Localization of Macrophages in the Human Lung via Design-Based Stereology

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ONLINE DATA SUPPLEMENT

Supplemental Materials

Methods

Human Lung processing

Studies used de-identified human lungs procured from organ donors by either Donor Alliance (Denver, CO, USA) or the International Institute for the Advancement of Medicine (Edison, NJ, USA). All deceased subjects were ventilated endotracheally for three days or less prior to organ donation. Lungs were shipped on ice in either Histidine-Tryptophan-Ketoglutarate (HTK) or University of Wisconsin (UW) solution, the same preservatives used for human organ transplantation. The IRB at National Jewish Health has determined that our experiments do not constitute human subjects research since the donors are deceased and all tissue is de-identified prior to receipt. Lung tissue was processed immediately upon arrival and always within 24 hours of death. The main pulmonary artery was perfused with phosphate buffered saline (PBS) until the venous output was clear. Subjects had no history of lung disease, including no emphysema or other smoking-related lung disease

Flow Cytometry

Fresh human lung tissue was dissected from the right upper lobe and defined as either "proximal" or "distal". Both tissue types contained predominantly alveolar parenchymal tissue, however "proximal" lung tissue also contained a bronchovascular bundle with a vessel and sub-segmental airway. Tissue was minced and then enzymatically digested for 30 min at 37°C in Collagenase D (1.5 mg/ml, Sigma-Aldrich) to liberate macrophages, as we have previously described (1, 2). Digested samples were passed through a 100 μm nylon filter and suspended in buffer containing HBSS (Sigma-Aldrich) with 0.3 mM EDTA (Sigma-Aldrich) and 0.2% FBS

(Invitrogen) for staining for multi-channel flow cytometry for 30 minutes with fluorescently-conjugated antibodies against CD45 (ThermoFisher), CD206 (BioLegend, San Diego, CA, USA), CD43 (ThermoFisher) and CD169 (BD Biosciences, San Jose, CA, USA) were included to identify macrophages, CD1c (BioLegend) to label dendritic cells, and CD3 (ThermoFisher), CD19 (BioLegend), CD15 (BioLegend) and DAPI (ThermoFisher) to exclude T cells, B cells, neutrophils and dead cells, respectively. To quantify macrophages via flow cytometry, 10,000 Flashred microsphere beads (5 µm, Bangs Laboratory, Fishers, IN, USA) were suspended in solution and run with flow cytometry, as previously described. FACS data were acquired using an LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (BD Biosciences).

CD169 (Siglec-1) positivity is also known to distinguish human AMs ($CD169^+$) from IMs ($CD169^-$) (3, 4), but our efforts to robustly visualize $CD169^+$ cells via microscopy were unsuccessful. We performed flow cytometry demonstrating high co-expression of CD43 and CD169 on AMs (**Figure E1B)** and subsequently found AMs ($CD206^+/CD43^+$) and IMs ($CD206^+/CD43^-$) to be easily distinguished visually within tissue sections. Because CD43 staining met our needs to robustly identify AMs in fluorescently stained tissue sections, further efforts to visualize CD169 (ie. by trialing additional CD169 antibody clones) were aborted.

In addition to stereology, we performed quantitative flow cytometry of digested lung to calculate the density of IMs (**Figure E5A**) and AMs (**Figure E5B**) in human lung tissue. Because of our interest in IM location, proximal lung tissue (containing alveolar tissue and a bronchovascular bundle) was compared to distal lung tissue (composed mostly of alveolar tissue). We measured fewer IMs in the lung digest by flow cytometry as compared to our estimate by stereology, with 0.15 (SD 0.14) million IMs / cm^3 and 0.11 (SD 0.09) million IMs / cm^3 in distal lung and proximal lung, respectively, as shown in **Figure E5A**.

