

Cytometry Time-of-Flight Identifies Alveolar Macrophage Subtypes in Acute Respiratory Distress Syndrome

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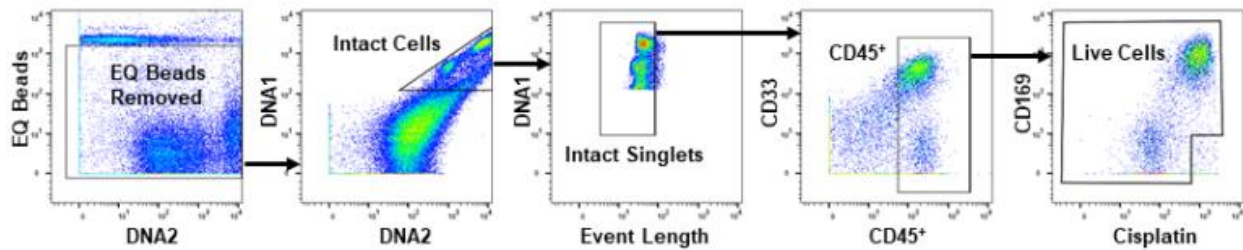
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Supplemental Data

Supplemental Figures

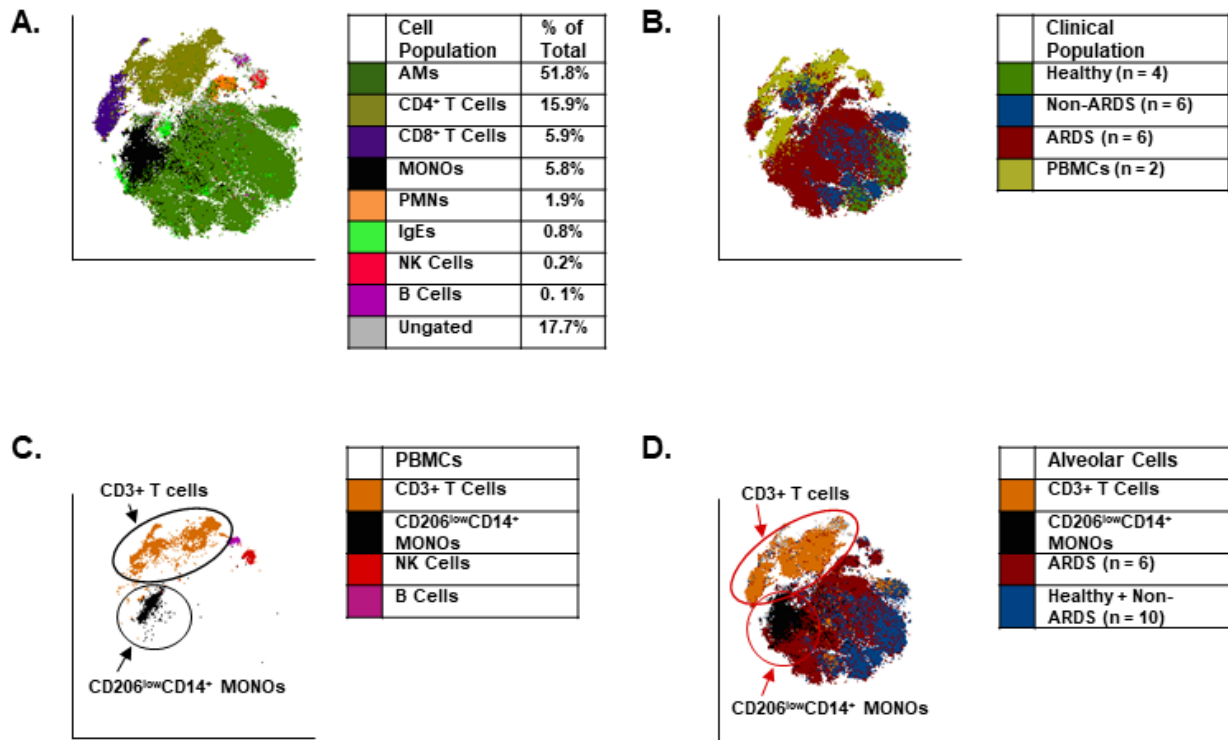
Figure S1.



