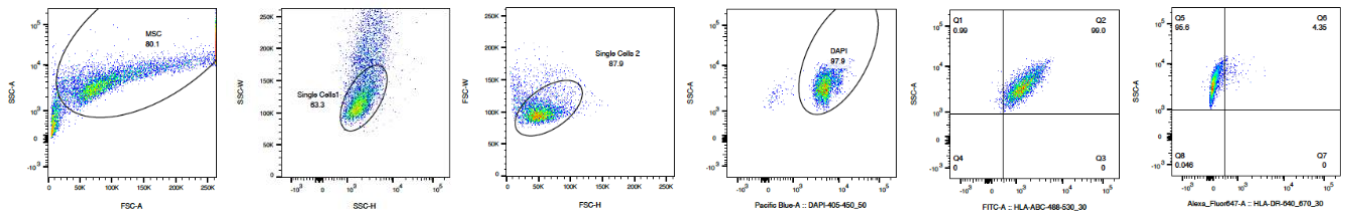

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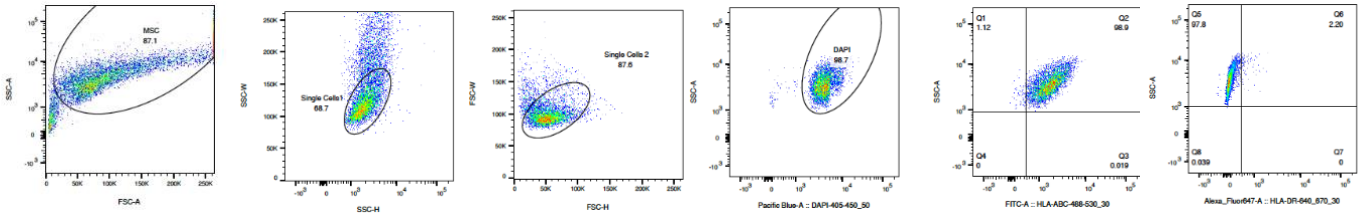

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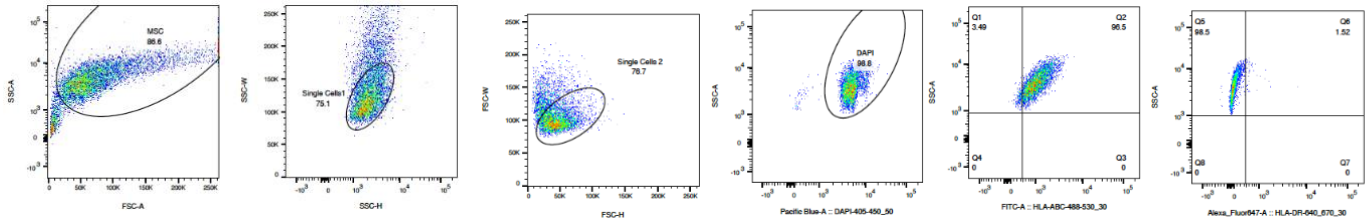

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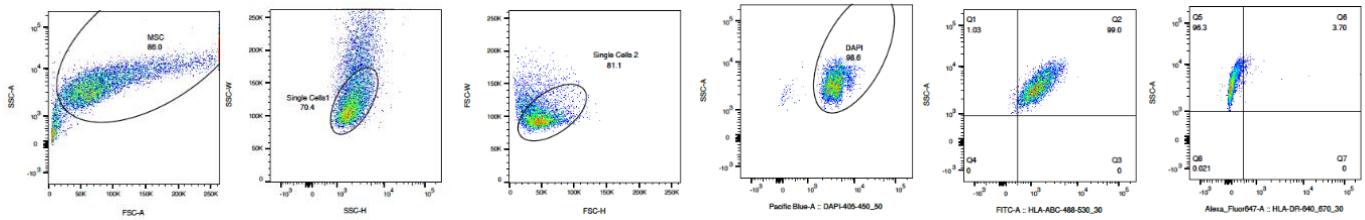

