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A Census of the Lung: CellCards from LungMAP

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Abstract

The human lung plays vital roles in respiration, host defense and basic physiology. Recent technological advancements such as single-cell RNA sequencing and genetic lineage tracing have revealed novel cell types and enriched functional properties of existing cell types in lung. The time has come to take a new census. Initiated from members of the NHLBI-funded LungMAP Consortium and aided by experts in the lung biology community, we synthesized current data into a comprehensive and practical cellular census of the lung. Identities of cell types in the normal lung are captured in individual cell cards with delineation of function, markers, developmental lineages, heterogeneity, regenerative potential, disease links and key experimental tools. This publication will serve as the starting point of a live, up-to-date guide for lung research at https://lungmap.net/cell-cards-browser/. We hope that this Lung CellCards will promote the community-wide effort to establish, maintain and restore respiratory health.

eTOC:

Sun et al. takes a cellular census of the normal human lung by accounting for not only the recent explosion of single cell datasets, but also by annotating function and developmental origin. It is intended as a blueprint practical guide to initiate harmonization of cell nomenclature across lung biology studies.

INTRODUCTION

The human lung is vital for survival starting at first breath. Serving as the primary gas exchange organ, an average adult lung is composed of ~480 million gas-exchange units called alveoli, comprising a surface area of ~130m^2 (Ochs, et al., 2004; Weibel, 2009). Inhaled air reaches alveoli through an elaborate branched network of conducting airways (Fig. 1). Once there, oxygen and CO_2 exchange occurs across the alveolar epithelium-capillary interface, before air is exhaled through the same airways. Cells of the immune system are also critical components of the lung with innate immune cell types taking up residence early in development. Other immune cells traverse across the vasculature in response to infection or injury. The lung is also wired by nerves of both the afferent and efferent types, indicating that this large organ serves as both the signaling source and effector target of the nervous system.

Lung disease is a leading cause of morbidity and mortality in the world. Its impact on human health is newly demonstrated by the devastation of the COVID-19 pandemic. Only in the coming years will we learn how this pandemic has injured the lungs of so many, and what the long term consequences are. In 2008, a key paper was published following an NHLBI workshop on the cellular composition of the lung (Franks, et al., 2008). In the years since, multiple groundbreaking technological advancements have occurred such as single-cell RNA sequencing (scRNA-seq), in vivo genetic lineage tracing, and newer methods of cell fate tracking generated through CRISPR/Cas9-based genome editing. These technologies have led to discoveries of not only new cell types, but also a more precise definition of cellular properties in lung. Several of these cell types are unique to the lung (e.g. alveolar type 2 or AT2 cells). Others, while having counterparts in other tissues (e.g. capillary endothelial cells), are customized to accommodate unique demands in the respiratory system. In particular, the impressive accumulation of scRNA-seq datasets has led to the putative identification of new cell types or states in the mouse and human lung. Some of these efforts have been directed towards surveying the normal lung (Raredon, et al., 2019; Reyfman, et al., 2019; Deprez, et al., 2020; Travaglini, et al., 2020), while others have interrogated abnormal lungs from devastating diseases such as idiopathic pulmonary fibrosis and cystic fibrosis (Reyfman, et al., 2019; Adams, et al., 2020; Habermann, et al., 2020; Carraro, et al., 2021). While the datasets reported in these studies are critical to our understanding of normal lung development and the response to injury and disease, there remains a significant need for conformational studies to better support and understand these findings. One of the core directives of the LungMAP Consortium is to utilize single cell techniques to map and characterize human lung cell lineages across the lifespan. However, as a group, we understand the limitations of evolving single cell technologies and have chosen to focus on cell types which have been validated with further experimentation in this CellCards document (Fig. 2). These confirmatory approaches include lineage tracing, organoid assays, and extensive subsequent gene and protein expression studies. As further studies are performed and reported, we hope and expect that additional validated cell types will be added to the present collection. Until such rigorous interrogation is performed, however, we have defined some of the novel cell populations that have emerged from single cell transcriptome studies under the entry of “heterogeneity and cellular states”.

While this document initiated from LungMAP investigators, the product is the result of a community-wide effort with extensive inputs from numerous leaders of lung biology. We have chosen to use a “cell cards” format, with the intent to provide comprehensive, streamlined, and easy-to-update information for practical assessment of lung cell types. Data are derived from both mouse and human lungs. We have utilized common names or terms to apply to well annotated cell types present in both the human and mouse lungs. In some instances, names or terms that have primarily been used for mouse cell types have been replaced with more generalizable names that can apply to multiple species, i.e. using the name secretory cell to encompass “club” cells, which is a term more specific to the mouse than human lung. We cover topics including cellular function, markers for identification, developmental origin, regenerative potential, and links to disease (Fig.3). In a section following the individual cards, we summarize emerging data from advanced proteomic and lipidomic approaches. While these streamlined cards are not intended to provide an in-depth assessment of lung cell types, in each section, we suggest key references for landmark primary studies and comprehensive reviews. For additional syntheses of existing cell type characteristics, we refer readers to several excellent reviews (Hogan, et al., 2014; Tata and Rajagopal, 2017; Basil, et al., 2020; Riccetti, et al., 2020; Ushakumary, et al., 2021).

This rigorously curated document is intended as the prototype of a frequently updated live version of Lung CellCards on the NHLBI-supported LungMAP.net website. We hope that this resource will promote lung research from both within the lung community and beyond.

As a vetted resource, the CellCards were created with the following considerations to limit repetition and enhance readability:

- A Table was generated for transcript and protein markers, as well as mouse genetic tools for lung structural cell types (Table 1).
- A figure was generated for immune cell type surface protein combinations for FACS and another for transcript markers (Fig. 6).
- Markers for each cell type were selected based on existing markers in the literature as well as recent data from single-cell RNAseq experiments. Unless specified, entries (markers, cellular properties, etc.) apply to both mouse and human lungs. Human-specific markers are delineated with the superscript “H”, and mouse-specific markers are delineated with the superscript “M”. Under “Developmental origin” and “Regenerative potential”, unless otherwise stated, properties are based on findings from mouse studies.
- To avoid repetition, under experimental validation: “standard approaches” refers to the following: immunofluorescence (IF) and immunohistochemical (IHC) staining; RNA in situ hybridization or RNAscope; bulk RNAseq of sorted cells, scRNA-seq.
- Given space limitation, we restricted references to landmark primary studies and comprehensive reviews, in which additional important studies can be found.

CELLCARDS BY LINEAGE

EPITHELIUM—The epithelium of the lungs and trachea are the externally facing cells of the respiratory system and as such have a wide variety of functions including gas exchange, clearance of foreign matters and pathogens, immunosurveillance, and transmission of external environmental cues to other cell types in the respiratory system (Fig. 4). The entirety of the respiratory epithelium is derived from the transcription factor NKX2–1-expressing endoderm specified in the early anterior ventral foregut (Kimura, et al., 1996; Minoo, et al., 1999). Early developmental events lead to the separation of the trachea and lung endoderm progenitors from the esophageal progenitors and rapid extension and branching of the primitive respiratory tree. Concurrent with these early events, the respiratory endoderm diverges along a proximal-distal axis to generate distinct progenitors essential for generating either airway or alveolar epithelium. After proximal-distal patterning, differentiation of the various mature epithelial lineages begins. These differentiation events have been characterized using histological analyses with multiple cell type-specific markers, cell lineage mapping techniques using indelible recombinase marking of both early and late endoderm/epithelial cell types, and most recently single-cell genomic analysis. While many of the distinct epithelial cell lineages in the mature lung have been identified, there remains much that is unknown about their cell fate relationships with each other during normal adult homeostasis and after injury. Questions regarding cellular plasticity versus stem/progenitor cell relationships have been reviewed previously (Hogan, et al., 2014; Tata and Rajagopal, 2017; Basil, et al., 2020). The cell lineages described below have been validated using multiple techniques including cell type-specific lineage tracing, loss- and gain-of-function genetic models, ex vivo models of cellular function including organoids, and single-cell genomic assessments (Fig. 2). We describe lung epithelial cells in the sequence of cell types in the airway, alveolar and glandular epithelium.

AIRWAY EPITHELIUM—Airway epithelial cells line the conducting airways of the trachea, bronchi and bronchioles. Some of these cell types are common while others are rare. Each is functionally specialized to moisturize the air, clear inhaled particles, serve as progenitors in repair, or sense aerosolized signals. Collectively, the airways conduct air to and from the gas exchange alveoli with the epithelium serving as a mucosal barrier to pathogens.

Basal cells: ***Morphological features:*** epithelial cells that underline luminal cells.

Function: serve as progenitors for other airway epithelial cells in homeostasis and repair.

Other names: none.

Markers: genes and proteins: *TP63, KRT5* (Table 1).

Location: reside in the basal layer of airway epithelium. In mouse, basal cells are common in the trachea and extrapulmonary main stem bronchi that are lined with cartilage. They are rare in intrapulmonary airways that are without cartilage (Yang, et al., 2018). In human, aside from trachea and extrapulmonary bronchi, basal cells are also found all along the

intrapulmonary airways, with diminishing number towards respiratory bronchioles (Fig. 4A).

Experimental validation: standard approaches; air-liquid interface culture, organoids (Rock, et al., 2009; Mou, et al., 2016); mouse-lineage tracing using *Trp63*^{creERT2}, *Krt5*^{creERT2}; iPSC-derived human basal cells (Hawkins, et al., 2020).

Developmental origin: in mouse, differentiated basal cells were first detected in the basal layer of airway epithelium based on their expression of Krt5 at E15.5. During development, they arise from naïve airway epithelial progenitors (Yang, et al., 2018). During adult homeostasis and injury repair, they are replenished primarily by surviving basal cells (Montoro, et al., 2018). In mice, when all basal cells are genetically depleted, secretory (club) cells can de-differentiate into basal cells (Pardo-Saganta, et al., 2015). In regions with submucosal glands, lost surface epithelium basal cells can be replaced by myoepithelial cells (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018).

Heterogeneity and cellular states: Krt13-expressing hillock cells in the mouse trachea (Montoro, et al., 2018); suprabasal, squamous cells and additional subsets from scRNAseq of the human airway (Deprez, et al., 2020; Carraro, et al., 2021).

Regenerative potential: basal cells are the primary airway progenitor cells. They can generate secretory and ciliated cells during homeostasis and following airway epithelium injury such as naphthalene injury (Hong, et al., 2004; Montoro, et al., 2018). In mouse following severe H1N1 influenza infection, rare Trp63+ basal progenitor cells, or lineage negative epithelial progenitors (LNEPs), can give rise to alveolar Krt5+ pods which act as an emergency response to re-establish barrier in the lung (Xi, et al., 2017; Yang, et al., 2018). In mouse following bleomycin injury, Trp63+ basal cells contributed less efficiently to alveolar Krt5+ pods than *Scgb1a1*^{creERT2} lineaged cells (Cassandras, et al., 2020).

Link to disease: basal cell hyperplasia is a common feature of COPD. Basal cells can serve as precursors for NSCLC (Ferone, et al., 2020).

Key references: (Rock, et al., 2009; McCauley, et al., 2018; Yang, et al., 2018; Hawkins, et al., 2020)

Secretory cells: ***Morphological features:*** columnar or cuboidal luminal epithelial cells that contain secretory granules. In mouse airways, they exhibit dome-shaped apical surface.

Function: keep the airway moist through production of secretoglobins into airway lumen.

Other names: Clara cells, club cells.

Markers: genes: *SCGB1A1, SCGB3A2*, protein: SCGB1A1 (Table 1).

Location: luminal layer of airway epithelium (Fig. 4D).

Experimental validation: standard approaches; air-liquid interface culture; organoids; mouse lineage tracing using *Scgb1a1*^{creERT2} which is active in most secretory cells, and also labels a subset of AT2 cells (Rawlins, et al., 2009b; Ray, et al., 2016).

Developmental origin: in mouse, during development, secretory cell progenitors were first detected at E14.5 based on *Scgb3a2* RNA expression (Guha, et al., 2012; Kiyokawa, et al., 2021). This is followed by the onset of *Scgb1a1* expression at E16.5. During adult homeostasis and injury repair, they arise from basal cells or other secretory cells (Pardo-Saganta, et al., 2015; Montoro, et al., 2018).

Heterogeneity and cellular states: in mouse, a subset of variant secretory or club cells express reduced level of *Cyp2f2*, encoding Cytochrome P450, rendering them resistant to naphthalene-induced cell death (Hong, et al., 2001; Guha, et al., 2012). These surviving cells serve as a primary source of reparative cells. In mouse following bleomycin injury, H2-K1^{hi} secretory-like cells have enhanced progenitor property and give rise to alveolar cells (Kathiriya, et al., 2020). Human and ferret lungs contain a SCGB3A2+ respiratory airway secretory cell (RASC) lineage unique to large mammal respiratory bronchioles, which are capable of regenerating AT2 cells (Basil et. al.-under revision).

Regenerative potential: secretory cells can self-renew and differentiate into ciliated cells during mouse airway development, homeostasis and airway epithelium injury repair (Rock, et al., 2009). Following severe lung injury such as bleomycin-induced damage, both *Sox2*^{creERT2}-lineaged cells as well as *Scgb1a1*^{creERT2}-lineaged cells can give rise to a small percentage of AT 1 and AT2 cells, as well as Krt5+ pods in the alveolar region (Yuan, et al., 2019; Cassandras, et al., 2020). As the common cell type that is lineage traced by both of these cre lines is secretory cells, these results suggest that secretory cells can give rise to a minority of alveolar epithelial cells following bleomycin-induced injury. The absolute number and precentage of alveolar epithelial cell generated from secretory cells after injury varies, depending on the severity and injury type including influenza and bleomycin based injuries (Ray, et al., 2016; Yuan, et al., 2019; Cassandras, et al., 2020; Kathiriya, et al., 2020). Use of the *Scgb1a1*^{creERT2} mouse line to lineage trace the contribution of secretory cells to the alveolar epithelium is confounded by the finding that this cre line marks a subset of AT2 cells at homeostasis in the uninjured lung (Rawlins, et al., 2009b; Ray, et al., 2016). The RASC lineage is capable of self-renewal and regenerating AT2 cells in the human lung (Basil et. al.-under revision).

Link to disease: can take on goblet cell characteristics and produce mucin in diseases such as asthma and chronic obstructive pulmonary disease (COPD).

Key references: (Giangreco, et al., 2009; Rock, et al., 2009; Pardo-Saganta, et al., 2015)

Ciliated cells: ***Morphological features:*** display multiple motile cilia on the apical surface.

Function: clear inhaled particles trapped by airway mucosal fluid.

Other names: multi-ciliated cells.

Markers: genes: *FOXJ1, RSPH1*; protein: FOXJ1, acetylated Tubulin (Table 1).

Location: luminal layer of airway epithelium (Fig. 4C).

Experimental validation: standard approaches; air-liquid interface culture; organoids; imaging beating of cilia, mouse lineage tracing using *Foxj1*^{creERT2} (Rawlins and Hogan, 2008).

Developmental origin: In mouse, ciliated cells were first detected in the airway epithelium based on their expression of FOXJ1 at embryonic day (E)15.5. During development, they arise from naïve airway epithelial progenitors (Rawlins and Hogan, 2008). During adult homeostasis and injury repair, they arise from either basal cells or secretory cells (Pardo-Saganta, et al., 2015; Montoro, et al., 2018).

Heterogeneity and cellular states: a subset of ciliated cells express *Miwi2* (Wasserman, et al., 2017). Single cell RNAseq of the human airway revealed early, mature and immunoregulatory subsets (Carraro, et al., 2021), as well as a transitional cell cluster termed deuterosomal cells between secretory and multi-ciliated cells (Deprez, et al., 2020).

Regenerative potential: no regenerative potential reported. Ciliated cells are terminally differentiated.

Link to disease: signature cell type affected in primary ciliary dyskinesia. Disruption of ciliated cell function is also observed in many complex lung diseases, e.g., COPD, cystic fibrosis, and asthma.

Key references: (You, et al., 2004; Rawlins and Hogan, 2008; Carraro, et al., 2021)

Goblet cells: ***Morphological features:*** large cells that contain mucus granules.

Function: in a normal lung, goblet cells secrete mucus to the luminal surface to trap inhaled particles. In diseases such as asthma and COPD, over production/secretion of mucus obstructs the airway, leading to reduced air conductance and air trapping.

Other names: mucus cells.

Markers: genes: *MUC5AC, SPDEF*; proteins: MUC5AC, AGR2 (Table 1).

Location: luminal layer of airway epithelium. They are rare or absent in normal mouse airways but present in normal human airways (Fig. 4B). Are induced to form after allergen exposure or injury of the mouse airways.

Experimental validation: standard approaches; PAS staining, air-liquid interface culture, organoids.

Developmental origin: during development, they likely arise from naïve airway epithelial progenitors, although this has not been directly studied as they are rare in mouse airways. During pathogenesis such as in asthma models, they arise primarily from secretory cells that acquire goblet cell characteristics.

Heterogeneity and cellular states: by scRNAseq, mouse tracheal goblet cells are separated into three subsets: immature goblet, goblet 1 expressing mucosal genes *Tff1, Tff2, Muc5ac* and *Muc5b*, and goblet 2 expressing lectin-like secreted protein genes *Dcpp1* and *Dcpp2* (Montoro, et al., 2018). In human, MUC5AC is produced by goblet cells in surface epithelium while MUC5B is produced by goblet cells in both surface epithelium and submucosal glands (Okuda, et al.,2019).