Preparation of Stereology Tissue

The whole right upper lobe (RUL) was isolated and used for all morphometry studies, with workflow summarized in **Figure E2**. The RUL was inflated with 1% (w/v) low melting-point agarose (ThermoFisher) at 40 °C with a constant pressure of 20 cmH_2O. Following gelation on ice, the reference volume of the whole, inflated RUL was measured via volume-displacement in phosphate buffered saline (5). Lung tissue was subsequently dissected via systematic uniform, random sampling for unbiased stereology, using accepted methods (6). Briefly, ten, unfixed, 1 cm cubic blocks per lung were randomly selected and immediately cryoembedded in Tissue-Tek O.C.T. compound (VWR, Radnor PA, USA) via submersion in 2-methyl butane (Sigma-Aldrich, St. Louis, MO, USA) chilled in liquid N_2. Frozen blocks were banked at -80°C in airtight containers until cryosectioning into 40 μm thick sections. For the purpose of blinding all subsequent analyses, slides were assigned a random identification number, shuffled, and banked in an airtight container at -80°C until immunostaining and subsequent analyses. Frozen slides were stained for immunofluorescence with DAPI, and fluorescently-conjugated antibodies against human CD206-PE (BioLegend), CD43-APC (ThermoFisher) and Elastin-FitC (Sigma-Aldrich), as follows. Frozen slides were immediately immersed in chilled 10% neutral buffered formalin (Sigma-Aldrich) for 15 minutes. Then, samples were rinsed in PBS for 15 minutes prior to blocking for 45 minutes with PBS containing 10% Donkey serum (Abcam) at 37°C. Next, antibodies and DAPI were placed on sample and incubated for an additional 45 min at 37°C. After rinsing in PBS, a 40 μm shim was placed between the tissue and coverslip and ProLong Diamond antifade mounting media (ThermoFisher) was applied prior to imaging.

Design-Based Stereology

Four-channel fluorescent three-dimensional Z-stack images were captured using an Olympus BX53 microscope (Tokyo, Japan) controlled by Visiopharm newCAST stereology software (Hoersholm, Denmark). Notably, elastin and DAPI staining in the overview image was visualized to manually define sub-regions of interest (ROI) as parenchyma, vessels, or airways for each slide as, as shown in **Figure E2F-G**, for subsequent sampling. Area point-counting was performed on randomly selected 10x images covering 40% of the total tissue area per slide to calculate the volume fraction of the following sub-tissues ($V/V_{subtissue}$): alveoli, alveolar septa vessel walls, vessel lumens, airway walls, and airway lumens, by

$$V/v_{subtissue} = \frac{\sum P_{subtissue}}{\sum P_{Total}}$$

Where $\Sigma P_{subtissue}$ is the number of area points per sub-tissue, per RUL and ΣP_{Total} is the total number of area points per RUL. The total volume of each sub-tissue ($V_{subtissue}$) was determined as,

$$V_{subtissue} = (V/v_{subtissue})(V_{RUL})$$

where V_{RUL} is the total RUL volume measured via water displacement.

Elastin and DAPI staining were visualized in an overview image to manually divide the lung into sub-regions of interest of parenchyma, vessels, or airways. Approximately 30 randomly positioned, high-power, 25 μm-thick 3D z-stacks were captured per region of interest per slide. Manual cell counting using the principle of the optical disector (7) determined the density of AMs and IMs per sub-tissue volume (ie. $\rho_{IM(subtissue)}$), by

$$\rho_{IM(subtissue)} = \frac{\sum N_{IM(subtissue)}}{SV(subtissue)}$$

Where $\Sigma N_{IM(subtissue)}$ is the total number of IMs manually counted per sub-tissue, per RUL. Equal antibody staining was verified throughout the tissue section (7) and IMs and AMs were counted within a 15 µm thick volume centered within each 25 µm thick Z-stack, thus yielding a 5 µm 'guard region' on either side of the sampled volume (8). It is accepted that approximately 150 - 200 counted objects per replicate are needed to make statistically valid observations about an organ when performing stereology (5). Accordingly, counting frame sizes and the number of images analyzed per sample were adjusted to target approximately 200 IMs or AMs counted per sub-tissue, per RUL. For example, an average of 190 (SD 130) IMs were counted within the alveola septa across all lungs. SV(subtissue) is the total sub-tissue volume sampled via the optical disector, per RUL, calculated as

$$SV(subtissue) = \frac{\sum P_{OD}(subtissue)}{\sum P_{OD}(Total)}(A_{dis})(N_{Dis})\left(\frac{h_{OD}}{(SF)}\right)$$