Initial Gating to Isolate Intact/Singlet/Live/CD45⁺ Cells for Downstream Analysis.

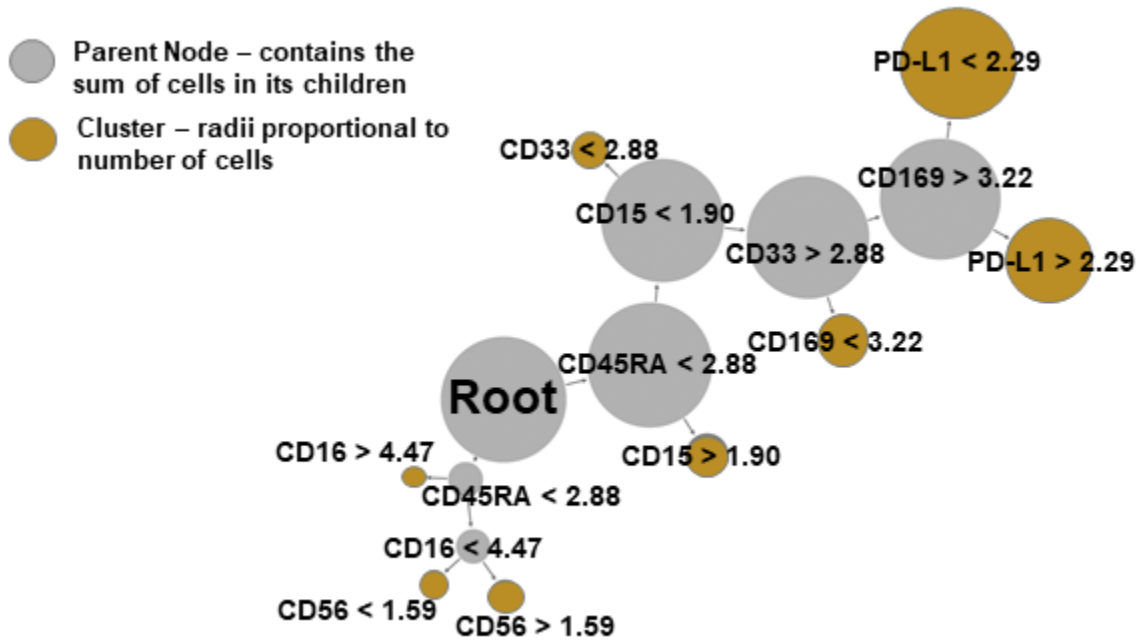
Normalization beads were excluded, and then intact cells were identified by gating on events with strong staining for the two isotopes of Ir intercalator. Further gating on the Event_Length parameter removed cell aggregates. Non-leukocytes and dead cells were eliminated with CD45 and cisplatin gating, respectively.

Figure S2.