Regenerative potential: unknown.

Link to disease: goblet cell metaplasia is a key feature of many airway diseases including COPD, asthma and CF.

Key references: (Chen, et al., 2009; Chen, et al., 2014a; Ostedgaard, et al., 2017)

Pulmonary neuroendocrine cells (PNECs): ***Morphological features:*** rare airway epithelial cells with dense core vesicles that contain neuropeptides and neurotransmitters. Present either as solitary cells or in clusters in neuroepithelial bodies (NEBs). Solitary cells are spindle-like in morphology while clustered cells are wedge-like in morphology with wider apical than basal surface.

Function: act as airway sensor. PNECs are stimulated by signals such as allergen, nicotine and mechanical stretch, and respond by secreting neuropeptides and neurotransmitters.

Other names: none.

Markers: genes: *ASCL1, GRP*^{H}(also termed Bombesin), *Calca*^{M}; proteins: GRP^{H}, CGRP^{M} (Table 1).

Location: reside in the tracheal and airway epithelium. In mice, NEBs are enriched at intrapulmonary airway branch points where inhaled particles congregate (Branchfield, et al., 2016b; Sui, et al., 2018). In human, clustered PNECs are less prevalent than in mice and their localization is less stereotypical (Fig. 4E,4L).

Experimental validation: standard approaches; air-liquid interface culture; organoids; mouse lineage tracing using *Ascl1*^{creERT2} (Kuo and Krasnow, 2015; Branchfield, et al., 2016b) *and Calca*^{creERT2} (Song, et al., 2012).

Developmental origin: in mouse, specified PNECs were first detected by ASCL1 antibody staining at E12.5 (Kuo and Krasnow, 2015; Noguchi, et al., 2015). They likely arise from naïve airway epithelial progenitors. In mouse trachea during homeostasis or after hypoxia exposure, PNECs can originate from basal cells (Montoro, et al., 2018; Shivaraju, et al., 2021).

Heterogeneity and cellular states: heterogeneity in the expression of neuropeptides, Notch2 expression and proliferative potential (Ouadah, et al., 2019).

Regenerative potential: can give rise to club and ciliated cells following airway injury (Hong, et al., 2001; Song, et al., 2012).

Link to disease: PNECs are required for allergen induced asthmatic response (Kuo and Krasnow, 2015; Noguchi, et al., 2015; Branchfield, et al., 2016b; Sui, et al., 2018). Increase in PNEC number has been documented in a wide-spectrum of human lung diseases.

Key references: (Kuo and Krasnow, 2015; Noguchi, et al., 2015; Branchfield, et al., 2016b; Sui, et al., 2018)

Tuft cells—*Morphological features:* rare airway epithelial cells that are spindle in shape with microvilli on the apical surface.

Function: have chemosensory function. Tuft cells respond to signals by releasing cytokines such as IL25 as well as leukotrienes.

Other names: solitary chemosensory cells, brush cells.

Markers: genes: *POU2F3, ASCL2, Dclk1*^{M}; proteins: POU2F3, DCLK^{M}.

Location: in mouse during homeostasis, primarily found in the tracheal epithelium (Gerbe, et al., 2009; Saunders, et al., 2013; Bankova, et al., 2018). Following influenza infection, ectopic tuft cells arise in intrapulmonary airwarys and near alveolar Krt5+ pods (Saunders, et al., 2013; Montoro, et al., 2018; Rane, et al., 2019).

Experimental validation: standard approaches; mouse lineage tracing using *Dclk1*^{creERT2} (Westphalen, et al., 2014); lineage reporters *Trpm5-GFP* (Saunders, et al., 2013; Bankova, et al., 2018).

Developmental origin: the origin of tuft cells in prenatal development has not been determined. During postnatal development and homeostasis, lineage tracing and labeling of proliferative cells indicated that tuft cells can originate from basal cells (Saunders, et al., 2013; Bankova, et al., 2018; Montoro, et al., 2018).

Heterogeneity and cellular states: by scRNA-seq, mouse trachea tuft cells are separated into three subsets: immature tuft cells, tuft 1 (sensory) and tuft 2 (inflammatory) (Montoro, et al., 2018).

Regenerative potential: unknown. In intestine, tuft cells can promote proliferation of adjacent epithelial cells by producing paracrine signals (von Moltke, et al., 2016).

Link to disease: tracheal tuft cells play a role in allergen response (Bankova, et al., 2018).

Key references: (Saunders, et al., 2013; Bankova, et al., 2018; Montoro, et al., 2018; Rane, et al., 2019)

Ionocytes—*Morphological features:* rare columnar epithelial cells.

Function: maintains airway fluid balance. In mouse, ionocytes are the primary cells in the surface airway epithelium that express *Cftr*, the chloride channel gene mutated in cystic fibrosis (Montoro, et al., 2018; Plasschaert, et al., 2018).

Other names: none.

Markers: genes: *FOXI1, ASCL3, Cft*^{M} (Table 1).

Location: airway epithelium.

Experimental validation: standard approaches; air-liquid interface culture; mouse lineage tracing using ***Ascl3***$^{eGfp\text{-}cre}$ (Bullard, et al., 2008); mouse reporter labeling using *Foxi1-GFP* (Montoro, et al., 2018).

Developmental origin: the origin of ionocytes in development has not been determined. During homeostasis, lineage tracing indicated that ionocytes can originate from basal cells (Montoro, et al., 2018).

Heterogeneity and cellular states: unknown.

Regenerative potential: unknown.

Link to disease: in the mouse trachea, ionocytes are the primary cell type that express the cystic fibrosis gene *Cftr*.

Key references: (Montoro, et al., 2018; Plasschaert, et al., 2018)

Bronchioalveolar stem cells (BASCs)—*Morphological features:* rare cuboidal lung epithelial cells that express both secretory and AT2 marker.

Function: progenitor cells that give rise to both airway and alveolar cells during repair (Kim, et al., 2005; Liu, et al., 2019; Salwig, et al., 2019).

Other names: none.

Markers: in mouse, co-express the AT2 marker *Sftpc* and secretory cell marker *Scgb1a1* (Kim, et al., 2005; Liu, et al., 2019; Salwig, et al., 2019) (Table 1). Unclear whether this cell type, or a similar cell type, is present in human lungs.

Location: in mouse, reside at the bronchioalveolar junction (BADJ), a structure found in the mouse but not human lung.

Experimental validation: standard approaches; mouse dual recombinases or split cre effector tracing using *Sftpc* and *Scgb1a1* drivers (Liu et al., 2019; Salwig et al., 2019).

Developmental origin: unknown.

Heterogeneity and cellular states: unknown.

Regenerative potential: can generate both alveolar and airway epithelial cells in lung repair in homeostasis and after injury (Kim, et al., 2005; Liu, et al., 2019; Salwig, et al., 2019).

Link to disease: BASCs were identified as a possible cell of origin for NSCLC (Kim, et al., 2005; Liu, et al., 2019; Salwig, et al., 2019).

Key references: (Kim, et al., 2005; Liu, et al., 2019; Salwig, et al., 2019)

ALVEOLAR EPITHELIUM

Alveolar epithelial cells line the alveoli. The large and squamous alveolar type 1 cells constitute ~95% of the surface area. In comparison, alveolar type 2 cells secrete surfactant to reduce surface tension and promote alveoli expansion. Together, they perform the core function of gas exchange.

Alveolar type 1 (AT1) cells—*Morphological features:* large flattened epithelial cells.

Function: essential for gas exchange by forming large and thin gas-diffusible interface juxtaposing capillary endothelium. Also act as a potent signaling hub regulating postnatal alveologenesis (Zepp, et al., 2021).

Other names: alveolar epithelial cell 1 (AEC1).

Markers: genes: *AGER*^{H}, *Hopx*^{M}, *Rtkn2*, proteins: AGER, HOPX (Table 1).

Location: alveoli (Fig. 4G,H,I; Fig. 5D).

Experimental validation: standard approaches; organoids; mouse lineage tracing using *Hopx*^{creERT2} (Jain, et al., 2015), *Ager*^{creERT2} (Chung and Hogan, 2018) and *Aqp5*^{cre} (Flodby, et al.,2010); fluorescence-activated cell sorting (FACS) using anti-HTI-56^{H} antibody (Dobbs, et al., 1999).

Developmental origin: in mouse, by *Hopx*^{creERT2} lineage tracing and anti-HOPX antibody staining, specified AT 1 cells were first detected at E15.5, likely originating from multipotent Nkx2–1+ distal stalk cells (Frank, et al., 2019; Zepp, et al., 2021).

Heterogeneity and cellular states: in mouse, there are Igfbp2+ and Igfbp2-AT1 subtypes (Wang, et al., 2018).

Regenerative potential: while previously considered terminally differentiated, they exhibit extensive ability to reprogram into AT2 cells after hyperoxic injury (Penkala, et al., 2021), and to a lesser extent after pneumonectomy (Jain, et al., 2015). In postnatal homeostasis and following injury, lost AT1 cells are replaced by AT2 cells (Barkauskas, et al., 2013).

Link to disease: reduced in number and function in COPD and BPD.

Key references: (Jain, et al., 2015; Wang, et al., 2018; Frank, et al., 2019)

Alveolar type 2 (AT2) cells—*Morphological features:* cuboidal alveolar epithelial cells with lamellar bodies and specialized microvilli.

Function: produce surfactant, a protein and lipid mixture that reduces surface tension to allow lung expansion during inhalation, as well as promotes host defense by assisting the killing of pathogens.

Other names: alveolar epithelial cell 2 (AEC2).

Markers: genes: *SFTPC, LAMP3*; proteins: SFTPC, ABCA3 (Table 1).

Location: alveoli (Fig. 4F,I-K).

Experimental validation: standard approaches; organoids; mouse lineage tracing using *Sftpc*^{creERT2} (Chapman, et al., 2011; Barkauskas, et al., 2013); FACS using anti-HTII-280^{H} antibody (Dobbs, et al., 1999).

Developmental origin: in mouse, specified AT2s can be detected by *Sftpc* RNA in situ and lineage tracing at E14.5-E15.5, likely originating from Sox9/Id2+ distal tip cells (Frank, et al., 2019; Zepp, et al., 2021). In postnatal homeostasis and following injury, lost AT2 cells are primarily replaced by remaining AT2 cells.

Heterogeneity and cellular states: AT2s harbor a progenitor sub-lineage (WNT-responsive Axin2+ alveolar epithelial progenitors or AEPs) that are critical for alveolar regeneration after injury (Nabhan, et al., 2018; Zacharias, et al., 2018).

Regenerative potential: a key driver of alveolar epithelial regeneration. Can self-renew as well as differentiate into AT1, at a low frequency during homeostasis, and at extensive levels after alveolar injury.

Link to disease: surfactant deficiencies leads to respiratory distress in neonates. AT2 dysfunction and senescence is associated with pulmonary fibrosis (Nureki, et al., 2018; Katzen and Beers, 2020). AT2 cells are an important cell of origin for NSCLC adenocarcinoma (Desai, et al., 2014).

Key references: (Barkauskas, et al., 2013; Nabhan, et al., 2018; Zacharias, et al., 2018; Frank, et al., 2019)

GLANDULAR EPITHELIUM

Submucosal glands (SMG) are a unique microorgan found in the connective tissues lining the large proximal cartilaginous airways and are a major source of the protective mucus layer critical to normal innate defense (Widdicombe and Wine, 2015) (Fig. 4M-R). Morphologically, SMGs are a branched tubule-acinar structure similar to the salivary gland, consisting of a single duct that has undergone multiple dichotomous branching events and then terminates in bulbous acini. The resulting mature SMG can be divided into four structural domains along the proximal-to-distal axis: the terminal ciliated duct, collecting ducts, mucous tubules and the distal serous acini (Fig. 4M). The terminal ciliated duct links the SMG to the surface airway epithelium and is lined with cell types that parallel the surface airway counterparts including ciliated, goblet, secretory, basal cells and ionocytes (Fig. 4O). Ciliated cells are lost at the first bifurcation into the collecting ducts. Collecting ducts are lined by epithelial cells that transition from columnar to cuboidal to flattened morphology along the proximal-to-distal axis (Tos, 1966; Meyrick, et al., 1969; Meyrick and Reid, 1970; Matsuba, et al., 1972; Widdicombe, 2019). These cells are rich in mitochondria, a cytoplasm easily highlighted by eosin, and have centrally positioned nuclei. Mucous

tubules are comprised of mucous cells while the most distal acini are comprised of serous cells. Surrounding the acini, mucous tubules, and collecting ducts are a thin layer of myoepithelial cells. The SMG is also known to be innervated and highly vascularized. In this section, we focus on specialized epithelial cells in the SMG that are distinct from those on the surface epithelium.

Terminal ciliated duct basal cells

Morphological features: single epithelial cell layer that may be pseudostratified.

Function: regenerate SMG and surface airway epithelial cells during normal homeostasis and following injury.

Other names: none.

Markers: aside from markers shared with surface airway basal cells, SMG terminal ciliated duct basal cells also express *VIM, SOX9* (Hegab, et al., 2011; Hegab, et al., 2012b; Goldfarbmuren, et al., 2020) (Table 1).

Location: these basal cells are present along the basement membrane of the ciliated duct that opens into the airway (Fig. 4Q).

Experimental validation: standard approaches (Borthwick, et al., 2001; Hegab, et al., 2011; Hegab, et al., 2012b; Anderson, et al., 2017); mouse lineage tracing using *Sox9*^{preERT2}*/R26-LSL-tdTomato* (Tata, et al., 2018); mouse reporter labeling using Krt5-eGFP strain (Schoch, et al., 2004); grafting experiments (Engelhardt, et al., 1995; Borthwick, et al., 2001; Hegab, et al., 2011).

Developmental origin: in humans, SMG ducts are first evident at ~10 weeks of gestation. In mouse at postnatal day (P)0, placodes lined by naïve epithelial cells evaginate from the developing tracheal epithelium. These TRP63+/KRT5+/KRT14+/SOX9+/LEF1+ naïve epithelial cells give rise to basal cells and the other cell types within the SMG (Rawlins and Hogan, 2005; Lynch, et al., 2016). These buds elongate distally and become surrounded by myoepithelial like cells that are ACTA2+KRT5+KRT14+ (Anderson, et al., 2017). Following lung injury, SMG basal cells can arise from both remaining basal cells or myoepithelial cells of the SMG (Hegab, et al., 2011; Anderson, et al., 2017; Lynch, et al., 2018).

Heterogeneity and cell states: scRNA-seq analysis identified multiple subtypes of SMG basal cells (Goldfarbmuren, et al., 2020). Bulk sequencing of mouse surface airway basal versus SMG duct basal cells revealed distinct expression profiles (Hegab, et al., 2011).

Regenerative potential: serve as progenitors capable of repopulating cells within the SMG and the tracheal/bronchial epithelium (Engelhardt, et al., 1995; Borthwick, et al., 1999; Rawlins and Hogan, 2005; Hegab, et al., 2011; Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018).

Link to Disease: This basal cell population is thought to contribute to the basal cell hyperplasia observed in smokers and gland hyperplasia observed in conditions like cystic fibrosis, asthma, and chronic bronchitis (Widdicombe and Wine, 2015). Depletion of this population may contribute to conditions including obliterative bronchitis (Swatek, et al., 2018).

Key references: (Hegab, et al., 2011; Hegab, et al., 2012a; Anderson, et al., 2017)

Myoepithelial cells (MECs)

Morphological features: flattened epithelial cells with elongated processes.

Function: provide structural support to acini to help maintain the hydrostatic pressure generated by fluid secretion. In response to stimulation by cholinergic agonists, myoepithelial cells contract to expel secreted contents of the glands. Following injury, these cells can regenerate multiple epithelial cell types within the SMG and on the airway surface.

Other names: none.

Markers: *KRT14, MYH11* (Anderson, et al., 2017; Goldfarbmuren, et al., 2020) (Table 1).

Location: myoepithelial cells are found on the basal side of acini, mucous tubules and are scattered along the perimeter of ducts (Fig. 4N,P).

Experimental validation: standard approaches (Lynch, et al., 2018; Goldfarbmuren, et al., 2020); mouse lineage tracing using *Acta2*^{creERT2} (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018) and *Myh11-creERT2* (Anderson, et al., 2017).

Developmental origin: myoepithelial cells are one of the earlier cell types that develop from naive SMG placode epithelium (Anderson, et al., 2017). Following lung injury, myoepithelial cells can arise from both basal and remaining myoepithelial cells of the SMG (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018).

Heterogeneity and cell states: unknown.

Regenerative potential: myoepithelial cells are capable of repopulating cells within the SMG and the tracheal/bronchial epithelium (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018).

Link to Disease: in patients with severe asthma, there is an increase in overall gland size and smooth muscle actin content of myoepithelial cells (Green, et al., 2010).

Key references: (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018; Goldfarbmuren, et al., 2020)

Mucous cells

Morphological features: cells with basally positioned nucleus and large densely packed electron opaque granules from the Golgi to the apical cell surface.

Function: in response to stimulation by basolateral neurotransmitters, these cells produce and secrete multiple mucins involved in host defense.

Other names: none.

Marker genes: *MUC5B, SPDEF* (Chen, et al., 2009) (Table 1). Mucous cells are identifiable by staining with the lectin Ulex Europaeus Agglutinin 1 (UEA-1)(Anderson, et al., 2017).

Location: line the mucous tubules that are located between the collecting ducts and distal serous acini (Fig. 4M,P,R).