Where ΣP_{OD}(subtissue) is the total number of sub-tissue area points manually counted within the optical disector per RUL. ΣP_{OD}(Total) is the total number of area points counted per optical disector per RUL, A_{Dis} is the area of the disector counting frame, N_{Dis} is the number of disector frames per RUL, h_{OD} is the height of the optical disector and SF is the shrinkage fraction of each tissue section. Finally, an unbiased estimate of the total number of IMs or AMs per tissue compartment, per RUL (ie. $\bar{N}_{\mathrm{IM(subtissue)}}$) was calculated, as

$$\bar{N}_{\mathrm{IM(subtissue}} = \left(\rho_{IM(subtissue)}\right)(V_{subtissue})$$

To assess the adequacy of our sampling approaches, we compared the coefficient of variation due to biologic variability (CV_{bio}) to the overall coefficient of variation (CV=SD/mean) for all $\rho_{IM(subtissue)}$ values using accepted methods (9), as

$$CV^2 = CV_{bio}^2 + CE^2$$

where the Coeffiecent of Error (CE) for the density of macrophages counted within in any one subcompartment, per RUL was calculated, as

$$CE = \left(\frac{SEM}{\bar{x}}\right)$$

SEM and $\bar{x}$ are the standard error of the mean and mean, respectively, density of macrophages within any one RUL replicate. For example, the CVbio for the IM density within the alveolar septa was 0.35, as compared to the overall CV of 0.42, indicating that the majority of our error (>80%) was due to biologic variability, rather than our sampling methods.

Supplemental References

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Supplemental Figure Legends

Figure E1. Flow Cytometry approaches to distinguish AMs and IMs. (A) Flow cytometry gating strategy to detect CD45+ single cells. (B) $CD206^{+}$ myeloid cells with CD169 expression plotted versus CD43. CD43+ AMs had a high degree of CD169 positivity. (C) of $CD206^{+}$ myeloid cell, only a small percentage (<5%) also expressed CD1c, a potential dendritic cell marker. (D) Immunofluorescent staining confirmed a low abundance of CD1c positive cells around the bronchovascular bundle shown in 4-colors (left) and then split into CD206 channel (PE, middle) and CD1c channel (APC, right).

Figure E2. Overview of organ sampling for stereology. (A) Healthy, whole, right human lung was obtained and then (B) the right upper lobe (RUL) is isolated. (C) The RUL is inflated with low melting point agar at 40°C at a constant pressure of 20 cmH_2O. Following volume displacement to measure reference volume, (D) the RUL was sectioned for systematic uniform random sampling and (E) a random selection of representative tissue blocks were obtained. Following cryoembedding, sectioning and immunostaining, (F-G) slides were scanned using Visiopharm stereology software and sub-regions of interest (ROI) were identified for (F) airways, outlined in green, (G) vessels, outlined in red, and parenchyma. Individual ROIs were subsequently randomly sub-sampled for the optical disector.

Figure E3. Sex did not influence IM location. (A) There was no difference in RUL volume by Sex. (B) Total IM number and (C) IM density in the RUL or tissue sub compartments were not influenced by sex.