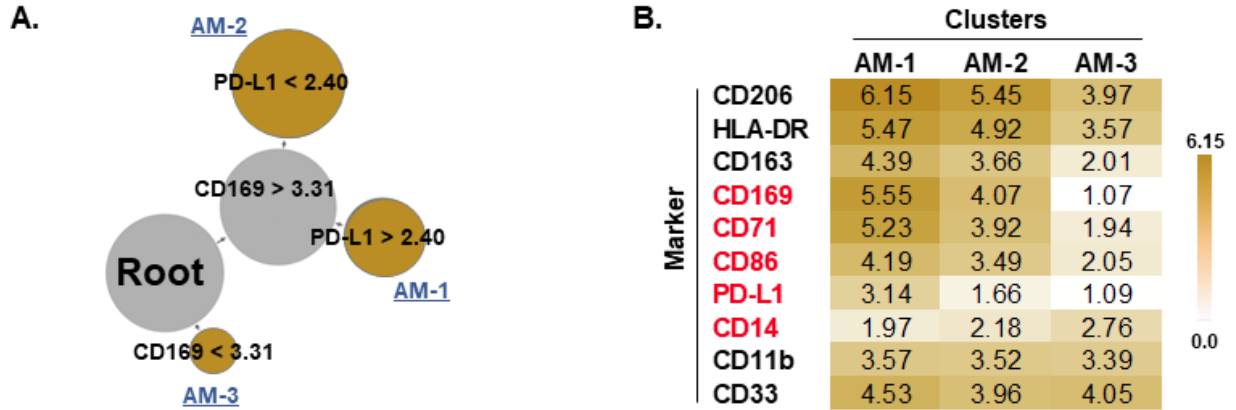
CyTOF Analysis of Leukocytes from Both Bronchoalveolar Lavage (BAL) Fluid and Peripheral Blood Mononuclear Cell (PBMC) Sample Sources. We combined equally sampled events from 2 PBMC samples obtained from healthy volunteers to our 16 HMC-BAL alveolar samples, and analyzed all events within a single viSNE plot. A) Concatenated viSNE plot generated from all 18 samples. The color of each dot represents its immune cell subset as designated by manual gating shown in Figure 1A. B) Concatenated viSNE plot of all cells colored per the clinical population from which the sample was procured. C) Concatenated viSNE plot of all cells with only PBMCs displayed. D) Concatenated viSNE plot of all cells with only alveolar cells displayed. CD3⁺ T cells and CD206^{low}/CD14⁺ MONOs from either the PBMCs or alveolar samples were phenotypically similar based on their respective cluster designations within the overall viSNE plot. AM = alveolar macrophage, IgE = eosinophil, basophil, and mast cells, MONO = monocyte, NK = natural killer cell, PMN = neutrophil, UNK = unclassified

Figure S3.



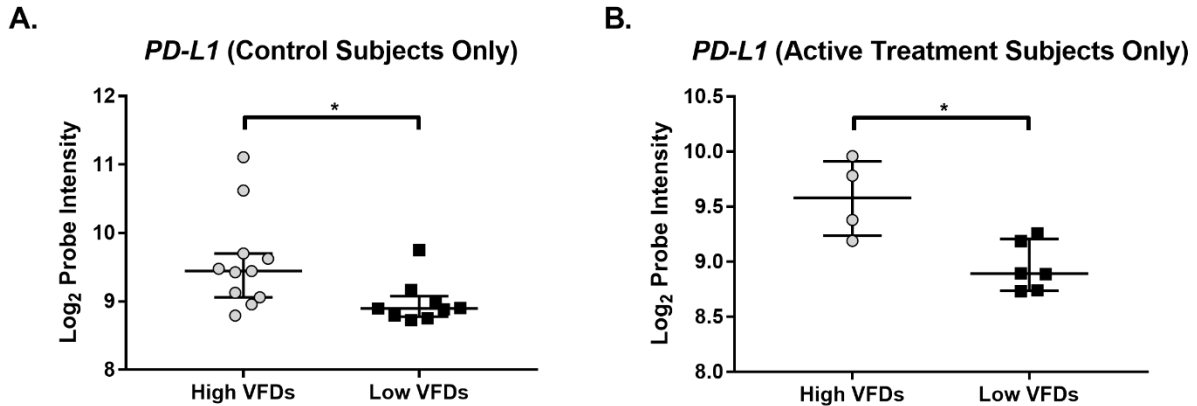
Unsupervised Clustering Identifies Novel Alveolar Myeloid Subtypes. Divisive Marker Tree (DMT) dendrogram displaying the eight myeloid clusters derived by κ NN-DE clustering (κ nearest neighbors = 30) of alveolar myeloid cells after exclusion of CD3⁺ T cells and CD206^{low}CD14^{high} MONOs. The DMT starts with the Root node encompassing all clusters, then progresses through successive binary divisions that are chosen to maximize the average uncentered Pearson correlation of each of the cluster expression profiles. For each division, the marker that has the largest variance-normalized difference between the two sister clusters is labeled along with the cut-off value.

Figure S4.