Experimental validation: standard approaches (Audie, et al., 1993; Hegab, et al., 2012b; Widdicombe and Wine, 2015; Anderson, et al., 2017; Lynch, et al., 2018; Goldfarbmuren, et al., 2020), air-liquid interface trans-well cultures (Finkbeiner, et al., 2010).

Developmental origin: during development, mucous cells arise from either basal or myoepithelial precursors of the SMG (Engelhardt, et al., 1995; Hegab, et al., 2011; Anderson, et al., 2017). Similarly following lung injury, mucous cells can arise from either basal or myoepithelial cells of the SMG (Hegab, et al., 2012b; Lynch, et al., 2018; Tata, et al., 2018).

Heterogeneity and cell states: unknown.

Regenerative potential: unknown.

Link to Disease: mucous cells of the SMG are a primary source of excess mucus production in conditions like asthma and chronic bronchitis (Widdicombe and Wine, 2015).

Key references: (Chen, et al., 2009; Widdicombe and Wine, 2015)

Serous cells

Morphological features: contain small electron dense granules and intracellular canaliculi.

Function: produce a variety of mucins, antimicrobial peptides and other molecules involved in host defense. In addition, serous cells are a main source of fluid from the gland, driven by secretion of bicarbonate and chloride.

Other names: none.

Markers: *LYZ, LTF* (Lee and Foskett, 2010; Hegab, et al., 2012b) (Table 1). Serous cells are also identifiable following staining with the lectin Dolichos Biflorus Agglutinin (DBA) (Anderson, et al.,2017).

Location: distal acini of the SMG (Fig. 4R).

Experimental validation: standard approaches (Borthwick, et al., 1999; Lee and Foskett, 2010; Hegab, et al., 2012b; Anderson, et al., 2017; Lynch, et al., 2018; Goldfarbmuren, et al., 2020) (Engelhardt, et al., 1992; Audie, et al., 1993); air-liquid interface trans-well

cultures (Finkbeiner, et al., 2010), fluid secretion assays (Lee and Foskett, 2010; Khansaheb, et al., 2011).

Developmental origin: during development, serous cells are derived from either basal or myoepithelial precursor cells of the SmG (Engelhardt, et al., 1995; Hegab, et al., 2011; Anderson, et al., 2017). Similarly following lung injury, serous cells are rederived from either basal or myoepithelial cells of the SMG (Hegab, et al., 2012b; Lynch, et al., 2018; Tata, et al., 2018).

Heterogeneity and cell states: unknown.

Regenerative potential: unknown.

Link to Disease: The lack of functional chloride channels encoded by mutations in CFTR are associated with reduced gland secretions from serous cells and mediate the pathogenesis observed in cystic fibrosis (OMIM:602421) (Widdicombe and Wine, 2015).

Key references: (Hegab, et al., 2012b; Widdicombe and Wine, 2015)

MESENCHYME

Starting from early development, the lung mesenchyme provides instructive cues to the epithelium to control proliferation, differentiation, and patterning along the proximal-distal axis. Classical tissue recombination experiments show that distal mesenchyme instructs distal epithelial specification, even when placed next to proximal epithelial cells (Shannon, 1994; Shannon, et al., 1998). The converse is true when proximal mesenchyme is placed next to distal epithelial cells. Subsequent studies have shown that reciprocal paracrine signaling between the mesenchyme and epithelium, so termed epithelium-mesenchyme crosstalk, coordinates the growth and differentiation of both cell populations (Shannon, 1994; Bellusci, et al., 1996; Bellusci, et al., 1997a; Bellusci, et al., 1997b; Naski, et al., 1998; Shannon, et al., 1998; Arman, et al., 1999; Weaver, et al., 1999). Throughout development, the pulmonary mesenchyme changes its paracrine and ECM-modulating functions to drive branching morphogenesis, sacculation and alveologenesis (Betsholtz, 1995; Olson and Soriano, 2011; Rock, et al., 2011; Zhang, et al., 2013; Endale, et al., 2017; Moiseenko, et al., 2017; Zepp, et al., 2021). In the adult lung, several mesenchymal cell types serve as niches and provide signals and ECM support for epithelial progenitors and their function in tissue regeneration (Lee, et al., 2017; Zepp, et al., 2017).

In mouse, the lung mesenchyme arises from a population of the lateral plate mesoderm called cardiopulmonary mesoderm progenitors (Peng, et al., 2013). Through multiple steps of development, specialized pulmonary mesenchymal cells emerge. Compared to the epithelial cell types, delineation of the different mesenchymal cell types remains less clear. As such, there is overlap between the different cell populations labeled using single gene reporter systems (Riccetti, et al., 2020). A recent study outlined the lineage progression of the multiple mesenchymal cell types (Zepp, et al., 2021). Aside from the mesenchymal cell cards outlined here, there are other cell groups identified from single cell studies or staining experiments that will require further validation to detail their unique characteristics

(Dahlgren, et al., 2019; Travaglini, et al., 2020). Here, we focus on established mesenchymal cell types and describe them in the proximal-to-distal sequence of their localization.

Airway smooth muscle cells (ASMCs)

Morphological features: spindled mesenchymal cells in bundles subjacent to the airway epithelium in either a continuous circumferential pattern (in non-cartilaginous airway) or in stripes connecting the ends of cartilages (in cartilaginous airways) (Hines, et al., 2013; Cieri, 2019).

Function: contract to control airway tone and size.

Other names: none.

Markers: genes: *ACTA2, DES, LGR6*; protein ACTA2/SMA (Table 1).

Location: subjacent to airway epithelium in airway wall (Fig. 5A).

Experimental validation: standard approaches, mouse lineage tracing using $Acta2^{creERT2}$ (Wendling, et al., 2009), $Lgr6^{creERT2}$ (Lee, et al., 2017).

Developmental origin: in mouse lung, first detected at E10.5 by *Acta2* expression. ASMCs are likely derived from cardiopulmonary mesoderm progenitor cells (Peng, et al., 2013; Ntokou, et al., 2015; El Agha, et al., 2017; Park, et al., 2019) and $Acta2^{creERT2}$ E12.5 lineage traced cells (Zepp, et al., 2021). *Wnt2* is necessary for the formation of ASMCs, but not vascular smooth muscle cells (Goss, et al., 2011).

Heterogeneity and cellular states: unknown.

Regenerative potential: direct regenerative potential unknown. Can serve as niche for regeneration of adjacent airway epithelium following injury (Rock, et al., 2011; Lee, et al., 2017; Volckaert, et al., 2017).

Link to disease: increase in cell number (hyperplasia) and cell size (hypertrophy) are found in asthma. Known to facilitate airway constriction in asthma.

Key references: (Lee, et al., 2017; Riccetti, et al., 2020).

Vascular smooth muscle cells (VSMCs)

Morphological features: spindled cells within the wall of pulmonary artery.

Function: contract to control pulmonary vessel tone and size.

Other names: none.

Markers: genes: *NTRK3, ITGA7*^{H}, *Cnn1*^{M}; protein: ACTA2/SMA (Table 1).

Location: prominent within the walls of pulmonary artery and a minor component of pulmonary vein (Fig. 5A,G).

Experimental validation: standard approaches, mouse lineage tracing using *Acta2*^{creERT2} (Sheikh, et al., 2015).

Developmental origin: in development, derived from the early (E8.5) cardiopulmonary mesoderm progenitors (Peng, et al., 2013) and the later (E12.5) *Acta2*^{creERT2} and *Pdgfrb*^{creERT2} expressing cells by lineage tracing (Zepp, et al., 2021). Unclear how cellular turnover during homeostasis is maintained.

Heterogeneity and cellular states: unknown.

Regenerative potential: unknown.

Link to disease: hyperplasia, hypertrophy and increase in constrictive property of VSMCs are linked to pulmonary hypertension. Source of signals for endothelium and vascular remodeling (de la Cuesta, et al., 2019).

Key references: (Greif, et al., 2012; Sheikh, et al., 2015; Steffes, et al., 2020)

Chondrocytes

Morphological features: round cells surrounded by thick matrix condensed together to form cartilage.

Function: give rise to cartilage that supports airway epithelium and prevent airway collapse at exhalation. Play a role in the development of tracheal epithelial cells (Hines, et al., 2013; Cieri, 2019).

Other names: cartilage cells.

Markers: gene: *COL2A1, HAPLN1*; protein: SOX9 (Table 1).

Location: in mouse, cartilage is only found in trachea and extrapulmonary bronchi. In human, airway cartilage is also present in intrapulmonary bronchi.

Experimental validation: standard approaches, mouse lineage tracing using *Col2a1*^{creERT2} (Hines, et al., 2019), alcian blue staining.

Developmental origin: in development, chondrocytes arise from SOX9+ cells in lung mesenchyme (Hines, et al., 2013).

Heterogeneity and cellular states: unknown.

Regenerative potential: unknown.

Link to disease: malformation of airway cartilage leads to tracheobronchomalacia. Altered airway cartilage pattern was identified as one of the earliest features of cystic fibrosis (Ogrinc, et al., 1998).

Key references: (Ogrinc, et al., 1998; Hines, et al., 2013; Chen, et al., 2014b)

Alveolar fibroblast 1 (AF1) cells

Morphological features: fibroblast-like morphology. Contain lipid granules in early postnatal mouse lung.

Function: a key source of signals such as FGF7 and FGF10 during lung development and postnatal homeostasis.

Other names: lipofibroblasts, matrix fibroblast 1.

Markers: genes: *Tcf21, Wnt2*^{M}; proteins: PLIN2/ADRP.

Location: alveolar mesenchyme (Fig. 5B).

Experimental validation: standard approaches, organoids, lineage tracing using *Tcf21*^{mcrem} (Park, et al., 2019), *Wnt2*^{creERT2} (Zepp, et al., 2021).

Developmental Origin: in mouse, these cells are derived from Wnt2+/Pdgfra+ precursors and obtain their mature phenotype by P3 (Zepp, et al., 2021).

Heterogeneity and cellular states: currently unknown but likely exists.

Regenerative potential: in mouse, proliferate in response to injury.

Link to disease: while some studies suggest that these cells can contribute to myofibroblasts in bleomycin model of lung fibrosis (El Agha, 2017), other studies using more specific cre drivers suggest they contribute to a minor fraction compared to Pdgfrb+ fibroblasts (pericytes-see below) (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019).

Key references: (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019; Ushakumary, et al., 2021)

Alveolar fibroblast 2 (AF2) cells

Morphological features: fibroblast-like morphology.

Function: a source of signals such as FGF7, FSTL1, WNT5A and IL6 that support AT2 cell proliferation and differentiation during postnatal lung maturation, homeostasis, and regeneration. Also a key source of ECM proteins.

Other names: matrix fibroblast 2, mesenchymal alveolar niche cell (MANC) (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019), type-2 associated stromal cell (TASC) (Chung, et al., 2018), adventitial fibroblast (Travaglini, et al., 2020).

Markers: genes: *MFAP5, SCARA5* (Table 1).

Location: alveolar mesenchyme (Fig. 5B).

Experimental validation: standard approaches, organoids. In mouse, tracing and isolation using *Axin2*^{creERT2};*Pdgfra*$^{H2B\text{-}GFP}$ approach (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019).

Developmental Origin: in development, these cells arise from cardiopulmonary progenitors cells together with several other lung mesenchymal cell types (Peng, et al., 2013; Zepp, et al., 2021).

Heterogeneity and cellular states: unknown but are a subset of Pdgfra+ mesenchymal cells.

Regenerative potential: proliferate after tissue injury. Axin2+Pdgfra+ AF2 cells supported AT2 cell organoid growth more efficiently than Wnt2+Pdgfra+ AF1 cells (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019).

Link to disease: unknown.

Key references: (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019; Ushakumary, et al., 2021; Zepp, et al., 2021).

Secondary Crest Myofibroblasts (SCMF)

Morphological features: elongated fibroblasts that contain long actin fibers and are contractile.

Function: a transient lineage that drives alveolar septa formation during alveologenesis, in part by receiving signals from AT 1 cells. Many disappear after alveologenesis through apoptosis, while those that remain in the adult lung no longer express markers such as ACTA2/SMA (Hagan, et al., 2019a; Zepp, et al., 2021).

Other names: myofibroblasts.

Markers: genes: $DACH2^{H}$, $Fgf18^{M}$; protein: ACTA2/SMA (Table 1).

Location: alveolar mesenchyme, underline nascent septal ridges and alveolar entrance rings (Fig. 5C,D) (Chen, et al., 2012; Li, et al., 2015; Branchfield, et al., 2016a; Hagan, et al., 2019b).

Experimental validation: standard approaches, mouse lineage tracing using $Acta2^{creERT2}$ (Wendling, et al., 2009; Zepp, et al., 2021); *Acta2* gene expression using the $Acta2^{DsRed}$reporter (Zepp, et al., 2021), $Pdgfra^{rtTA}$;*tetO-cre* (Li, et al., 2018); $Pdgfra^{creERT2}$ (Zepp, et al., 2021) and $Fgf18^{creERT2}$ (Hagan, et al., 2019a) all at early postnatal stages when these cells are present as contractile cells.

Developmental origin: in mouse, first detected at E15.5 using $Acta2^{DsRed}$ reporter (Zepp, et al.,2021) or later by ACTA2+ staining in the alveolar region at the onset of alveologenesis at P3 (Chen, et al., 2012; Li, et al., 2015; Branchfield, et al., 2016a; Hagan, et al., 2019b). Lineage labeling in prenatal stages indicate that these cells originate from Pdgfra+ and Acta2+ lung mesenchyme as early as E15.5 (Moiseenko, et al., 2017; Li, et al., 2018; Zepp, et al., 2021).

Heterogeneity and cellular states: unknown.

Regenerative potential: as this is a transient lineage, their role in neonatal repair and regeneration remains unclear.

Link to disease: change in myofibroblast number and characteristics contributes to disruption of septa formation and alveolar simplification in BPD (Popova, et al., 2014; Branchfield, et al., 2016a).

Key references: (Chen, et al., 2012; Li, et al., 2015; Branchfield, et al., 2016a; Hagan, et al., 2019b; Ushakumary, et al., 2021; Zepp, et al., 2021)

Pericytes

Morphological features: fibroblast-like cells, often with long processes.

Function: adhere to the endothelium by gap, tight, and adherens junctions, enabling the endothelium to retain a tight barrier (Hung, et al., 2019).

Other names: mural cells, Axin2+ Myofibroblast Precursors (AMPs) (Torday and Rehan, 2016; Zepp, et al., 2017; Park, et al., 2019).

Markers: genes: *TRPC6, LAMC3*; protein:CSPG4 (NG2), PDGFRb (Table 1).

Location: adjacent to blood vessels and alveolar capillaries.

Experimental validation: standard approaches, organoids, mouse lineage tracing using *Pdgfrb*^{creERT2} (Cuervo, et al., 2017).

Developmental origin: arise from *Pdgfrb*^{creERT2} lineage tracing population during development (Zepp, et al., 2021).

Heterogeneity and cellular states: unknown.

Regenerative potential: unknown.

Link to disease: lineage-tracing using *Foxd1-cre* or *Cspg4*^{creERT2} labeled pericytes show that they can proliferate and express ACTA2 following bleomycin-induced injury (Rock, et al., 2011; Hung, et al., 2013; Wilson, et al., 2018).

Key references: (Chen, et al., 2012; Barkauskas, et al., 2013; Hung, et al., 2013; Lee, et al., 2017; Zepp, et al., 2017; Kato, et al., 2018; Biasin, et al., 2020; Zepp, et al., 2021)

Mesothelial cells

Morphological features: flat cells with epithelial morphology even though they are derived from mesenchymal cells.

Function: encase and protect the lung, and provide lubricant surface to allow smooth sliding between lung versus other organs and chest wall. Source of signaling molecules such as FGF9 during development and cytokines such as IL33 during homeostasis (Yin, et al., 2011; Mahlakoiv, et al., 2019).

Other names: lung pleura.

Markers: genes: *WT1, UPK3B, FREM2*; protein: WT1 (Table 1).

Location: external cell layer that wraps around the lung.

Experimental validation: standard approaches, mouse lineage tracing using *Wt1*^{creERT2}*(von* Gise, et al., 2016).

Developmental origin: in mouse starting at E10.5, WT1+ mesothelial cells emerge from the mesodermal lineage (Que, et al., 2008).

Heterogeneity and cellular states: unknown.

Regenerative potential: during development, undergo epithelium-to mesenchyme transition and contribute to lung mesenchymal cells such as vascular smooth muscles and pericytes (Que, et al., 2008; von Gise, et al., 2016).

Link to disease: WT1 + mesothelial cells contribute to fibrotic myofibroblasts in models of fibrosis (Sontake, et al., 2015; Sontake, et al., 2018). Has1^{hi} fibroblasts in regions of fibrosis express WT1, but it is unknown if these cells arose from WT1+ mesothelium or WT1-mesenchymal progenitors (Habermann, et al., 2019).