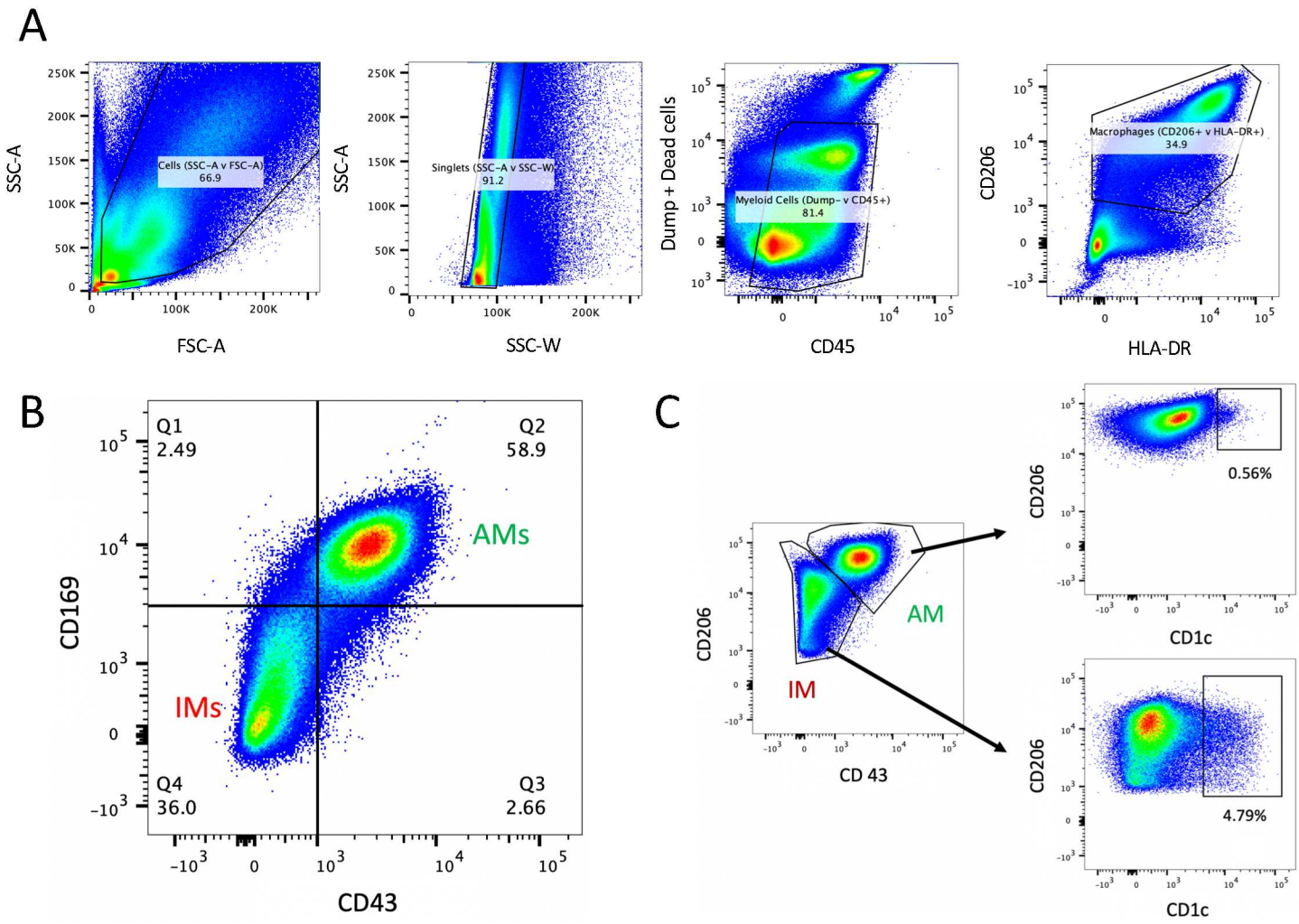
Figure E4. Age did not influence IM location. Age did not influence IM density in the (A) alveolar septi, (B) airway walls, or (C) vessel walls.