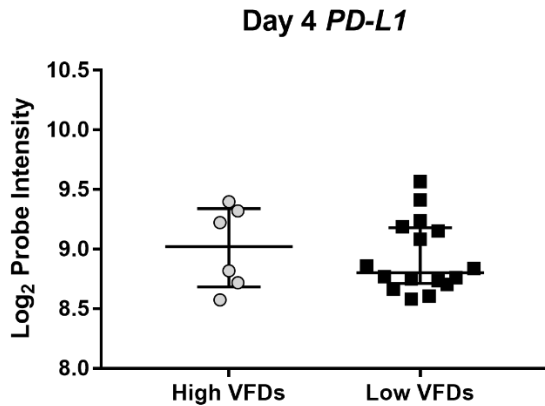
Unsupervised Clustering of CD33⁺CD71⁺CD163⁺ AMs Validates the Presence of Alveolar Myeloid Subtypes. A) Divisive Marker Tree (DMT) dendrogram displaying the three AM clusters derived by κ NN-DE clustering (κ nearest neighbors = 30) of CD33⁺/CD71⁺/CD163⁺AMs. B) Heatmap displaying the median marker intensities (asinh(marker intensity/5) scale) of the most informative markers for each subtype identified and displayed in panel A. The markers colored in red were carried forward for further analysis.

Figure S5.



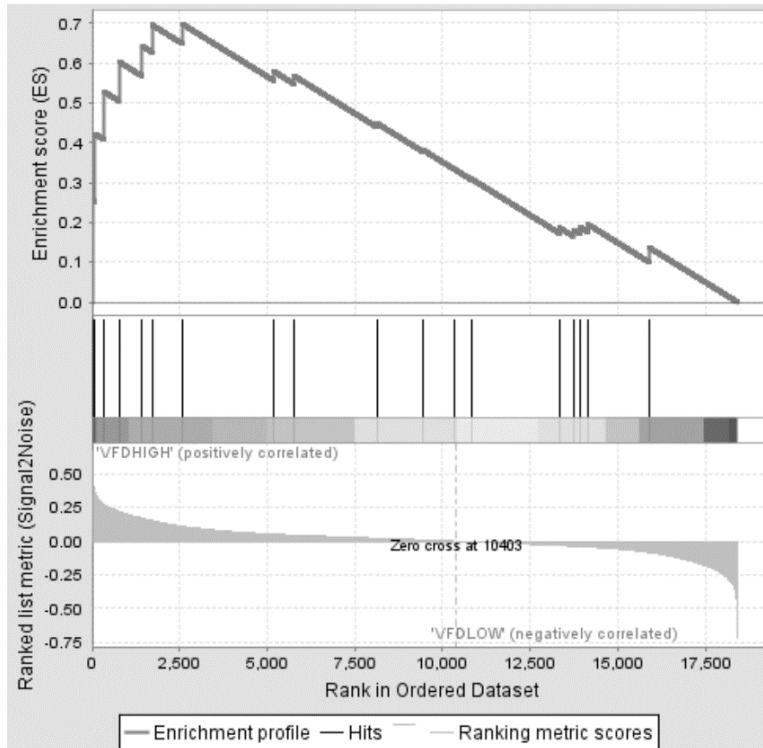
AM *PD-L1* Gene Expression is Associated with Ventilator-Free Days (VFDs) in Subjects with ARDS Treated with Placebo or Fish Oil. AM gene expression was measured by microarray analysis from a cohort of patients enrolled in a randomized controlled trial comparing subjects treated with placebo versus fish oil (ARDS-AMGE) < 48 hours after ARDS onset. A) AM expression of *PD-L1* in subjects randomized to the placebo group (High VFDs: $n = 11$ versus Low VFDs: $n = 9$). B) AM expression of *PD-L1* in subjects randomized to the active drug (fish oil) group (High VFDs: $n = 4$ versus Low VFDs: $n = 6$). High VFDs = VFD ≥ 18 and Low VFDs = VFD < 18. mRNA normalized log₂ probe intensity for each transcript is expressed as individual values and median \pm IQR for each group. For each gene, comparisons were made with a Mann-Whitney test. * = $p < 0.05$

Figure S6.