Key references: (Que, et al., 2008; Sontake, et al., 2015; von Gise, et al., 2016; Sontake, et al., 2018)

ENDOTHELIUM

Pulmonary endothelium facilitates gas exchange between air and blood, delivers nutrients and growth factors to the lung, plays key roles in inflammation, tissue fluid clearance, blood clotting, and serves as source of angiocrine signals for homeostasis and injury repair. The pulmonary vasculature in humans is composed of both pulmonary and bronchial circulations. The pulmonary circulation delivers deoxygenated blood from the right cardiac ventricle to the alveoli through the pulmonary artery and returns oxygenated blood to the left atrium through the pulmonary vein (Fig. 5E). The bronchial circulation originates from the aorta and provides oxygen and nutrient-rich blood to lung structural cells including conducting airways and surrounding tissues. The pulmonary lymphatic system consists of lymphatic vessels, nodes and blunt-ended capillaries, regulating host defense and tissue fluid clearance. Endothelial cells are heterogenous and are comprised of arterial, venous, lymphatic and microvascular/capillary endothelial cells (Fig. 5E), all of which share the expression of cell surface adhesion molecules CD31 (PECAM) (Fig. 1B,F; Fig. 5G,H) and CDH5 (VE-Cadherin), as well as the transcription factor ERG and signaling molecule PDGFB (Kalna, et al., 2019). In mouse, they can be traced using *Cdh5*^{creERT2} and *Pdgfb*^{creERT2}, each label all endothelial cells (Bazigou, et al., 2011; Cai, et al., 2016). Based on scRNAseq studies in mice, pulmonary endothelial cells express a number of genes including *Grtpl, Adrbl, Scn7a, Tmem100, Foxfl* and lncRNA *Fendrr* that are unique to the lung and are not expressed in endothelial cells of other organs (Paik, et al., 2020). For

established lung endothelial cell populations, we describe them in the sequence of cell types in the arterial and venous vessels, lymphatic vessels, and alveolar capillaries.

Arterial endothelial cells

Morphological features: thin flattened cells lining the interior surface of arteries and arterioles.

Function: conduct blood flow from the heart to the lung, regulate inflammatory responses, synthesize and secrete growth factors, regulate hemostasis and coagulation.

Other names: none.

Markers: genes: *GJA5, DKK2*; protein: VWF (also stains vein) (Table 1).

Location: pulmonary arteries are located near bronchi and bronchioles as part of the broncho-vascular bundles.

Experimental validation: standard approaches. In mouse, $Bmx^{creERT2}$ labels arterial endothelium with the exception of smaller arterioles (Ehling, et al., 2013). $Sox17^{creERT2}$ preferentially labels arteries than veins, and it also labels capillary endothelial cells (Liao, et al., 2009).

Developmental origin: at the initiation of lung development, they arise from cardiopulmonary progenitor cells (Peng, et al., 2013). Later on, they arise from endothelial progenitor cells (Bolte, et al., 2018). They can also arise from proliferation of existing arterial endothelial cells (Whitsett, et al., 2019).

Heterogeneity and cellular states: unknown.

Regenerative potential: can self-renew in mice (Ingram, et al., 2005; Zengin, et al., 2006).

Link to disease: linked to pulmonary arterial hypertension.

Key references: (De Val and Black, 2009; Peng, et al., 2013; Corada, et al., 2014; Ren, et al., 2019)

Venous endothelial cells

Morphological features: thin flattened cells lining the interior surface of veins and venules.

Function: conduct blood flow from the lung to the heart, regulate inflammatory responses, synthesize and secrete growth factors, regulate hemostasis and coagulation.

Other names: none.

Markers: genes: $ACKR1^{H}$, $HDAC9^{H}$, $Slc6a2^{M}$; proteins: VWF (also artery), Endomucin (also capillaries, but not artery) (Table 1).

Location: in proximal lung, pulmonary veins are located next to the secondary bronchi and pulmonary arteries. In distal lung, pulmonary veins do not accompany smaller bronchioles and arteries but travel alone through the connective tissue septa.

Experimental validation: standard approaches; mouse reporter using *Nr2f2*^{lacZ} (Coup-TFII-lacZ) knock-in allele labels venous endothelial cells (You, et al., 2005).

Developmental origin: at the initiation of lung development, they arise from cardiopulmonary progenitor cells (Peng, et al., 2013). Later on, they arise from endothelial progenitor cells (Bolte, et al., 2018). They can also arise from proliferation of specified venous endothelial cells (Ren, et al., 2019).

Heterogeneity and cellular states: unknown.

Regenerative potential: can self-renew in mice (Bolte, et al., 2018; Ren, et al., 2019).

Link to disease: linked to Pulmonary Veno-Occlusive Disease (PVOD), Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACDMPV).

Key references: (You, et al., 2005; De Val and Black, 2009; Corada, et al., 2014; Neal, et al.,2019)

Lymphatic endothelial cells

Morphological features: thin flattened cells lining the interior surface of lymphatic vessels (Figure 1 C).

Function: maintain interstitial fluid homeostasis, conduct fluid away from lung interstitium, regulate immune responses.

Other names: lymphatics.

Markers: genes: *PROX1, MMRN1*; proteins: LYVE1 (Table 1).

Location: found along trachea, near airways, in the alveolar region and on the pleura.

Experimental validation: standard approaches, lineage tracing with *Prox1*^{creERT2} (Bazigou, et al., 2011).

Developmental origin: in mouse, specified lymphatic endothelial cells were first detected at E9.5 as Prox1-positive cells located within endothelium of the cardinal vein (Yang, et al., 2012). Their specific developmental origin is unknown.

Heterogeneity and cellular states: unknown.

Regenerative potential: can self-renew (Cui, et al., 2017).

Link to disease: linked to Lymphangiectasis, Lymphangioma, Lymphangioleiomyomatosis (LAM), Lymphatic malformation syndromes include Generalized Lymphatic Anomaly

(GLA, also known as lymphangiomatosis), Gorham-Stout disease (GST) and Kaposiform lymphangiomatosis (Itkin and McCormack, 2016).

Key references: (Srinivasan, et al., 2014; Yao, et al., 2014; Stump, et al., 2017; Reed, et al.,2019)

Capillary 1 (CAP1) cells

Morphological features: thin flattened cells lining the interior surface of alveolar microvasculature.

Function: tissue perfusion and gas exchange with the external environment.

Other names: general capillary cells (gCAPs)

Markers: **genes:** $IL7R^{H}$, $Aplnr^{M}$, $Gpihbp1^{M}$ (Table 1).

Location: form the microvascular network together with, and more proximal to Capillary 2 cells in the alveolar region based on makers PLVAP (CAP1) and CAR4 (CAP2) staining (Ren, et al., 2019; Ellis, et al., 2020; Gillich, et al., 2020; Kalucka, et al., 2020; Niethamer, et al., 2020).

Experimental validation: standard approaches, mouse lineage tracing with $Aplnr^{creERT2}$ (Gillich, et al., 2020).

Developmental origin: these cells arise from Cdh5+ endothelial progenitors located in the lateral mesoderm at the beginning of lung development (Peng, et al., 2013). Later on, they arise from $Aplnr^{creERT2}$ lineage-traced cells, which can also give rise to Capillary 2 cells (see below) (Gillich, et al., 2020).

Heterogeneity and cellular states: in the early postnatal mouse lung, in KIT^{+} cells which contain CAP1 cells, FOXF1-$GFP^{+}KIT^{+}$ subset exhibited higher colony forming potential than FOXF1-GFP^{-} KIT^{+} subset (Wang, et al., 2021).

Regenerative potential: $Aplnr^{creERT2}$-lineaged cells give rise to both CAP1 and CAP2 cells after elastase lung injury (Gillich, et al., 2020). Adoptive transfer of FOXF1-$GFP^{+}KIT^{+}$ cells which are enriched for CAP1 cells with endothelial progenitor cell potential, increased angiogenesis by differentiating into capillary, arterial and venous endothelium in a mouse model of alveolar capillary dysplasia with misalignment of pulmonary veins (Wang, et al., 2021).

Link to disease: linked to alveolar capillary dysplasia with misalignment of pulmonary veins, bronchopulmonary dysplasia, pulmonary capillary hemangiomatosis.

Key references: (Ren, et al., 2019; Ellis, et al., 2020; Gillich, et al., 2020; Kalucka, et al., 2020; Niethamer, et al., 2020) (Wang, et al., 2021).

Capillary 2 (CAP2) cells

Morphological features: thin flattened cells lining the interior surface of alveolar microvasculature.

Function: tissue perfusion and gas exchange with the external environment.

Other names: aerocytes, alveolar capillary cells (aCAPs), $Car4^{+}$ capillary endothelial cells, $CD34^{hi}$ endothelial cells (mouse only).

Markers: **genes:** *EDNRB, HPGD*^{H}*, Apln*^{M}*, Car4*^{M}; proteins: EDNRB, CA4.

Location: form the microvascular network together with and more distal to CAP1 cells in the alveolar region based on makers PLVAP (CAP1) and CAR4 (CAP2) staining (Ren, et al., 2019; Ellis, et al., 2020; Gillich, et al., 2020; Kalucka, et al., 2020; Niethamer, et al., 2020); located in close apposition to AT1 epithelial cells to facilitate gas exchange.

Experimental validation: standard approaches; $CD34^{hi}$ FACS (mouse) (Niethamer, et al., 2020), mouse lineage tracing with *Apln*^{creERT2} (Gillich, et al., 2020).

Developmental origin: $Car4^{+}$/$Ednrb^{+}$ cells appear just before birth and mark a subset of capillary endothelium enriched in expression of angiogenic factors (Ellis, et al., 2020). Aplnr-lineage labeled cells can give rise to CAP2 cells (Ren, et al., 2019; Ellis, et al., 2020; Gillich, et al., 2020; Kalucka, et al., 2020; Niethamer, et al., 2020).

Heterogeneity and cellular states: unknown.

Regenerative potential: in mouse, one study shows regenerative Car4/Ednrb/$CD34^{hi}$ cells in alveoli after influenza injury (Neithamer 2020), whereas a different study showed that *Apln*^{creERT2} lineage-traced cells do not proliferate after acute elastase lung injury in the mouse (Gillich, et al.,2020).

Link to disease: linked to Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACDMPV), Bronchopulmonary Dysplasia (BPD), Pulmonary Capillary Hemangiomatosis (PCH).

Key references: (Ren, et al., 2019; Ellis, et al., 2020; Gillich, et al., 2020; Kalucka, et al., 2020; Niethamer, et al., 2020)

IMMUNE

The lung constitutes the largest surface of the body that is exposed to the outside environment. Pathogens can enter the lung either with inhaled air through the epithelial layer or via bloodstream through the endothelium. Cellular immunity is conducted by both immune cell types that reside in the lung interstitium, as well as immune cell types that patrol the extensive lung vasculature, ready to be recruited in response to infection or injury. Lung immune cells belong to either the innate or adaptive immune systems. In addition to defense against infection or injury, immune cells also play critical roles in tissue homeostasis.

Here, we will focus on immune cells in the healthy lung. Early in fetal development, the lung is seeded with immune cells that originate in the yolk sac, fetal liver or bone marrow (Holt and Jones, 2000; Tan and Krasnow, 2016; Ivanovs, et al., 2017; Dzierzak and Bigas, 2018; Ghosn, et al., 2019; Popescu, et al., 2019; Ardain, et al., 2020; Park, et al., 2020). Compared to lung structural cells in the epithelium, mesenchyme and endothelial lineages, immune cells are more dynamic in their gene expression and position. Thus, the entries of the cell cards in this section have been tailored to these cells. In addition to gene expression, cell surface molecules are widely used in FACS to study lung immune cells (Fig. 6). We describe lung immune cells in the myeloid lineage, followed by the lymphoid lineages (Fig. 2A).

Alveolar macrophages (AMs)

Morphological features: largest immune cells in lung, cytoplasm filled with lipid from surfactant.

Function: critical for surfactant turnover, phagocytosis of inhaled pathogens and particles. While they can present antigens to adaptive immune cells, their antigen presentation capacity is poor compared to other macrophages or dendritic cells.

Other names: aMac.

Markers: genes: *SIGLEC1*^{H}, *SiglecF*^{M}, *ABCG1, FABP4, PPARG, MARCO* (Fig. 6B); proteins: CD45, CD11b, CD11c, CD64, CD163, CD206, HLA-DR (Fig. 6A)

Location: luminal surface of alveoli in close proximity to epithelial cells.

Experimental validation: bulk RNAseq of sorted AMs in mice (Sajti, et al., 2020), flow cytometry with HLA-DR/CD64/CD68/CD206 in humans and SiglecF/CD11c/CD64 in mice (Fig. 6A) (Misharin, et al., 2013; Yu, et al., 2016; Hume, et al., 2020); parabiosis, clodronate depletion, adoptive transfer. Lineage tracing using *Itgax-cre* (*Cd11c-cre*, also target dendritic cell) (Caton, et al., 2007), *Lyz2*^{creERT2} also target interstitial macrophages and neutrophils (Canli, et al., 2017). Genetic ablation with *Slco2b1*$^{flox/DTR}$ crossed with *Lyz2*^{cre} that labels all macrophage populations (Chakarov, et al., 2019).

Developmental origin: in mouse, they arise from fetal liver macrophage precursors (Guilliams, et al., 2013; Gomez Perdiguero, et al., 2015; Hoeffel, et al., 2015; Tan and Krasnow, 2016).

Heterogeneity and cellular states: unknown in normal healthy lung. In disease states, recruited macrophages join the resident AMs. In human fibrotic lungs, influenza, bacteria infected or LPS treated lungs, there is heterogeneity in AM profile (Mould, et al., 2019; Reyfman, et al., 2019).

Regenerative potential: regenerates itself during homeostasis and disease.

Link to disease: linked to infection, fibrosis, asthma, COPD and lung cancer (Misharin, et al., 2017). Key cell type in pulmonary alveolar proteinosis (Trapnell, et al., 2019).

Key references: (Ginhoux and Guilliams, 2016; Allard, et al., 2018)

Interstitial macrophages (IMs)

Morphological features: smaller in size than AMs, with smoother surface, irregularly shaped nucleus and numerous cytoplasmic vacuoles (Sabatel, et al., 2017).

Function: present antigens and induce adaptive immunity.

Other names: iMac.

Markers: genes: *C1QA, C1QB, C1QC, HLA-DPA1, SLC40A1* (Fig. 6B); proteins: CD11b, CD64, CD206 (lower expression than aMAC), HLA-DR (Fig. 6A).

Location: by pan-macrophage marker staining, they are found in the lung interstitium around airways, vessels and nerves.

Experimental validation: bulk RNAseq of sorted IMs in mice (Sajti, et al., 2020), flow cytometry with CD206/CD64/CD68 in mice (Misharin, et al., 2013; Yu, et al., 2016; Hume, et al., 2020) (Fig. 6A). Mouse genetic lineage tracing using *Lyz2*^{creERT2} (also labels AMs and neutrophils) (Canli, et al., 2017).

Developmental origin: mixed origin, arise from embryonic yolk-sac or postnatal bone marrow (Tan and Krasnow, 2016). Parabiosis studies suggested that they can be replenished from blood monocytes in adults (Sabatel, et al., 2017).

Heterogeneity and cellular states: mouse lung: 3 different subpopulations based on surface markers: LYVE1^{lo}MHCII^{hi}CX3CR1^{hi} (LYVE1^{lo}MHCII^{hi}) and LYVE1^{hi}MHCII^{lo}CX3CR1^{lo} (LYVE1^{hi}MHCII^{lo}) (Gibbings, et al., 2017; Chakarov, et al., 2019). Based on function: phagocytic and non-phagocytic subtypes and IL-10 producing subtype (Sabatel, et al., 2017).

Regenerative potential: unknown.

Link to disease: important in LPS induced lung inflammation (mouse model). LYVE1^{hi}MHCII^{lo} IMs can ameliorate lung fibrosis (Chakarov, et al., 2019). Increased in numbers in the lung of smokers (Hume, et al., 2020). Decrease in number in asthma (Draijer, et al., 2017).

Key references: (Gibbings, et al., 2017; Schyns, et al., 2018; Chakarov, et al., 2019; Sajti, et al., 2020)

Inflammatory monocytes (iMONs)

Morphological features: smaller than macrophages, no other morphological distinctions.

Function: extravasate readily to sites of injury/inflammation, assemble antigens, and reenter circulation to present to other immune cells without differentiating into macrophages (Jakubzick, et al., 2013)..

Other names: classical monocytes.

Markers: genes: *S100A8, S100A9, CD14, VCAN* (Fig. 6B); proteins: CD11b, CD14, do not express CD16 (Fig. 6A).

Location: reside in blood vessels, retained even after extensive perfusion. Can extravasate to sites of injury/inflammation.

Experimental validation of cell type: flow cytometry markers: human CD14+CD16− (Fig. 6A), mouse LY6C+.

Developmental origin: bone marrow.

Heterogeneity and cellular states: circulating iMONs versus iMONs recruited to tissue.

Regenerative potential: cannot self-renew, live ~2 days and are continuously replaced. Can give rise to macrophages or patrolling monocytes once they extravasate into the interstitium. Recruited inflammatory monocytes promote lung regeneration following pneumonectomy (Lechner, et al., 2017).

Link to disease: CX3CL1-CX3CR1 induced changes in iMONs have been shown in fibrotic lungs (Misharin, et al., 2017).

Key references: (Jakubzick, et al., 2013; Misharin, et al., 2017)

Patrolling monocytes (pMONs)

Morphological features: smaller than macrophages, no other morphological distinctions.

Function: patrol endothelium. In other organs, pMONs are known to remove damaged cells from the vasculature (Carlin, et al., 2013).

Other names: non-classical monocytes.

Markers: genes: *CDKN1C, PTP4A3, HES4, TNFRSF8* (Fig. 6B); protein: CD11b, CD14 (dimmer than iMONs), CD16 (Fig. 6A)

Location: reside in the blood vessels and retained even after extensive perfusion. Engage in long-range migration along endothelium.