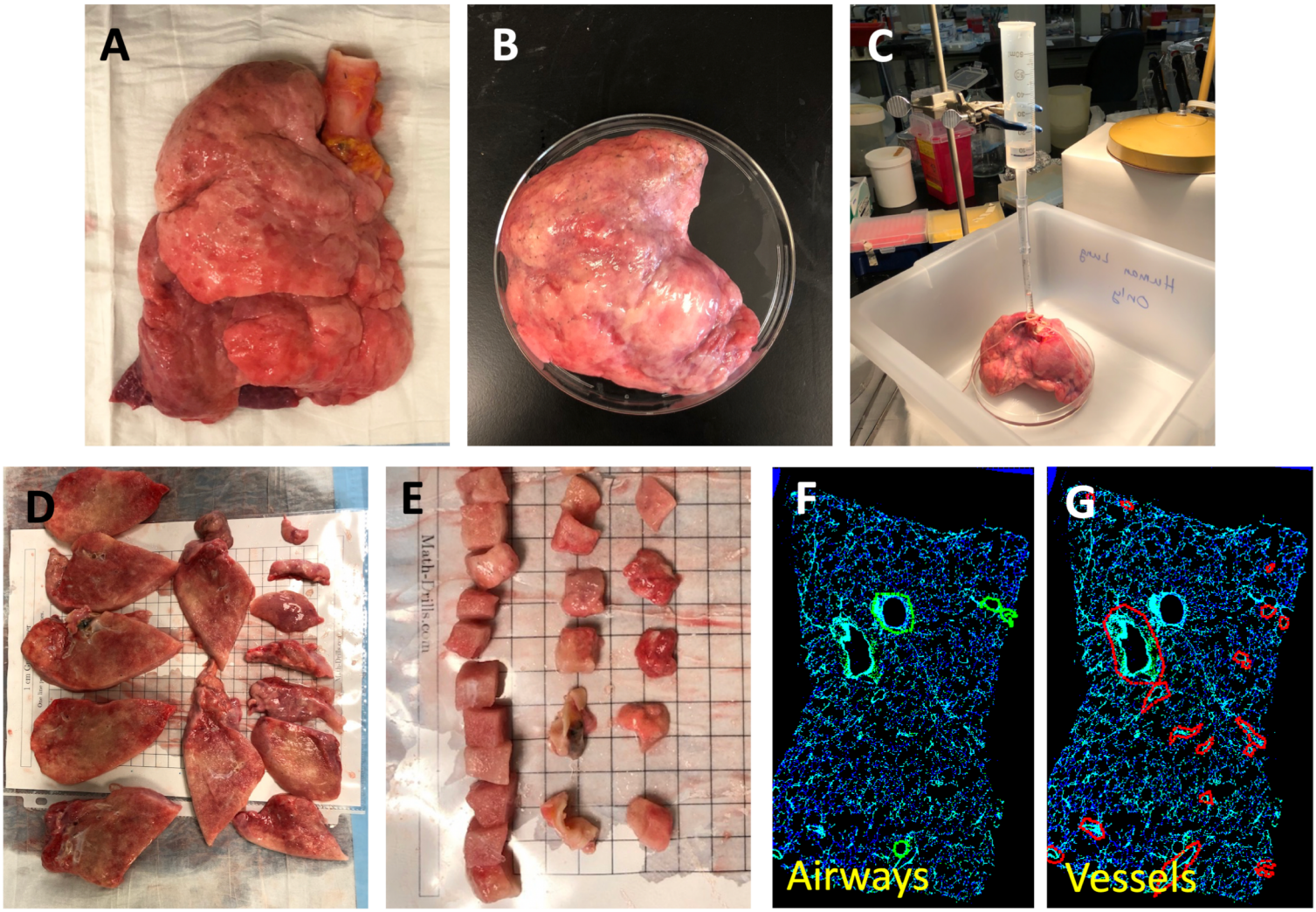
Figure E5. Flow Cytometry of whole lung digest to quantify (A) IM density and (B) AM density in proximal and distal lung tissue. Y-axis plotted as $\log_{10}$ scale.