Day 4 Alveolar Macrophage (AM) PD-L1 Gene Expression is not Associated with Ventilator-Free Days (VFDs) in Subjects with ARDS. AM gene expression was measured in a cohort of patients enrolled in a randomized controlled trial comparing subjects treated with fish oil versus placebo (ARDS-AMGE). Samples used in this analysis were collected on Day 4 after ARDS onset. High VFDs = VFD \geq 18 ($n = 6$) and Low VFDs = VFD $<$ 18 ($n = 16$). mRNA normalized \log_2 probe intensity for each transcript is expressed as individual values and median \pm IQR for each group. $p = 0.75$ comparing the medians between High and Low VFDs using a Mann-Whitney test.

Figure S7.



The REACTOME_PD1_SIGNALING Gene Set is Enriched in Alveolar Macrophages from High Ventilator-Free Day (High VFD) Compared with Low VFD Subjects in Acute Respiratory Distress Syndrome (ARDS). Each vertical line in the middle portion of the plot represents one of the probes in the REACTOME_PD1_SIGNALING gene set. The left-to-right position of each line indicates the relative position of the probe within the rank ordering of the 18,415 probes on the microarray based on the most upregulated probe in the High VFD (High VFD = VFD \geq 18, $n = 15$) versus Low VFD (VFD Low = VFD $<$ 18, $n = 15$) group. The top portion of the plot shows the running enrichment score (ES) as the analysis proceeds along the entire transcriptome. The ES reflects the degree in which each gene from the gene set is overrepresented or underrepresented along the entire transcriptome. The bottom portion of the plot displays the ranked list metric along the entire transcriptome, which reflects the degree of a gene's correlation with a phenotype. The REACTOME_PD1_SIGNALING gene set is enriched in High VFD compared with Low VFD subjects as evidenced by the increased number of black lines on the left side of the plot and the positive enrichment score marked by the ES line ($p < 0.001$). Plot was generated with GSEA (<http://software.broadinstitute.org/gsea/index.jsp>).

Supplemental Tables

Table S1. CyTOF Marker Panel

TARGET	CLONE	LABEL	VENDOR
IL-1R	Polyclonal	166Er	FDM
CD3	UCHT1	154Sm	FDM
CD4	RPA-T4	145Nd	FDM
CD8A	RPA-T8	162Dy	FDM
CD11B (MAC-1)	ICRF44	167Er	FDM
CD11C	Bu15	147Sm	FDM
CD14	M5E2	151Eu	FDM
CD15 (SSEA-1)	W6D3	164Dy	FDM
CD16	3G8	209Bi	FDM
CD19	HIB19	142Nd	FDM
CD25 (IL-2R)	BC96	159Tb	In-house
CD33	WM53	169Tm	FDM
CD38	HIT2	144Nd	FDM
CD45	HI30	89Y	FDM
CD45RA	HI100	143Nd	FDM
CD54	HA58	170Er	FDM
CD56 (NCAM)	NCAM16.2	149Sm	FDM
CD71	OKT-9	175Lu	FDM
CD80 (B7-1)	2D10.4	161Dy	FDM
CD86	IT2.2	150Nd	FDM
CD103	Ber-ACT8	160Gd	In-house
CD123 (IL-3R)	6H6	153Eu	In-house
CD127 (IL-7RA)	A019D5	172Yb	In-house
CD163	GHI/61	165Ho	FDM
CD169	7-239	158Gd	FDM
CD183 (CXCR3)	G025H7	156Gd	FDM
CD194 (CCR4)	205410	173Yb	In-house
CD196 (CCR6)	G034E3	141Pr	FDM
CD197 (CCR7)	G043H7	152Sm	In-house
CD206 (MMR)	15-2	168Er	FDM
CD274 (PD-L1)	29E.2A3	148Nd	FDM
CD279 (PD-1)	EH12.2H7	155Gd	FDM
CD294 (CRTH2)	BM16	163Dy	FDM
FCERI	AER-37 (CRA-1)	171Yb	In-house
HLA-DR	L243	174Yb	FDM
SIGLEC 8	7C9	176Yb	In-house

FDM: Fluidigm

Table S2. All Median Myeloid Marker Intensities for Each Subtype Identified by κ NN-DE in HMC-BAL.