Experimental validation of cell type: flow cytometry markers: human $CD14^{dim}CD16^{+}$ (Fig. 6A), mouse $Ly6c^{-}$.

Developmental origin: bone marrow, can arise from iMONs.

Heterogeneity and cellular states: unknown.

Regenerative potential: cannot self-renew, live ~10 days and are continuously replaced from bone marrow.

Link to disease: in other organs, pMONs play a role in inflammation resolution (Carlin, et al., 2013).

Key references: (Carlin, et al., 2013; Sajti, et al., 2020)

Dendritic cells (DCs)

Morphological features: round to oval cell bodies with well-developed ER and multiple fine dendrites.

Function: at baseline the lung is populated by 3 types of DCs with distinct functions:

Plasmacytoid DC (pDC) are highly effective in sensing intracellular viral or self-DNA and RNA mainly via Toll-like receptors (TLRs). They in turn secrete a large amount of type I interferon to recruit lymphocytes and NK cells.

Classical DC subset 1 (cDC1) cross-present antigens to CD8+ T cells and produce IL-12 that support Th1 and cytotoxic responses.

Classical DC subset 2 (cDC2) can uptake, process and present antigens on MHC-I or -II molecules. They migrate to lymph nodes to cross-present to $CD4^+$ T cells and can also support Th1, Th2, and Th17 polarization. cDC2 are more superior antigen presenter than pDC or cDC1.

Other names: cDC1 is also called myeloid dendritic cell 2 (mDC2), cDC2 is also called myeloid dendritic cell 1 (mDC1). Classical DCs are also called conventional DCs.

Markers: genes (Fig. 6B):

pDC: *CLEC4C, LILRA4, IRF7, PLD4.*

cDC1: *FLT3, CLEC9A, ZBTB46.*

cDC2: *CD1C, CD1E, FCGR2B, CLEC10A.*

protein (Fig. 6A):

pDC: CD123, CD303 (CLEC4C), HLA-DR

cDC1: CD141, CD163, CD370 (CLEC9A), HLA-DR

cDC2: CD1c, CD11b, CD14, CD172a (SIRPA), HLA-DR

Location: can be found below the epithelial cells of both the airway and alveoli with dendrites protruding into the air space to sample aerosol contents. After antigen uptake they migrate to the nearest lymph node (Patel and Metcalf, 2018).

Experimental validation: standard approaches; FACS: for human markers see above and Fig. 6A; mouse markers CD45R (B220), CD45RA, Ly-6C, Siglec-H, and BST2 (CD317). ZBTB46 is a transcription factor highly specific for cDCs. *Batf3*$^{-/-}$ mice lack mDC2s.

Developmental origin: pDCs develop from both common dendritic cell progenitors and LY6D^{hi}CD2^{hi} lymphoid progenitors. pDC development requires transcription factor IRF8, whereas pDC identity relies on TCF4 (Cisse, et al., 2008; Rodrigues, et al., 2018; Dress, et al., 2019). Both cDC1s and cDC2s develop from the common dendritic cell progenitor, with cDC1s dependent on IRF8 and cDC2s dependent on IRF4 (Schlitzer, et al., 2013).

Heterogeneity and cellular states: during steady state, CD11c^{dim} pDCs are found in the conducting airways. During inflammation, CD11b+ monocyte-derived DCs, expressing LY6C and FceRI, are recruited. Heterogeneity of cDC-1 and cDC-2 are not known.

Regenerative potential: unknown. Short lived and replaced by blood-borne progenitors.

Link to disease: in animal model, pDCs triggered proinflammatory response can exacerbate asthma (Chairakaki, et al., 2018).

Key references: (GeurtsvanKessel and Lambrecht, 2008; Veres, et al., 2011; Patel and Metcalf, 2018; Rodrigues, et al., 2018; Balan, et al., 2019; Musumeci, et al., 2019)

Neutrophils

Morphological features: smallest cells among granulocytes, characteristic multilobed nucleus joined by thin strands, cytoplasm contains azurophilic granules with microbicidal agents.

Function: patrol for signs of microbial infections and respond to pathogens through three antimicrobial actions: phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs). In addition, neutrophils can modulate the activities of neighboring cells and contribute to the resolution of inflammation, regulate macrophages for long-term immune responses, actively participate in diseases including cancer, and play a role in innate immune memory (Rosales, 2018).

Other names: granulocytes, polymorphonuclear cells (PMNs).

Markers: genes: *IL1B, CCR2, CSF1R*; proteins: CD11b, CD15, in mice Ly6g (Fortunati, et al., 2009; Misharin, et al., 2013).

Location: marginalized pool in the capillary bed of the lung during steady state, recruited to the parenchyma of airways and alveoli during inflammation and infection.

Experimental validation: standard approaches. Neutrophils can be depleted by anti-Gr1 and anti-Ly6G antibody treatment. Genetic mouse models deficient in neutrophils include: hMrp8-Dtr mice, *Mrp8-cre;Mcl1$^{fl/fl}$* mice, *Lyz2^{cre};Mcl1$^{f/fl}$* mice, *Csf3r(Gcsfr)$^{-/-}$* mice, *Cxcr2$^{-/-}$* mice, *Gfi1* hypomophic mutant Genista mice (Stackowicz, et al., 2020).

Developmental origin: bone marrow derived. Neutrophil development starts from granulocyte/monocyte progenitors which differentiate into myeloblasts, promyelocytes and myelocytes. Next, myelocytes give rise to non-proliferating metamyelocytes, band cells and finally mature neutrophils (Ng, et al., 2019).

Heterogeneity and cellular states: different maturational stages: metamyelocytes, band cells and mature neutrophils. During infection, the proportion of immature forms increases in blood.

Regenerative potential: none, die shortly after recruitment to lung.

Link to disease: linked to chronic inflammatory diseases (e.g. asthma, ARDS, COPD, etc) and cancer (Uribe-Querol and Rosales, 2015).

Key references: (Fortunati, et al., 2009; Misharin, et al., 2013; Uribe-Querol and Rosales, 2015; Rosales, 2018; Ng, et al., 2019; Stackowicz, et al., 2020)

Basophils

Morphological features: irregular blunt surface, condensed chromatin, and polylobed nuclei. Human basophils contain a large number of dense granules and are highly basophilic. In contrast, murine basophils lack obvious granules and are polymorphic and moderately basophilic (Lee and McGarry, 2007).

Function: basophils produce antimicrobial extracellular traps and regulate group 2 innate lymphoid cell (ILC2) responses (Lee and McGarry, 2007; Siracusa, 2016; Cohen, et al., 2018; Kabashima, et al., 2018; Inclan-Rico, et al., 2020; Marone, et al., 2020; Vivanco Gonzalez, et al., 2020). In addition, they produce effector molecules in response to stimuli such as inflammatory mediators (histamine, serotonin, platelet-activating factor, and leukotriene), cytokines (IL-4, IL-5, IL-6, IL-9, IL-13, and IL-15), and chemokines (CCL3, CCL4, CCL12, and CXCL12) (Siracusa, 2016; Inclan-Rico, et al., 2020).

Other names: none.

Markers: genes: *MS4A2, TPSAB1, TPSB2, KIT, GATA2* (Fig. 6B); proteins: FCER1B, CD23, CD123, and lack of expression of CD117 (c-Kit) (Fig. 6A). In mice, basophils are identified by their expression of CD90, FcεRIa, CD200R, and CD49b and lack expression of CD117 (c-Kit) (Siracusa, 2016).

Location: resident basophils reside in the alveoli (Cohen, et al., 2018).

Experimental validation: standard approaches; FACS (Fig. 6A)

Developmental origin: bone marrow. Development starts as hematopoietic stem cells commit to the myeloid lineage. Later, they develop into granulocyte/monocyte precursor cells, and then to basophil/mast cell precursors, followed by basophil precursor cells that represent the direct precursors of terminally differentiated basophils.

Heterogeneity and cellular states: unknown in lung. Four subpopulations have been characterized in blood (Vivanco Gonzalez, et al., 2020)

Regenerative potential: no self-renewal potential (Yamanishi and Karasuyama, 2016).

Link to disease: unknown in lung.

Key references: (Lee and McGarry, 2007; Siracusa, 2016; Cohen, et al., 2018; Kabashima, et al., 2018; Inclan-Rico, et al., 2020; Marone, et al., 2020; Vivanco Gonzalez, et al., 2020)

Mast Cells

Morphological features: abundant granules in the cytoplasm, characteristic metachromatic staining with aniline dyes.

Function: stimulated to degranulate when they encounter antigen immunoglobulin E (IgE) antibody bound to high affinity IgE receptor, FcεRI. They then release chemical and biological mediators including histamine, ATP, prostaglandin, leukotriene, cytokines and angiogenic factors (de Souza Junior, et al., 2017; Ren, et al., 2020).

Aliases and acronyms: MC_{TC}, mastocyte.

Markers: genes: *MS4A2, TPSAB1, TPSB2, KIT, GATA2* (Fig. 6B); proteins FCER1B, CD117 (c-Kit) and lack of expression of CD123 (Fig. 6A).

Location: rare in human lung. When observed, they are found in bronchial epithelium, bronchial lamina propria or adjacent to blood vessels.

Experimental validation: standard approaches; FACS (Fig. 6A), mast cell-deficient $Kit^{W/Wv}$ mice (Nakano, et al., 1985).

Developmental origin: bone marrow derived, SCF is the major growth factor essential for mast cell survival.

Heterogeneity and cellular states: each anatomical compartment of the lung harbors site-specific mast cell populations. Can be classified based on the content of their granules, tryptase only, chymase only or mixed. Connective tissue type mast cells (MC_{TC}): IL-4+, IL-13+, TPSAB1+, CMA1+, CPA3+. Tissue resident mast cells (MC_T): IL-5+, Il-6+, TPSAB1+, CMA1-, CPA3+.

Regenerative potential: unknown; long-lived cells.

Link to disease: found in higher numbers in asthma, BPD, COPD lungs. Also linked to IPF, ARDS, pulmonary hypertension, lung neoplasia.

Key references: (Kabashima, et al., 2018; Ravindran, et al., 2018; Komi, et al., 2020)

Innate lymphoid cells (ILCs)

Morphological features: lymphoid cell morphology.

Function: similar to Th cells in function but lack T cell receptor. There are three types of ILCs:

Innate lymphoid group 1 cells (ILC1): are involved in type I immune responses and host defense against viruses, intracellular microbes, and tumor cells. ILC1s respond to cytokines

including IL-12, IL-18, and IL-15, and produce IFNγ and GM-CSF. Unlike NK cells, ILC1s are not cytotoxic.

Innate lymphoid group 2 cells (ILC2): are involved in type 2 immune responses and host defense against extracellular parasites, for example, helminths like N. brasiliensis and allergens. In response to cytokines such as IL-25, TSLP, and IL-33, ILC2s secrete IL-4, IL-5, IL-9, IL-13, amphiregulin (AREG) to repair damaged tissue.

Innate lymphoid group 3 cells (ILC3): possess similar function as Th17 cells and defend against extracellular microbes such as bacteria and fungi in lung. ILC3s can respond to IL-23, IL-1α, IL-1β, IL-7, TL1A, and prostaglandin E2 (PGE2). These cells produce GM-CSF, IL17, IL-22, TNF-α, and lymphotoxin α/β to promote epithelial stem cell proliferation.

Other names: Nuocytes (ILC2).

Markers: genes: *FCGR3A, KLRB1, GZMB, PRF1* (Fig. 6B)

proteins (Fig. 6A)

ILC1s: in human CD127(IL-7Rα), CXCR3, and transcription factor (TF) T-bet and lack expression of TF eomesodermin (Eomes). In mouse, CD90.2, ILC1s express NKp46, NK1.1, CD49A, and T-bet, but lack Eomes.

ILC2s: in human CD127 (IL-7Rα), ST2, CD117, CRTH2, and TF GATA3. In mouse, CD90.2, CD127, CD117, CD90, and ST2.

ILC3s: in human CD127, IL23R1, CD56, and TFs, RORyT, and AHR. In mouse CD90.2, CD127, CD117, CD90, NKp46 and TFs T-bet, RORyT, and AHR (Yudanin, et al., 2019).

Location: resident in the airways, alveolar mucosa, and perivascular niches (ILC2 and ILC3). Can travel to lymph nodes for antigen presentation (ILC3) (Dahlgren, et al., 2019; Oherle, et al., 2020).

Experimental validation: standard approaches; FACS (Fig. 6A) (Eberl, et al., 2015b). In steady-state naïve mice, ILC2s can be labeled using $Il5^{cre}$ mice (von Moltke, et al., 2016); ILC3s can be labeled using $Rorc^{cre}$ mice (Eberl, et al., 2004).

Developmental origin: ILCs develop from a common lymphoid progenitor (CLP), which further differentiates into a shared ILC precursor termed a-lymphoid precursor (αLP). Further differentiation of αLP generates different precursors with more restricted lineage repopulation capacity. Among them, the common helper ILC precursor (CHILP) give rise to ILC1, ILC2, and IlC3 (Constantinides, et al., 2014).

Heterogeneity and cell states: scRNAseq and ATACseq studies demonstrate considerable heterogeneity in transcriptome and epigenome in a tissue-dependent fashion (Yudanin, et al.,2019). Between ILC subtypes, the different ILCs can transdifferentiate from one to another (Vonarbourg, et al., 2010; Bal, et al., 2016). Within individual ILCs, pulmonary

ILC2s exhibit two distinct subsets, lung resident natural ILC2s (nILC2s) and inflammatory ILC2s (iILC2s) recruited from gut to lung (Huang, et al., 2015; Huang, et al., 2018). ILC3s also show two subsets: natural cytotoxicity receptor-positive (NCR^+) ILC3s or NCR negative (NCR^-) ILC3s.

Regenerative potential: ILCs do not recirculate and are maintained predominantly via local self-renewal rather than through replenishment from blood-derived ILCs or their precursors from circulation and bone marrow (Gasteiger, et al., 2015).

Link to disease: ILC1s are implicated in host defense against influenza H1N1 and intracellular bacterial pathogens. ILC2s play a critical role in allergen response and respiratory viral, bacterial, and helminthic lung infections (Monticelli, et al., 2011; Barlow, et al., 2012). ILC3s are critical in respiratory viral (H1N1 influenza) and bacterial infections (K. pneumoniae, S. pneumoniae, and P. aeruginosa) (Chen, et al., 2011).

Key references: (Eberl, et al., 2004; Di Stefano, et al., 2009; Vonarbourg, et al., 2010; Chen, et al., 2011; Monticelli, et al., 2011; Barlow, et al., 2012; Klose, et al., 2013; Constantinides, et al., 2014; Eberl, et al., 2015b; Gasteiger, et al., 2015; Huang, et al., 2015; Zuo, et al., 2015; Bal, et al., 2016; Silver, et al., 2016; von Moltke, et al., 2016; Huang, et al., 2018; Dahlgren, et al., 2019; Schneider, et al., 2019; Yudanin, et al., 2019; Oherle, et al., 2020; Mazzurana, et al., 2021)

Natural Killer cells (NK cells)

Morphological features: large granular lymphocyte.

Function: NK cells are cytotoxic cells involved in type I immunity. They are the innate counterpart of CD8+ T cells. Similar to CD8+ T cells, NK cells employ the perforin/granzyme pathway, TRAIL, FAS/FASL interactions to kill cells. NK cells are also able to secrete IFNγ.

Other names: none.

Markers: genes: *SPTSSB, NKG7, KLRD1, KLRC1* (Fig. 6B); proteins: CD11b, CD56, CD94, NKG2A, NKG2B (Fig. 6A)

Location: in circulating blood and in tissue.

Experimental validation: standard approaches; FACS (Fig. 6A). While NK cells have similarities with ILC1s, they differ by the expression of eomesodermin, which is present in NK cells (Eberl, et al., 2015a).

Developmental origin: common lymphoid progenitors differentiate into common innate lymphoid progenitors which give rise to NK progenitors.

Heterogeneity and cellular states: CD56 bright versus dim. Conventional NK cells that circulate versus tissue-resident NK cells.

Regenerative potential: none.

Link to disease: viral infection, lung cancer.

Key references: (Spits and Cupedo, 2012; Spits, et al., 2013; Artis and Spits, 2015; Eberl, et al., 2015a)

T cells

Morphological features: lymphoid morphology.

Function: there are multiple T cell types with distinct functions.

$CD4^+$ (helper) T cells: help B cells mount antibody responses, co-stimulate DC, and support CD8+ T cells, perform direct cytotoxic functions, stimulate macrophages. Can form immunological memory.

CD8+ (cytotoxic) T cells: kill viral infected cells. A subset of the CD8 memory precursor cells in the lung will differentiate into tissue-resident memory T cells.

Th1: defend against viruses (influenza, RSV), M. tuberculosis, and fungi.

signature cytokines: IFNg, IL-2, TNFa

transcription factors: TBX21, STAT4

Th2: activated by allergen. Produce IL13 and induce airway hyperresponsiveness, goblet cell metaplasia, and mucus hypersecretion. Activate eosinophils through IL5 production. Defend against helminth parasites.

signature cytokines: IL-4, IL-5, IL-13

transcription factors: GATA3, STAT6

Th17: either protective or pro-inflammatory depending on infectious agent.

signature cytokines: IL-17

transcription factors: RORC, STAT3

Treg: control excess inflammation and maintain homeostasis.

signature cytokines: IL-10

transcription factors: FOXP3, STAT5

Mucosal-associated invariant T cells (MAIT): capable of mounting immediate response to bacteria by recognizing microbial ligands in conjunction with non-classical MHC-related protein MR1

NKT, iNKT, γδTcells: bridge innate and adaptive immunity.