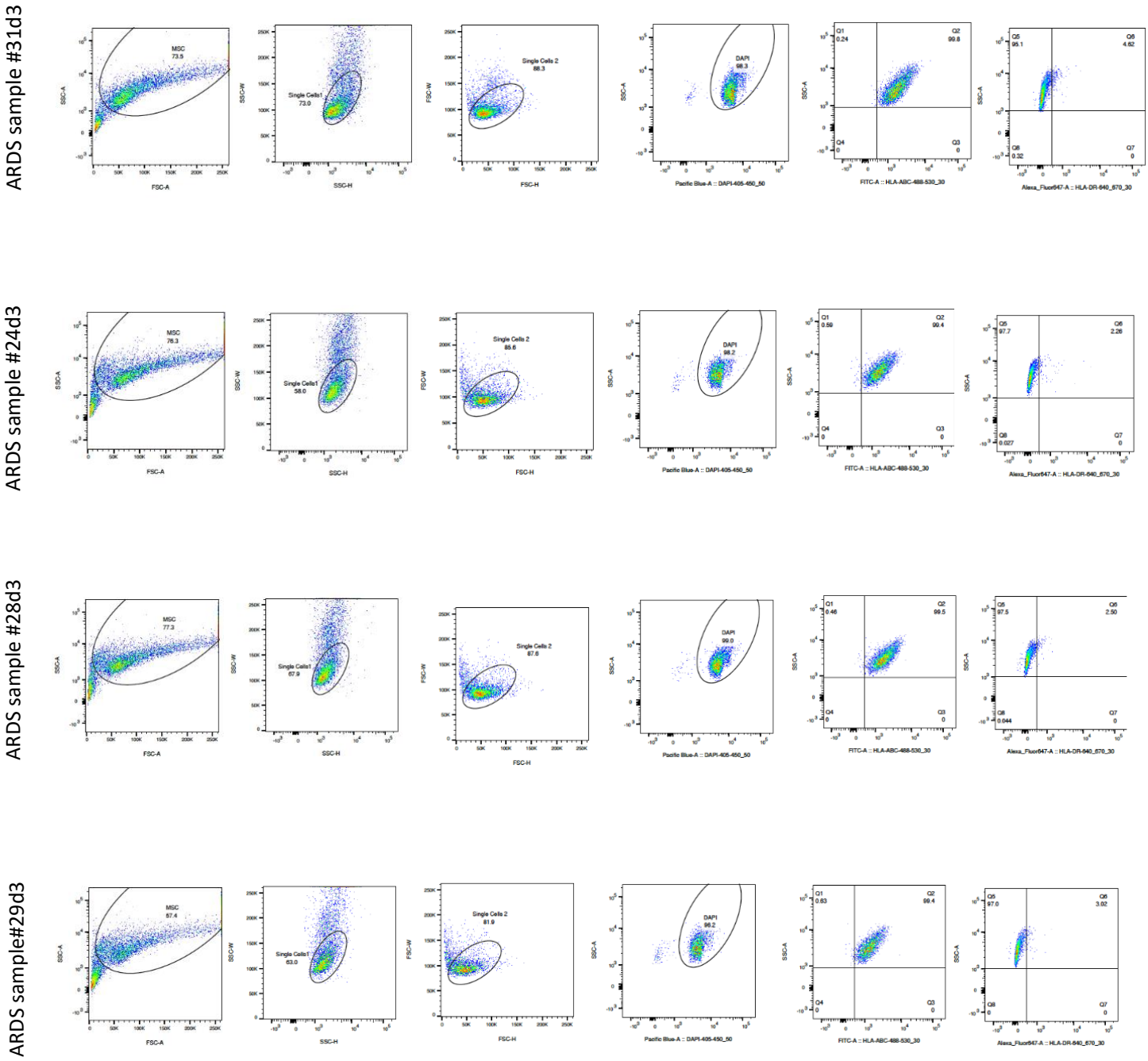

Figure S4. All HLA genes and pseudogenes that were measured on hMSCs post BALF exposure. Using ingenuity software of HLA genes (**A**) expressed by ARDS-exposed (left) and healthy control BALF (right) samples were compared to PBS-exposed hMSCs. Data are presented as mean of log2 fold change. Abbreviations: HC, healthy control; ARDS, acute respiratory distress syndrome; PBS, phosphate-buffered saline; HLA, human leukocyte antigen. (**C**) The gating strategy and dot plots on all samples analyzed for HLA-DR and HLA-ABC surface markers.