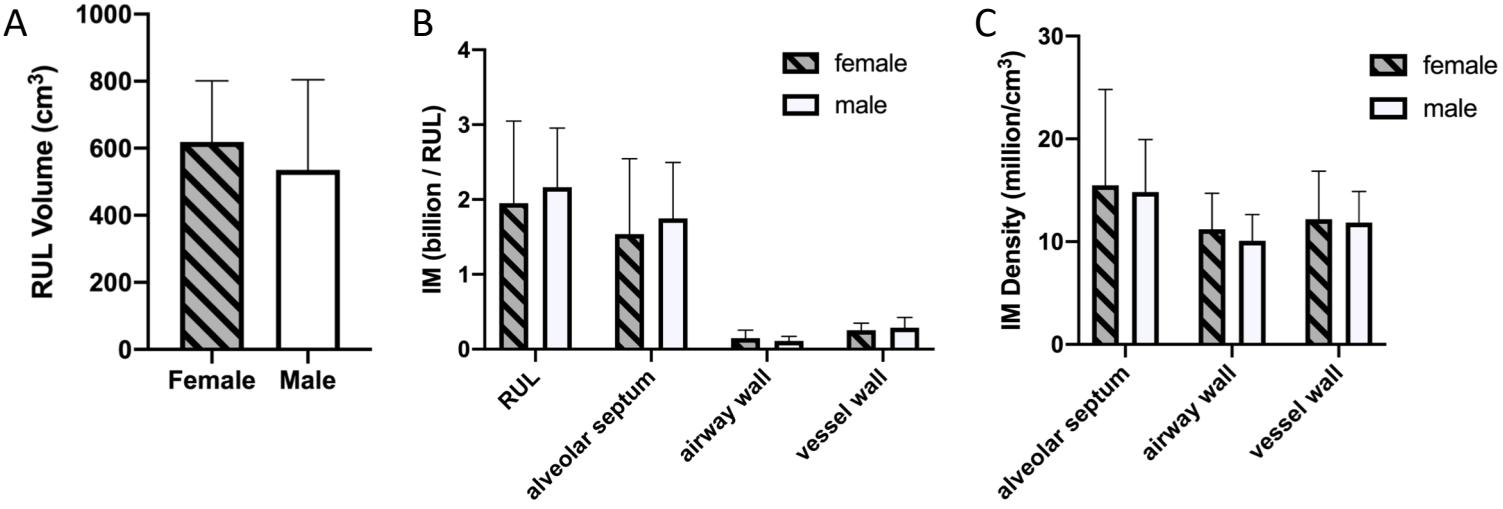
Table E1. Antibodies

Target	Antibody ID	Clone ID	IF Dil	Flow Cytometry Dil	Host Organism	Conjugate	Company	Catalog #
CD206*	AB_571910	15-2	1:50	1:100	Mouse	PE	Biolegend	321105
CD206	AB_10899411	15-2	-	1:100	Mouse	PerCP	BioLegend	321122
CD43*	AB_11219080	eBio84-3C1	1:25	1:100	Mouse	APC	Thermo Fisher	17-0439-42
CD1c	AB_1953227	L161	-	1:100	Mouse	PE/Cy7	BioLegend	331515
CD1c	AB_10719956	L161	1:25	-	Mouse	APC	BioLegend	331524
CD169#	AB_2189029	7-239	1:25	1:100	Mouse	PE	BioLegend	346004
CD45	AB_2744400	HI30	-	1:100	Mouse	BUV395	BD Biosciences	563791
HLA-DR	AB_399974	L243	-	1:100	Mouse	APC-Cy7	BD Biosciences	3357906
CD3	AB_1272055	OKT3	-	1:100	Mouse	eFluor 450	Thermo Fisher	48-0037-42
CD19	AB_2073118	HIB19	-	1:100	Mouse	Pacific Blue	BioLegend	302232
CD15	AB_2105359	W6D3	-	1:100	Mouse	Pacific Blue	BioLegend	323024
Elastin*	AB_1848109	Poly	1:100	-	Rabbit	Unconjugated	Sigma-Aldrich	HPA018111
Cytokeratin 7	AB_1079181	Poly	1:50	-	Rabbit	Unconjugated	Sigma-Aldrich	HPA007272
Rabbit IgG*	AB_2340597	Poly	1:250	-	Donkey	FitC	Jackson	711-096-152

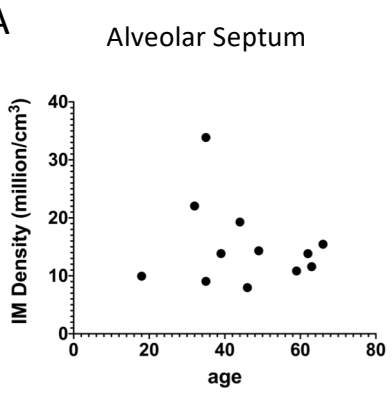
*Denotes antibody used for all stereology studies, #Denotes CD169 antibody unable to resolve AMs by microscopy
Antibody ID refers to https://antibodyregistry.org/
Abbreviations: Immunofluorence Staining (IF), Polyclonal antibody (Poly), Dilution (Dil), Identification (ID)


A
SSC-A
FSC-A
Cells (SSC-A v FSC-A)
66.9
SSC-W
Singlets (SSC-A v SSC-W)
91.2
Dump + Dead cells
CD45
Myeloid Cells (Dump- v CD45+)
81.4
CD206
HLA-DR
Macrophages (CD206+ v HLA-DR+)
34.9
B
CD169
CD43
Q1
2.49
Q2
58.9
Q4
36.0
Q3
2.66
AMs
IMs
C
CD206
CD 43
AM
IM
CD1c
0.56%
4.79%
D
Nuclei
CD206
CD1c
Elastin
100 µm
CD206
CD1c