Tissue resident effector memory T cells: provide local immune protection after reinfection. The majority of T cells in the human lung are memory phenotype (both CD4+ and CD8+T cells). These cells are CD45RA–CCR7–. They are different from blood TEM by expressing CD69. CD69+CD8+T cells also express CD103.

Other names: none.

Markers: genes: (Fig. 6B), proteins: (Fig. 6A)

Naïve: CD3E, MAL, CCR7

Central memory: CD3E, CD40LG

Effector memory: CD3E, CXCR6

T regulatory: FOXP3, CD3E, CTLA4, FANK1

MAIT: CD3E, KLRB1, IL7R

NKT: CD3E, CCL5

Location: found in the circulation and can be recruited to airway and alveoli. They do not recirculate.

Experimental Validation: genetic knockout models (T cell receptors, cytokines, chemokines, transcription factors), antibody-mediated depletion experiments, parabiosis experiments, cell transfer experiments, reporter gene animal models.

Developmental origin: precursors originate in the bone marrow and mature in the thymus.

Heterogeneity and cellular states: $CD4^+$ T cells are composed of at least five distinct populations that display different surface markers, produce different effector cytokines and regulated by different transcription factors. T helper (Th)1, Th2 cells, and Th17 cells are well established cell states.

Regenerative potential: memory T cells can undergo clonal expansion. Other T cells have no self-renewal potential. Average half-life of a few weeks. Repopulated by blood borne cells.

Link to disease: linked to inflammatory lung diseases (e.g. Bronchopulmonary Dysplasia and fibrosis), infectious diseases, and cancer.

Key references: (Paul, et al., 2019; Snyder and Farber, 2019; Imanishi and Saito, 2020; Khan, 2020; Zemmour, et al., 2020; Bugya, et al., 2021; Wen, et al., 2021; Williams, et al., 2021).

B cells

Morphological features: no distinct morphological feature is used in characterization.

Function: produce antibodies and cytokines. Support and activate T helper cells.

Other names: plasma blasts, antibody secreting cells, antigen presenting B cells.

Markers: genes (Fig. 6B):

B cells: MS4A1, BANK1

Plasma cells: SdC1, CD38

Proteins (Fig. 6A) all expressing CD19, CD20, CD79;

Naïve B cells: CD25-, CD69-, HLA-DR-

Effector B cells: CD25+, CD69+, HLA-DR+

Plasma cells: CD38+, CD69-, CD138+, HLA-DR-

Location: found in circulation and can be recruited into parenchymal regions.

Experimental validation: standard approaches. In mouse, CD19 genetic ablation, transgenic B cell receptor animals, depletion studies.

Developmental origin: bone marrow.

Heterogeneity and cellular states: subsets of B effector (Be) cells can express transcription factors and cytokines akin to CD4 T cells (e.g. Be1, Be2, Be17 and Breg).

Regenerative potential: none.

Link to disease: increased in inflammation (COPD, allergies), produce autoantibodies in systemic sclerosis and IPF.

Key references: (Rickert, et al., 1997; Kato, et al., 2013; Tsuneto, et al., 2014; Chiu and Openshaw, 2015; Lam and Baumgarth, 2019; Sanz, et al., 2019; Allie and Randall, 2020; Grasseau, et al., 2020; Wang, et al., 2020b).

PROTEOMIC, LIPIDOMIC AND METABOLIC STUDIES OF THE LUNG

A variety of biological mechanisms directly impact the abundance of proteins relative to their transcripts, e.g., post-transcriptional regulation, different protein half-life, change in subcellular localization, etc. Lipids and metabolites are products of interactions between genes, transcripts, proteins, and the environment. In LungMAP, we have made initial efforts to delineate protein, lipid, and metabolomic pan-lineage markers at increasing resolutions using LC-MS/MS. Below, we summarize previous efforts and aggregated insights (Bandyopadhyay, et al., 2018; Kyle, et al., 2018; Du, et al., 2019) (Proteomics: https://lungmap.net/breath-omics-experiment-page/?experimentTypeId=LMXT0000000015&experimentId=LMEX0000000661; Lipidomics: https://lungmap.net/breath-omics-experiment-page/?experimentTypeId=LMXT0000000005&experimentId=LMEX0000001622)

Of the 3,320 proteins quantified, proteins unique or enriched in the pan-epithelial population include CD36/EPCAM and CDH1/E-Cadherin (Supplemental Table 1, link above). We also identified markers for ciliated cells RSPH1 and the Tektin family proteins (TEKT1, TEKT2, TEKT3, TEKT4); markers for goblet cells AGR2; the polymeric immunoglobulin receptor

(PIGR) located at the apical surface of serous cells; the AT1 markers AGER/RAGE; the AT2 markers LAMP3, ABCA3, SFTPC. Proteins unique to or enriched in mesenchymal cells included pericyte marker PDGFR-β; or smooth muscle/myofibroblast factor TAGLN/SM22. Proteins unique or enriched in the pan-endothelial population include CD31/PECAM, CDH5/VE-Cadherin, PCDH1, CLDN5 and ICAM2 (Halai, et al., 2014). We also identified markers of capillary cells (e.g., SLC6A4, FCN3, CA4) and the venous cell marker EPHB4. Proteins unique or enriched in immune cells include the pan-immune marker CD45/PTPRC, and the pan-myeloid marker FCER1G. We also identified the panmacrophage marker CD68; alveolar macrophage transcription factor SPI1/PU.1; monocyte protein S100A8/A9, dendritic cell protein ribonuclease 6.

Less is known about lipid markers for the different pan-lineage populations in lung (Nakayasu, et al., 2016; Kyle, et al., 2018). Using a recently developed bioinformatic tool, Lipid Mini-On (Clair, et al., 2019), we uncovered structural traits enriched in the lipids over-represented in the different sorted cell populations (link above). Of the 286 lipid species quantified, 51 were more abundant in epithelial cells. They are enriched in long-chain fatty acids such as palmitic acid (16:0), and phosphatidylcholines with a total of 32 and 34 carbons in the fatty acid chains. As expected, epithelial lipids were enriched in phosphatidylcholines (PC) and phosphatidylglycerols (PG) that are the most abundant lipids found in pulmonary surfactants, e.g. PC(16:0/16:0), pC(14:0/16:1) PG(16:0/18:1). For the panendothelial population, 72 are over-represented. The endothelial lipids are enriched in ceramides, phosphatidylserines, phosphatidylethanolamines, and phosphatidylinositol lipids containing the fatty acid 20:4 (likely arachidonic acid). For the pan-mesenchymal population, 19 lipid species were unique or enriched in mesenchymal cells. The mesenchymal lipids were enriched in triglycerides with medium-chain fatty acids (C8-C12) and C16 or C18 saturated fatty acids (no double bonds). Finally, the 51 lipids over-represented in the pan-immune population are enriched in polyunsaturated fatty acids (most of which are 20:4 fatty acids), triglycerides that contain long-chain fatty acids, and ether-linked phosphatidylcholines. Interestingly, the lipid data also show that immune cells contain a lesser-known phospholipid and structural isomer of PGs, bis(monoacylglycerol)phosphates. Subcellularly, bis(monoacylglycerol)phosphates are localized to late endosomes and lysosomes (Kyle, et al., 2018).

CONCLUDING REMARKS

This collection of cell cards is intended as a foundational resource to unify and boost lung research, and will be posted as a live document on LungMAP.net and frequently updated. We anticipate that the number of cell cards will grow as new results become available. For example, cell types such as intrinsic neurons, glial cells and bronchial vascular endothelial cells will be defined as the number of cells captured by single-cell RNAseq increases. New cell types may also be elevated from currently listed cell states if evidence supports their persistence and distinguished function. Future studies will also iteratively refine these cell cards, generating additional markers from integrated analysis of multiple single-cell datasets. Currently, several cell types cannot be definitively traced using single gene lineage tracing tools (Table 1) (Riccetti, et al., 2020). The identification of more refined marker genes, along with the generation of intersectional dual recombinase tools (Liu et al., 2019; Salwig

et al., 2019), should provide the wider lung community the ability to better characterize current and emerging cell lineages and states. Single-cell resolution spatial transcriptomics will reveal if heterogeneity within a cell type can be spatially distinguished. In addition, spatial data will generate new hypotheses for how ligand-receptor interactions may dictate cell fate specification and progenitor/niche collaboration. Epigenomic technologies such as single-nucleus ATACseq will reveal cell type resolved chromatin landscape and can be integrated with single cell transcriptomic data to better define the gene regulatory network that drives these cell fates (Wang, et al., 2020a; Zepp, et al., 2021).

Another dimension of knowledge growth will come from understanding of species differences. For example, it has been noted that basal cells in human are found all along the airway including intrapulmonary bronchi and bronchioles, while they are restricted to trachea and main stem bronchi of the mouse respiratory system (Rock, et al., 2010). Additional cellular differences in airway structure, bronchial circulation, immune investment will continue to be documented as the depths of study increases. Extensive gene expression differences are emerging from single cell RNAseq studies of the human, mouse, rat, ferret, pig and primate lungs, building the repertoire of data that could populate a new entry of “species differences” in future versions of cell cards (Raredon, et al., 2019). These cross-species cellular and molecular diversity will inform how lung function evolves and adapts to organismal size, energy demand and environment.

While focusing on healthy lungs here, these cell cards will serve as the foundation for comparisons with diseased lungs. Such comparison will yield changes in developmental lung diseases, a current focus of the NHLBI LungMAP consortium effort. These comparisons will also extend to chronic diseases of the adult lung including COPD and IPF. Overall, we hope that these cell cards will stimulate collaboration throughout the broad pulmonary research community, and help to build bridges to other tissue-focused mapping efforts.

METHODS

Histology and Immunofluorescence

7μm frozen embedded tissue sections of 4% paraformaldehyde fixed human lung tissue were equilibrated to room temperature and rehydrated in PBS. Slides were then re-fixed with 4% PFA for 5 minutes and washed in PBS. 5μm thick paraffin embedded sections of 10% formalin fixed human lung tissue were melted at 60°C for two hours, following rehydration through xylene and alcohol, and finally in PBS. Tissue sections were stained with H&E according to standard protocols. PIGR Immunohistochemistry was performed according to standard laboratory protocols. For immunofluorescence, antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) by microwaving. Slides were blocked for 2 hours at room temperature using either 4% normal goat serum (Jackson Immuno Research Laboratories) or 4% normal donkey serum (Jackson Immuno Research Laboratories) in PBS containing 0.2% Triton X-100, and then incubated with primary antibodies diluted in blocking buffer for approximately 16 hours at 4°C. Primary antibodies were listed in the key resources table. Appropriate secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 555/568, or Alexa Fluor 633/647 (Thermo Fisher Scientific) were used at a dilution of 1:200 in blocking

buffer for 1 hour at room temperature. Nuclei were counterstained with DAPI (1 μg/ml) (Thermo Fisher Scientific). Sections were mounted using ProLong Gold (Thermo Fisher Scientific) mounting medium and coverslipped.

Confocal microscopy

Tissue sections stained by immunofluorescence were imaged on an inverted Nikon A1R confocal microscope (×20, 60X magnification) NA 1.27 objective using a 1.2 AU pinhole. Maximum intensity projections of multi-labeled Z stack images obtained sequentially using channel series across the sections were generated using Nikon NIS-Elements software. Brightfield images were captured using a Zeiss Axio ImagerA2 microscope utilizing Axiovision software.

Human lung single-cell data analysis

Single-cell data of Human Lung Cell Atlas (https://hlca.ds.czbiohub.org/) was downloaded and 10x sequencing data was further used for marker gene discoveries. Library size of each cell was normalized to 1 million and then log2 transformation was applied. To get better annotations of immune cells, especially lymphocytes, we applied Azimuth label projection strategy using human PBMC as the reference (https://azimuth.hubmapconsortium.org/) (Hao, et al., 2020). Average normalized expression values were calculated for each cell type and Morpheus was used for heatmap visualization (https://software.broadinstitute.org/morpheus/). Cell ontology terms were mapped to cell annotations manually (Meehan, et al., 2011). IGSF21+ dendritic cells from the original publication were reannotated as interstitial macrophages due to high levels of interstitial macrophages markers (such as C1QA, IGSF21 and SLC40A1), and low expression levels of dendritic cell markers (such as CD1C and CLEC9A). Basophil/Mast cells were reannotated as mast cells since they are mainly CD11c- (ITGAX), CD117+ (KIT), CD123- (IL3RA) cells.

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A Census of the Lung: CellCards from LungMAP

DEVELOPMENTAL-CELL-D-21-00414R2

Highlights:

- Rich single cell and lineage tracing data necessitate a new census of lung cell types
- Lung CellCards serves as a curated, up-to-date, practical resource for lung research
- CellCards annotates developmental origin, cellular function, regenerative potential etc.
- Lung CellCards serves as a starting point for harmonization of lung nomenclature

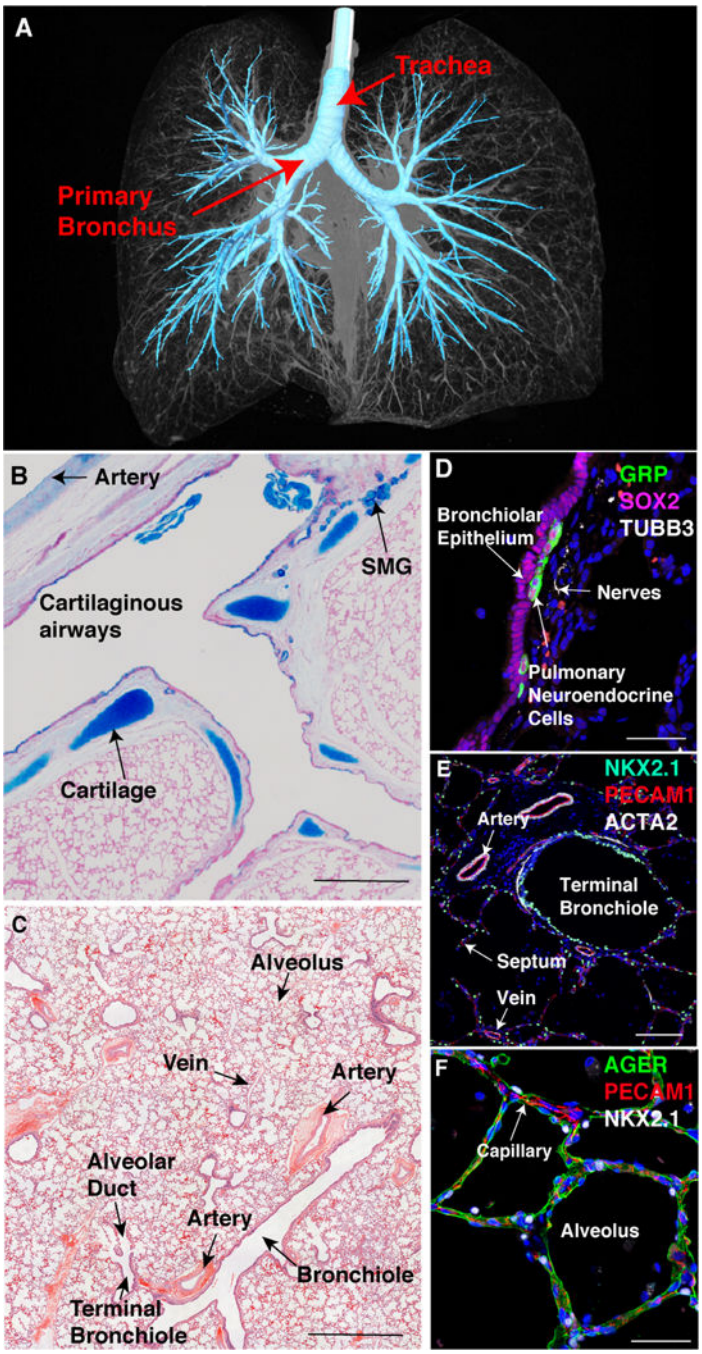

Figure 1: Overall lung structure and regional niches.

(A) Computer tomography of an intact human lung with the trachea and conducting airways highlighted. **(B)** A tile scan of a section of the normal human lung after alcian blue staining identifies proximal airway structures. SMG (submucosal gland). **(C)** A tile scan of a distal section of normal human lung after H& E staining identifies bronchial and alveolar structures. **(D-F)** Lung sections were stained with cell-selective markers and imaged by immunofluorescence confocal microscopy to identify diverse cells within airways and alveolar regions. Most conducting airway epithelial cells express SOX2 (D). Clusters of

GRP^+ pulmonary neuroendocrine cells were localized along the airways, innervated by $TUBB3^+$ nerves (D). $NKX2.1^+$ identifies epithelial cells in airways and alveoli (E, F). Smooth muscle and myofibroblasts express ACTA2 in bronchioles, pulmonary arteries, pulmonary veins, and alveolar septa (E,F). $AGER^+$ alveolar type I cells (F) line the lumen of the alveoli. Scale bars are 1000 mm (B, C), 40 mm in (D, F) and 100 mm in (E).