Marker	Clusters								
	AM-1	AM-2	AM-3	UNK	PMN	NK cells	pDC	cDC	
CD206	6.08	5.27	4.17	1.77	1.79	0.67	0.90	1.03	
HLA-DR	5.38	4.78	3.64	1.59	1.40	0.76	3.42	2.62	
CD163	4.33	3.35	1.76	0.90	1.38	0.55	0.40	0.93	
CD169	5.50	3.91	0.95	1.82	1.28	0.55	1.13	2.66	
CD71	5.16	3.72	1.87	0.94	1.39	0.93	2.07	1.16	
CD86	4.13	3.36	2.18	0.83	0.61	0.91	0.74	0.62	
PD-L1	3.05	1.54	1.09	0.57	0.44	0.61	0.48	0.63	
CD14	1.95	2.08	3.09	0.35	0.40	0.25	0.44	0.26	
CD11b	3.49	3.35	3.55	0.56	2.96	0.52	0.52	1.13	
CD15	1.81	1.41	0.94	0.43	3.36	0.48	0.49	0.48	
CD56	1.20	0.78	0.66	0.41	0.19	2.96	0.22	0.55	
CD123	3.64	4.44	2.59	1.08	1.07	0.39	1.56	0.96	
CD45RA	1.35	0.90	0.98	0.67	0.62	3.78	4.80	5.14	
CCR6	2.53	1.82	0.99	1.14	2.14	0.54	2.46	8.25	
CD33	4.43	3.80	4.11	1.34	1.44	0.62	1.11	0.98	
CD45	4.03	3.42	3.77	3.47	3.06	3.12	3.69	3.25	
CD54	3.99	3.88	4.36	1.48	1.56	1.47	2.76	1.00	
CCR4	3.00	2.77	2.22	0.84	0.93	0.35	1.78	1.21	
CD38	2.69	1.92	2.44	0.90	0.71	2.55	2.88	2.16	
CD4	2.64	1.92	1.59	1.20	0.32	0.60	0.93	1.32	
CD11c	2.72	2.55	2.89	0.70	1.13	0.49	0.77	1.10	
IL-1R	2.93	2.50	2.25	0.58	2.04	0.47	0.58	2.57	
CD16	2.47	2.39	1.29	1.28	3.07	2.34	0.88	8.05	
CD25	2.55	1.55	0.54	0.72	0.90	1.16	1.88	1.86	
CXCR3	2.31	1.48	0.97	1.46	1.19	0.88	1.49	1.80	
Siglec-8	2.41	1.59	0.84	0.48	0.70	0.38	0.70	0.78	
CD80	2.10	1.31	0.76	0.76	0.67	0.44	0.71	0.54	
CD103	2.30	1.82	0.99	1.34	1.17	0.98	0.98	0.83	
FceR1a	1.56	1.70	2.06	0.56	0.69	0.40	1.07	0.63	
CCR7	1.19	1.89	1.48	0.40	1.04	0.33	1.51	0.35	
CD3	1.35	1.92	0.84	1.35	0.33	0.28	0.63	0.19	
CD8	1.00	0.94	0.87	0.63	0.53	0.73	0.25	0.35	
CD127	1.53	1.44	1.29	0.93	0.53	0.34	0.69	0.34	
CRTH2	1.26	0.81	0.58	0.38	1.57	0.33	0.30	0.42	
PD-1	0.95	0.88	0.42	0.94	0.36	0.26	0.46	0.33	

All values are expressed on the $\text{asinh}(\text{raw intensity}/5)$ scale; AM = alveolar macrophage, cDC = conventional dendritic cell, NK = natural killer cell, pDC = plasmacytoid dendritic cell, PMN = neutrophil, UNK = unclassified