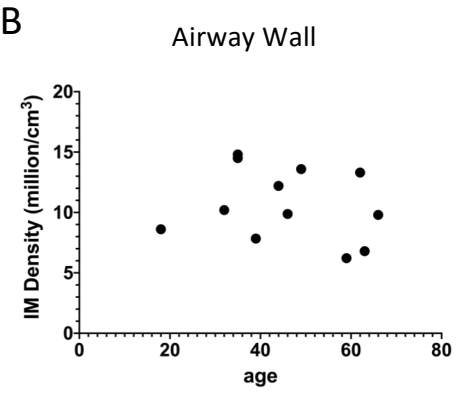

A
B
C
D
E
F
G
Airways
Vessels


A
RUL Volume (cm3)
1000
800
600
400
200
0
Female
Male
B
IM (billion / RUL)
4
3
2
1
0
female
male
RUL
alveolar septum
airway wall
vessel wall
C
IM Density (million/cm3)
30
20
10
0
female
male
alveolar septum
airway wall
vessel wall

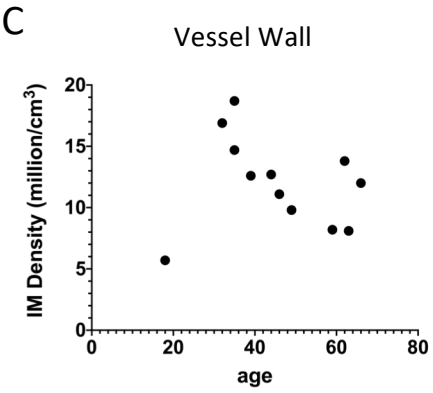
A

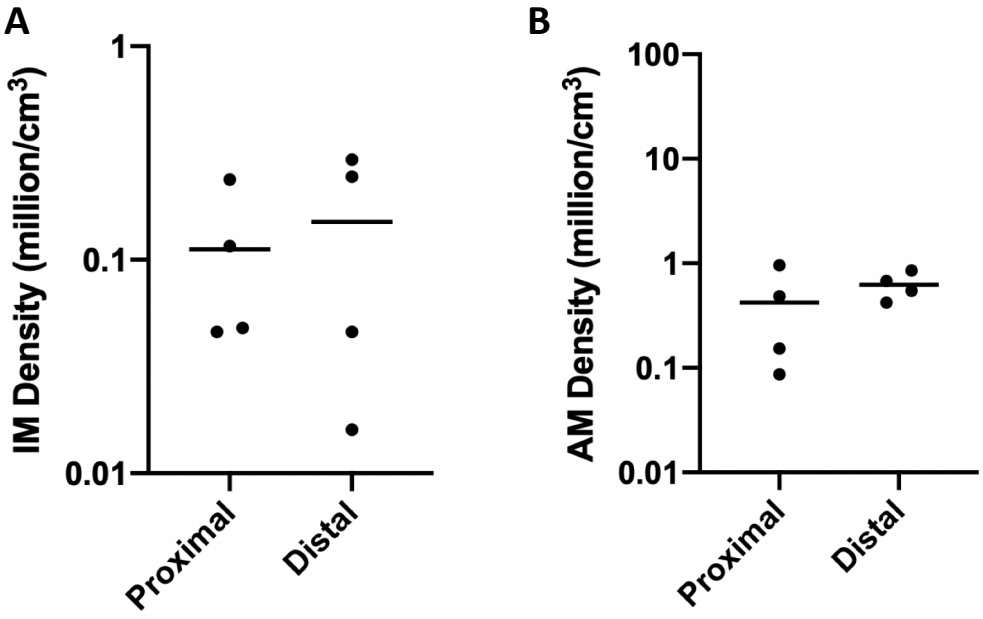

Alveolar Septum
IM Density (million/cm3)
0
10
20
30
40
0
20
40
60
80
age

B


Airway Wall
IM Density (million/cm3)
0
5
10
15
20
0
20
40
60
80
age

C


Vessel Wall
IM Density (million/cm3)
0
5
10
15
20
0
20
40
60
80
age


A
IM Density (million/cm3)
1
0.1
0.01
Proximal
Distal
B
AM Density (million/cm3)
100
10
1
0.1
0.01
Proximal
Distal