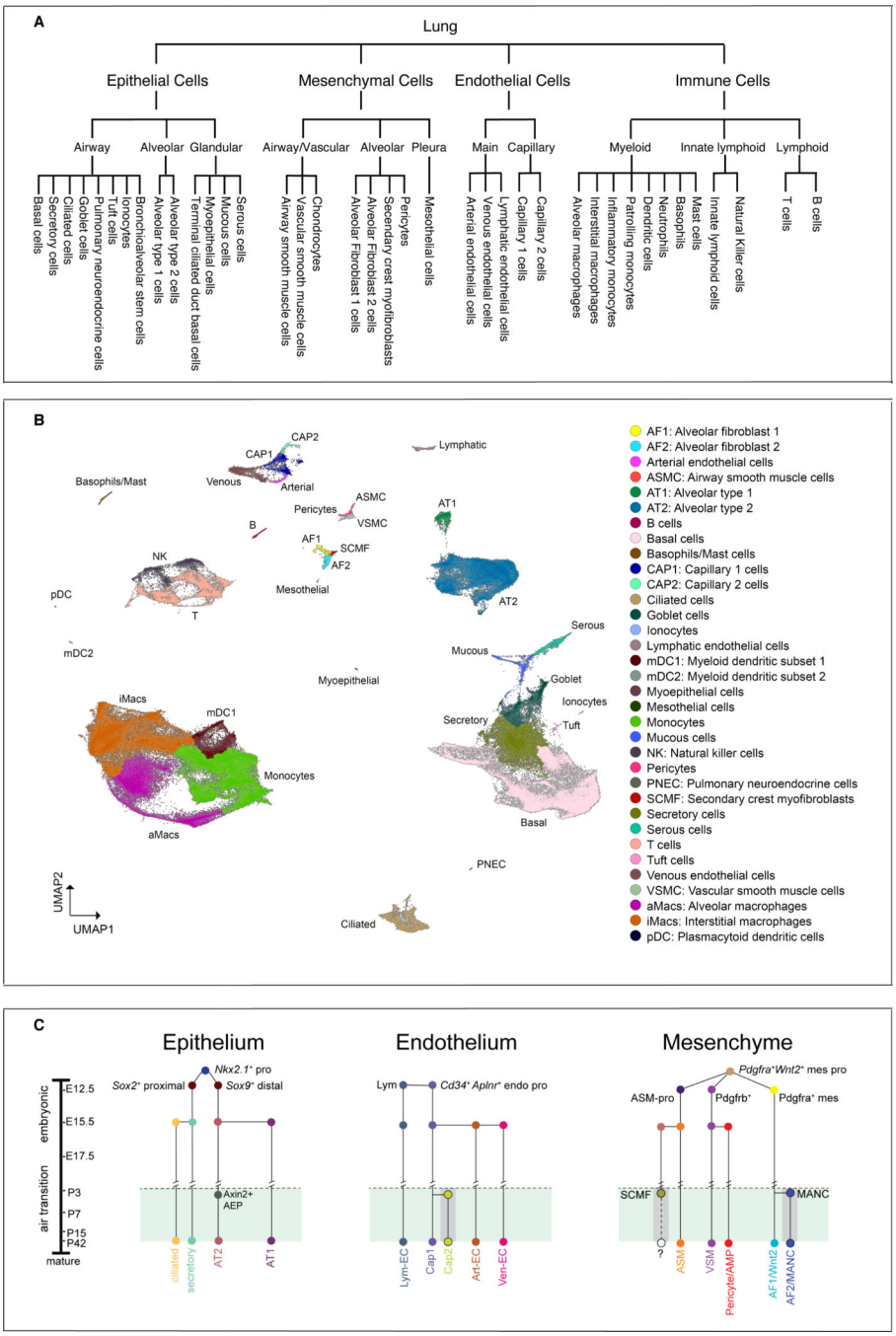

Figure 2: Lung cell types by region, by single cell clustering and by lineage.

(A) A schematic of cell types featured in CellCards, delineated by general regions of the lung where they reside. **(B)** UMAP visualization of human lung single cells (n=259,565) colored by their predicted cell identities. Data were from collected 5 published scRNA-seq cohorts (Reyfman, et al., 2019; Adams, et al., 2020; Deprez, et al., 2020; Goldfarbmuren, et al., 2020; Habermann, et al., 2020). Data integration and analysis were performed using Monocle 3 (Cao, et al., 2019). **(C)** Cell circuitry dendrogram showing developmental cell lineage relationships described in the various resident cell types of the mouse lung. This

does not include all identified cell types or states in the various scRNA-seq analysis, rather those that have been confirmed across mouse development using complementary techniques including lineage fate mapping techniques and high resolution imaging across developmental time points. Adapted from (Zepp, et al., 2021), with additional input from (Rawlins, et al., 2009a; Zepp, et al., 2017; Zacharias, et al., 2018; Frank, et al., 2019; Gillich, et al., 2020).

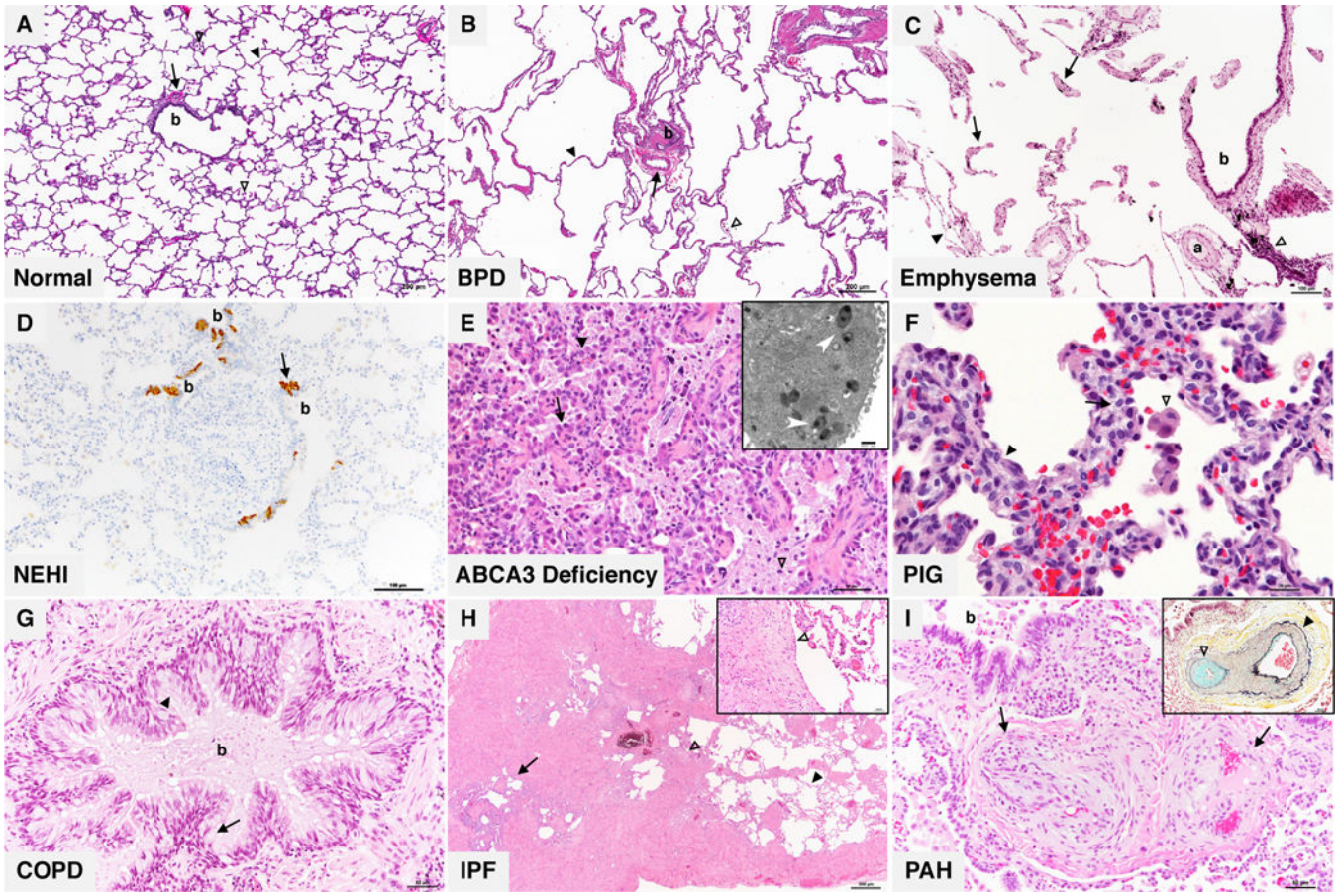

Figure 3: Histology of normal and diseased human lung.

(A) Normal infant lung: Bronchiole (b) and accompanying artery (arrow) with surrounding alveoli (black arrowhead). Scattered alveolar macrophages (white arrowheads) are present within the alveolar spaces. **(B)** Bronchopulmonary dysplasia (BPD): large simplified alveoli separated by thin alveolar septa (black arrowhead) in the absence of significant airway injury. Enlargement of the alveoli can be appreciated when comparing to the size of the bronchiole (b) and accompanying artery (arrow). Accumulation of macrophages (white arrowhead) are frequently present. **(C)** Emphysema: isolated or “free floating” segments of viable alveolar septal tissue (arrows) are the characteristic histologic finding in emphysema. Lymphocytic inflammation with admixed macrophages containing anthracotic black pigment (white arrowhead) present adjacent to the bronchiole (b) and its accompanying artery (a) is a frequent finding in emphysema. **(D)** Neuroendocrine cell hyperplasia of infancy (NEHI): increased bombesin immunoreactive pulmonary neuroendocrine cells (PNEC) (arrow) within bronchioles (b) are the key diagnostic histologic feature of NEHI. NEHI is diagnosed in lung biopsies free of pathologic findings indicative of other disorders such as architectural disruption, diffuse or advanced airway injury, inflammation, and significant vascular changes. **(E)** Surfactant deficiency associated with *ABCA3* mutation: diffuse AT2 cell hyperplasia (arrow) lining thickened alveolar septa (black arrowhead) and pulmonary alveolar proteinosis features including abundant granular eosinophilic material with admixed foamy macrophages (white arrowhead). Electron microscopic analysis

(inset) reveals the characteristic electron dense inclusions within abnormal lamellar bodies described as resembling “fried eggs” (white arrows). **(F)** Pulmonary interstitial glycogenosis (PIG): alveolar septal expansion (black arrowhead) by glycogen laden mesenchymal cells with vacuolated cytoplasm and indistinct cell borders (arrow). Alveolar macrophages (white arrowhead) are also present. **(G)** Chronic obstructive pulmonary disease (COPD): large conducting bronchus (b) with increased goblet cells (arrow) and decreased ciliated cells (arrowhead) lining a mucus filled lumen. Chronic bronchitis and emphysema are the two cardinal features of smoking related COPD. **(H)** Idiopathic pulmonary fibrosis (IPF): nonuniform collagenous interstitial fibrosis associated with cystically dilated airways (arrow), fibroblastic foci (white arrowheads) alternating with areas of preserved alveoli with thin septa (black arrowhead). **(I)** Pulmonary arterial hypertension (PAH): muscular artery (arrows) with marked intimal cellular proliferations resulting in nearly total luminal occlusion and formation of multiple small vascular spaces. Another muscular artery in the same lung (inset) with prominent medial vascular smooth muscle hyperplasia (black arrowhead) and intimal cellular proliferation and fibrosis (white arrowhead) highlighted by Movat pentachrome stain.

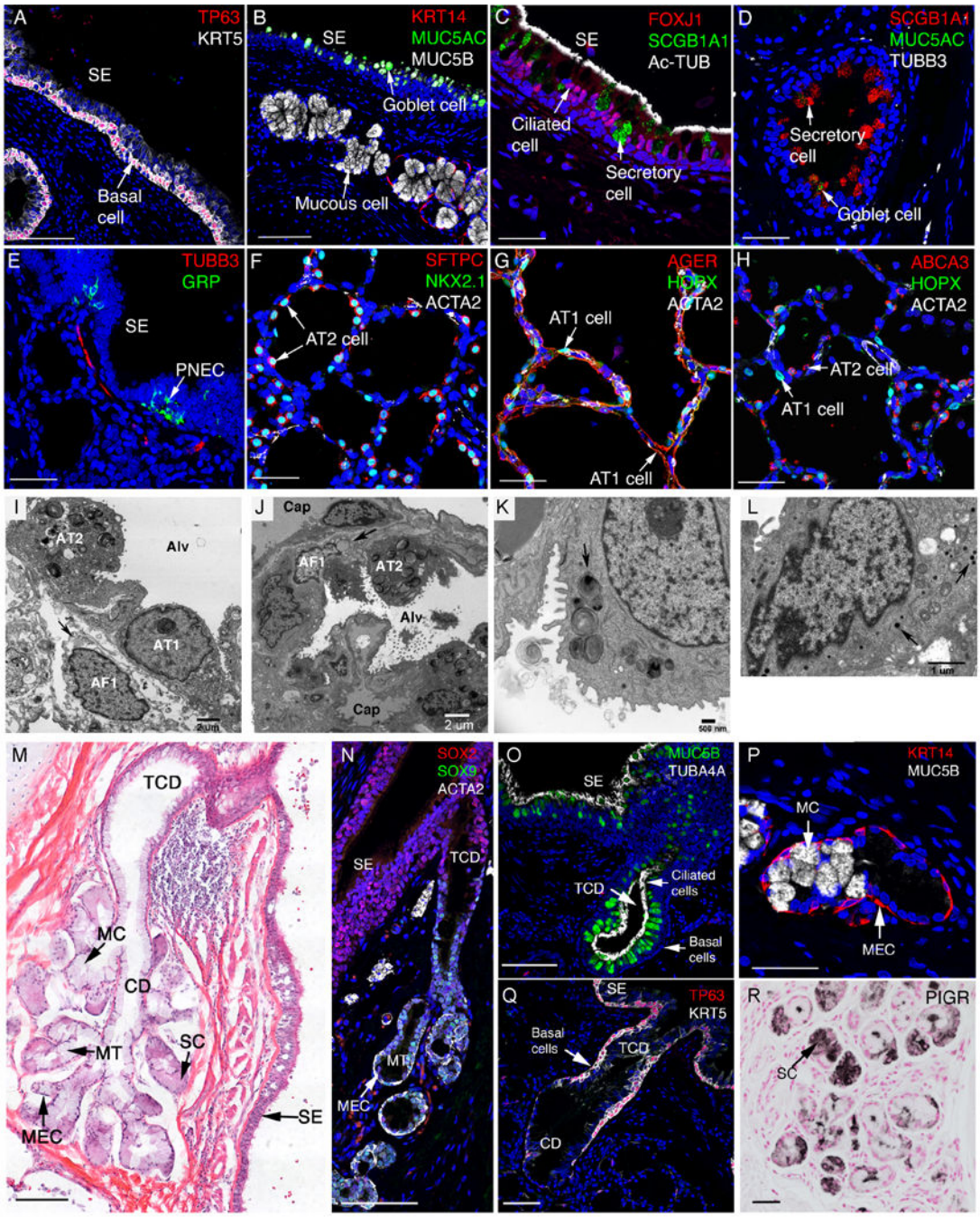

Figure 4: Epithelial cells in the human lung.

Lung sections were stained and imaged by immunofluorescence confocal microscopy **(A)** Trachea and larger conducting airways are lined by a pseudostratified epithelium comprised of TP63 and KRT5 stained basal cells. **(B)** Goblet cells expressing MUC5AC reside in surface epithelium (SE) while mucous cells expressing MUC5B reside in submucosal glands, adjacent to KRT14 stained myoepithelial cells. **(C)** SCGB1A1^{+} secretory and FOXJ1^{+}, Ac-TUB^{+} ciliated cells intersperse along the airway. **(D)** A few SCGB1A1^{+} cells also express goblet marker MUC5AC. **(E)** Pulmonary neuroendocrine cells (PNEC)

are found in clusters forming neuroendocrine bodies (NEB) that stain for GRP which are innervated by TUBB3^{+} nerves. **(F-H)** Alveolar regions are lined by AT 1 cells (AGER^{+}, HOPX^{+}) and AT2 cells (SFTPC^{+}, NKX2.1^{+}, and ABCA3+). AT 1 cells are closely opposed to capillary endothelial cells for efficient gas exchange. AT2 cells secrete pulmonary surfactant lipids and protein into the alveolus. ACTA2 stains alveolar myofibroblasts (F-H). **(I)** Alveolar septa with AT1 and AT2, capillaries and an alveolar fibroblast 1 cell (lipofibroblast) containing a lipid droplet (arrow). Alv (alveolar lumen), TEM X6900. **(J)** Alveolar septa with AT2, alveolar fibroblast 1 (lipofibroblast), capillaries (cap) lined with endothelial cells (en). Alv (alveolar lumen), TEM X2900. **(K)** Normal lamellar bodies in a AT2 cell, including ones with projection cores (arrow). TEM X12400. **(L)** A bronchiolar neuroendocrine cell containing multiple dense-core granules (arrows). TEM X19500. **(M)** H & E staining of a bronchial submucosal gland. Glands open to the airway lumen or surface epithelium (SE). TCD: terminal ciliated ducts; MC: mucous cell; CD: collecting ducts; MT: mucous tubules; SC: MEC: myoepithelial cells. **(N)** SMG cells in the ducts and most epithelial cells lining conducting airways express SOX2. SOX9 is selectively expressed in SMG epithelial cells. **(O)** Pseudostratified, terminal ciliated ducts (TCD) are lined by ciliated cells (TUB4A4^{+}) and goblet cells (MUC5B^{+}). **(P)** Collecting ducts are lined by mucous cells (expressing MUC5B but not MUC5AC). **(Q)** Pseudostratified, terminal ciliated ducts (TCD) are lined by basal cells (TP63^{+}KRT5^{+}). **(R)** Peripheral regions of the SMG are lined by serous cells (sc) expressing PIGR. Acini and ducts of the SMG are surrounded by myoepithelial cells (MEC) expressing ACTA2^{+} and KRT14^{+} (M, N, P). Scale bars: A, B (100 mm), C-H (40 mm), M, N and Q (100 mm), O and P (40 mm), R (50 mm).

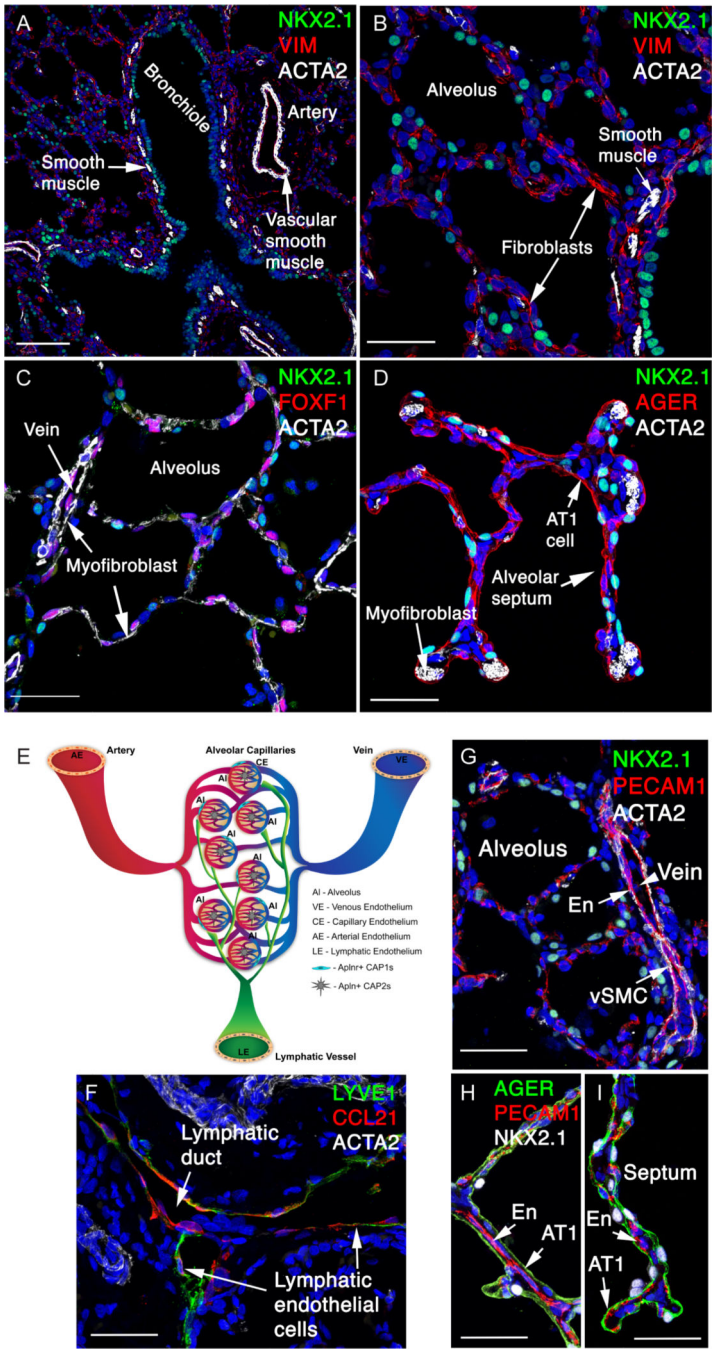

Figure 5: Mesenchymal and endothelial cells in the human lung.

(A,B) ACTA2+ airway smooth muscle cells line the NKX2–1+ bronchiled epithelium. ACTA2+ vascular smooth muscle cells line an artery. Vimentin (VIM^+) stains a subset of fibroblasts. **(C,D)** ACTA2+ myofibroblasts in alveolar septae in close relationship to AT 1 cells ($AGER^+$). $FOXF1^+$ fibroblasts and endothelial cells are shown (C). **(E)** A schematic of the main types of pulmonary endothelial cells. Large vessels are lined by arterial endothelium (AE), venous endothelium (VE) and lymphatic endothelial (LE) cells. The alveolar microvasculature consists of two types of capillary endothelial cells: $Aplnr^+$

CAP1 (gCAPs) and Apln^{+} CAP2 (aCAPs). **(F)** LYVE1^{+} and CCL21^{+} lymphatic endothelial cells line the lymphatic ducts. **(G)** PECAM1^{+} endothelial cells (En) are identified in vein and alveolar region, adjacent to vascular smooth muscle cells (vSMC). **(H,I)** PECAM1^{+} endothelial cells (En) in the alveolar region are shown adjacent to AGER^{+} AT1 epithelial cells in the alveoli, facilitating gas exchange. Scale bars: A (100 mm), B-D (40 mm), F-I (40 mm).

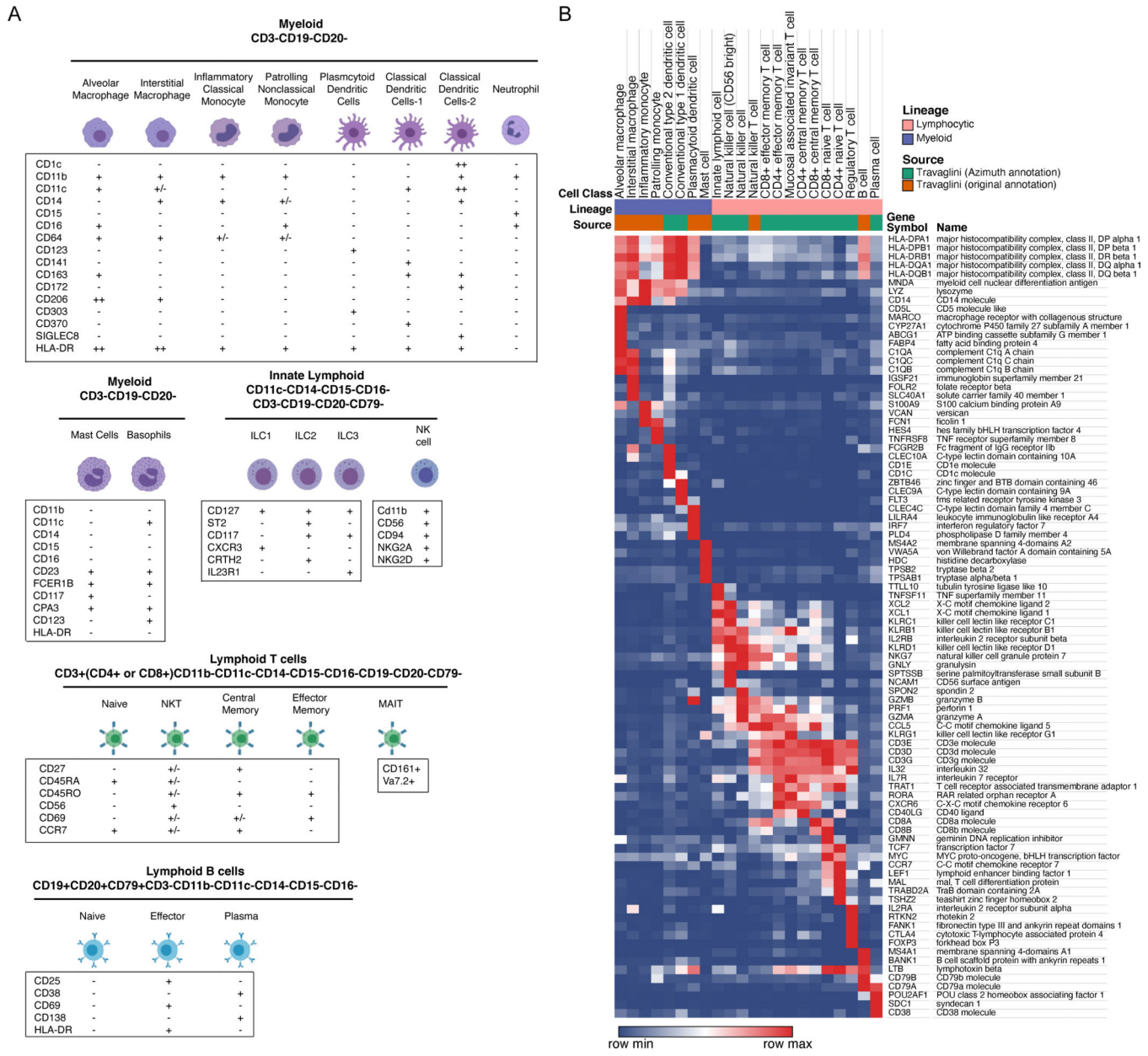

Figure 6. Immune cells of the human lung.

(A) Depiction of lung immune cell types featured in CellCards. They can be delineated based on surface protein expression, with all immune cells expressing CD45. Macrophages are CD11b+; dendritic cells are CD11c+; T cells are CD3+ and B cells are CD19+. Specialized T and B cell states can be identified using additional markers. **(B)** Heatmap shows expression levels of representative marker genes of lung immune cells using single cell RNAseq data (Travaglini, et al., 2020). Both original annotations and Azimuth-projected annotations were used for cell designations (Hao, et al., 2020). IGSF21+ dendritic cells and basophil/mast cells from the original publication were renamed as interstitial macrophages and mast cells based on the transcriptional profile. Cell ontology labels were used to better

illustrate identities of cells. Average normalized values (log_2(CPM+1)) for each cell type were used in the heatmap.

Table 1.

Markers and cre lines:

Cell lineage	Cell type	Marker Genes[#]	Surface Protein Genes[#]	Antibodies	Cre lines*
Epithelium	Basal cell	*TP63, KRT5*	*NGFR* [H], *TRPC6*[H]	TRP63, KRT5	*Trp63*^{creERT2}(Lee, et al., 2014) *Krt5*^{creERT2}(Van Keymeulen, et al., 2011)
Epithelium	Secretory cell	*SCGB1A1, SCGB3A2*	*SLC4A7*[H], *SCUBE2*[H]	SCGB1A1	*Scgb1a1*^{creERT2}(Rawlins, et al., 2009b)
Epithelium	Ciliated cell	*FOXJ1, RSPH1*	*CDHR3, CDHR4*	FOXJ1, Acetylated-Tubulin	*Foxj1*^{creERT2}(Rawlins and Hogan, 2008)
Epithelium	Goblet cell	*MUC5AC, SPDEF*	*PCDH7*[H], *SLC4A 11*[H]	MUC5AC, AGR2	
Epithelium	Pulmonary neuroendocrine cell	*ASCL1, GRP*[H], *Calca* [M]	*NRXN1*[H], *CDH18H*	GRP[H]/ Bombesin, CGRP[M]	*Ascl1*^{creERT2}*(Km*, et al., 2011) Ca/ca^{creERT2}(Song, et al., 2012)
Epithelium	Tuft cell	*POU2F3, ASCL2, Dclk1* [M]	*TRPM5* [M]	POU2F3, DCLK[M]	*Dc/k1*^{creERT2}(Westphalen, et al., 2014) *Pou2f3*^{creERT2}(McGinty, et al., 2020)
Epithelium	lonocyte	*FOXI1, ASCL3, Cftr* [M]	*CFTRM*		Asc/*3*$^{EGFP\text{-}Cre}$(Bullard, et al., 2008)
Epithelium	Bronchoalveolar stem cell			Co-express low level SFTPC and SCGB1A1	Dual recombinases or split cre effector (Liu, et al., 2019; Salwig, et al., 2019)
Epithelium	Alveolar type 1 cell	*AGER*[H], *RTKN2, Hopx* [M]	*AGER, SEMA3B HTI-56*[H] *(MAB)*	AGER, HOPX	Hopx^{creERT2}(Jain, et al., 2015) *Ager*^{creERT2}(Chung and Hogan, 2018) Aqp5^{cre}(Flodby, et al., 2010)
Epithelium	Alveolar type 2 cell	*SFTPC, LAMP3*	*KCNJ15 HTII-280*[H] *(MAB)*	SFTPC, ABCA3	*Sftpc*^{creERT2}(Chapman, et al., 2011 ; Rock, et al., 2011)
Epithelium	Ductal basal cell	*VIM, SOX9*		TRP63, KRT5	*Trp63*^{creERT2}(Lee, et al., 2014) *Krt5*^{creERT2}(Van Keymeulen, et al., 2011)
Epithelium	Myoepithelial cell	*KRT14, MYH11*		ACTA2/SMA	*Acta2*^{creERT2} (Anderson, et al., 2017; Lynch, et al., 2018; Tata, et al., 2018) *Myh11-creERT2* (Anderson, et al., 2017). (Both are also active in smooth muscles and myofibroblasts.)
Epithelium	Mucous cell	*MUC5B, SPDEF*		MUC5B	
Epithelium	Serous cell	*LYZ, LTF*			
Mesenchyme	Airway smooth muscle cell	*ACTA2, DES, LGR6*	*LGR6*	ACTA2/SMA	*Lgr6*^{creERT2}(Snippert, et al., 2010) *Acta2*^{creERT2}(Moiseenko, et al., 2017; Zepp, et al., 2021)
Mesenchyme	Vascular smooth muscle cell	*NTRK3, ITGA7, Cnn1* [M]	*ITGA7, NTRK3*	ACTA2/SMA	*Acta2*^{creERT2}(Moiseenko, et al., 2017; Zepp, et al., 2021)
Mesenchyme	Chondrocytes	*COL2A1, HAPLN1*		SOX9	*Co/2a1*^{creERT2}(Zhu, et al., 2008)
Mesenchyme	Alveolar fibroblast 1	*TCF21, WNT2*	*PCDH15H*	PLIN2/ADRP	*Tcf21*^{mercremer} (Park, et al., 2019) *Wt2*^{creERT2}(Zepp, et al., 2021)
Mesenchyme	Alveolar Fibroblast 2	*MFAP5, SCARA5*	*CDON*		

Cell lineage	Cell type	Marker Genes[#]	Surface Protein Genes[#]	Antibodies	Cre lines*
Mesenchyme	Secondary crest myofibroblast cell	*DACH2*^{H} , *Fgf18*^{M}	*ITGBL1*^{H}	ACTA2/SMA	*Fgf18c*^{reERT2}(Hagan, et al., 2019a) *Pdgfrar*^{rTA}(Li, et al., 2018) *Pdgfra*^{creERT2} (Chung, et al., 2018) Acta2^{creERT2}(Moiseenko, et al., 2017; Zepp, et al., 2021)
Mesenchyme	Pericyte	*TRPC6. LAMC3*	*TRPC6*	CSPG4/NG2 PDGFRb	*Pdgfrb*^{creERT2} (Cuervo, et al., 2017)
Mesenchyme	Mesothelium	*WT1, UPK3B, FREM2*		WT1	*Wt1*^{creERT2}(Zhou, et al., 2008)
Endothelium	Arterial endothelial cell	*DKK2, GJA5*	*EFNB2*	VWF (also vein)	*Bmx*^{creERT2} (not active in small arterioles) (Ehling, et al., 2013) *Sox17*^{creERT2} (also labels capillaries) (Liao, et al., 2009)
Endothelium	Venous endothelial cell	*ACKR1*^{H}, *HDAC9*^{H} *Slc6a2*^{M}	*ACKR1*	VWF (also artery) Endomucin (also capillaries, but not artery)	
Endothelium	Lymphatic endothelial cell	*PROX1, MMRN1*	*LYVE1, NRP2*	LYVE1	*Prox1*^{creERT2} (Bazigou et al., 2011)
Endothelium	Capillary cell 1	*IL7R*^{H} , *Aplnr*^{M} , *Gpihbp1*^{M}	*IL7R*^{H} , *Aplnr*^{M}		*Aplnr*^{creERT2} (Gillich, et al., 2020)
Endothelium	Capillary cell 2	*EDNRB, HPGD*^{H} , *Apln*^{M} , *Car4*^{M}	*EDNRB*	EDNRB, CA4^{M}	*Apln*^{creERT2} (Gillich, et al., 2020)

KEY RESOURCES TABLE

REAGENT	SOURCE	IDENTIFIER
Antibodies		
ABCA3	Seven Hills Bioreagents	WMAB—17G524
ACTA2	Sigma-Aldrich	A5228
AGER	R&D Systems	AF1145
CCL21	R&D Systems	AF366
FOXF1	R&D Systems	AF4798
FOXJ1	Thermo Fisher Scientific	14-9965-02
GRP	Thermo Fisher Scientific	20073
HOPX	Santa Cruz Biotechnology	SC-30216
LYVE1	ABCAM	AB36993
KRT5	Biolegend	PRB-160P-200
KRT14	ABCAM	AB7800
MUC5AC	ABCAM	AB3649
MUC5B	Santa Cruz Biotechnology	SC-20119
NKX2.1	Seven Hills Bioreagents	RB TTF-1 1231
PECAM1	R&D Systems	BBA7
SCGB1A1	Lifespan Bioscience	LS-B6822
SOX2	Santa Cruz Biotechnology	SC-365823
SOX9	Millipore	AB-5535
TP63	Biocare	CM163
TUBA4A	Sigma Aldrich	T7451
TUBB3	Biolegend	801201
VIM	Santa Cruz Biotechnology	SC-7557