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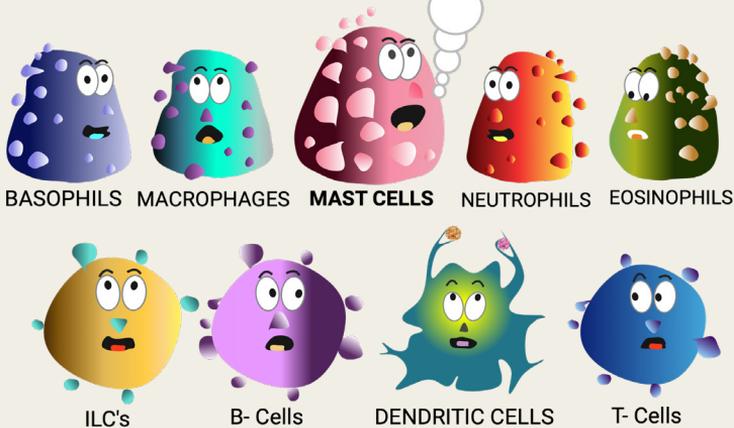
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Lung Microenvironments Influence on Mast Cell Plasticity and Spatial Distribution of Immune cells

PREMKUMAR SIDDHURAJ

DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY

*You may stay silent on increasing pollution,
deforestation and antibiotic abuse,
but we wont*



FYI: Your immune cells are stern environmental activists,
they act whether you want it or not !!



Lung Microenvironments Influence on Mast Cell Plasticity and Spatial Distribution of Immune cells

by

Premkumar Siddhuraj



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DOCTORAL DISSERTATION

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Faculty opponent

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Title and subtitle Lung Microenvironments Influence on Mast cell Plasticity and Spatial Distribution of Immune cells		Sponsoring organization
Abstract Inflammatory airway diseases and lower respiratory tract infections are leading causes of severe illness, disability and death worldwide. Much of today's hypotheses about the underlying inflammatory processes and pathogenic contribution by immune cells come from in vitro experiments or animal models. As a consequence, our knowledge of the actual immunopathological events in real patient tissues is surprisingly limited. To grasp the true nature of the disease it is therefore important to assess the phenotypes and interplay of immune cells under disease-relevant in vivo conditions. The overarching aim of the present study is to investigate how lung microenvironments dictate immune cell phenotypes, plasticity and their spatial coordination in health and airway diseases. Focus is on mast cells, a key player in immune surveillance and tissue homeostasis, and cells associated with type 2 immunity. The methodological focus is on innovative computer integrated histology-based approaches that have been applied on human lung explant models, humanized mouse models (NSG-SGM3) and clinically relevant lung tissue samples from patients with COPD, CF, IPF, and COVID-19. Individuals with no history of chronic lung diseases served as control. Papers 1 and 2 profoundly alter the view on mast cell protease heterogeneity by showing that, rather than the prevailing division of mast cells into binary MCT and MCTc protease profiles, both the mRNA expression and protein granule storage of tryptase, chymase and carboxypeptidase A3 are under a fine-tuned regulation by the local tissue microenvironment. These studies also provide strong indications that individual proteases may be selectively released already at healthy base-line conditions and then further up-regulated in COPD, IPF, CF, and COVID-19. Paper 3 represents the first extensive exploration of eosinophils, basophils, and the type 2 immunity surrogate marker GATA3 across multiple anatomical sites in COPD-affected lungs. Computerized quantification and spatial analysis revealed a surprisingly patchy eosinophil distribution that co-localized with GATA3, basophil, and ILC2-containing type 2 microenvironments. Importantly, the occurrence of eosinophils, basophils and patchy type 2 microenvironments correlated with disease severity. In Paper 4 a cutting-edge humanized mouse model (NSG-SGM3) was used to show that sophisticated highly spatially organized human leukocyte tissue patterns spontaneously develop in mouse lung and lymphoid tissues. Although some of the patterns had a striking resemblance to human COPD, confounding species cross reactivity events must be taken into account before establishing humanized immune system mouse models for any human disease. In a nutshell, the present thesis reveals several new important aspects of mast cell protease plasticity, type 2 immunity, and leukocyte pattern formation and how these features are dynamically controlled by the local tissue microenvironment in health and disease. As such, the thesis also provides a powerful example of the need for "direct" explorations of patient tissues in order to understand the true complexity of inflammatory diseases and infections.		
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Date 2021-04-28

For a randomly assembled strand of DNA to be able to write this thesis, it required 3.5 billion years of evolution!

So, don't get stressed when things are slow in life, because life is programmed to be slow! ☺

- Premkumar Siddhuraj

Lung Microenvironments Influence on Mast Cell Plasticity and Spatial Distribution of Immune cells

by

Premkumar Siddhuraj

Main supervisor: Professor, Jonas Erjefält, Lund University.

Co supervisor: Professor, Leif Bjermer, Lund University.



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To My Parents

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List of Papers

1. **Siddhuraj, P.**, Clausson, C. M., Sanden, C., Alyamani, M., Kadivar, M., Marsal, J., Erjefält, J. S. (2021). Lung Mast Cells Have a High Constitutive Expression of Carboxypeptidase A3 mRNA That Is Independent from Granule-Stored CPA3. *Cells*, 10(2). doi:10.3390/cells10020309
2. **Siddhuraj,P.**, Caroline Sanden., Michiko Mori., Jimmie Jönsson, Carl-Magnus Clausson, Elin Rönnerberg , Jonas Larsson, S Agatheeswaran, Karl Swärd, Richard Ingemansson, Nilsson G.,Thais Maud, Marissa Dohnnikoff, Leif Bjermer, Jonas S. Erjefält* . Environmental-Induced Mast Cell Plasticity Creates Infinite Tryptase and Chymase Profiles and Altered Protease Phenotypes in Chronic Lung Diseases and COVID-19
3. Jogdand, P., **Siddhuraj, P.**, Mori, M., Sanden, C., Jonsson, J., Walls, A. F., Erjefält, J. S. (2020). Eosinophils, basophils and type 2 immune microenvironments in COPD-affected lung tissue. *Eur Respir J*, 55(5). doi:10.1183/13993003.00110-2019
4. **Siddhuraj,P.**, S Agatheeswaran, Cozzolino O, J Jönsson, R Bhongir ,Kasetty G ,Jain MV, C.M Clausson, A Egesten, M Kadivar, J Marsal, J Larsson, L Bjermer, C Sanden, and Erjefält J.S*. Development of Highly Organized Human Leukocyte Infiltration Patterns and Human Lymphoid Tissue in Lungs of Humanized Human Immune System (HIS) Mice

Popular Science Summary

Do you know we take roughly 500 million breaths in our lifetime? Which we often do taken for granted, but little we know of the countless actions our lungs perform during every breathe. When we breathe, our lungs take the oxygen from the air and transport it into the blood and meanwhile also clear the dust and dangerous pathogens that settle in the air sacs of your lungs. What are air sacs? well, it is the place (small balloon like structures) where the gas exchange occurs and another fun fact, if you unfold all of the air sacs in your lungs, you can cover half of a tennis court! If you think you are working hard, think again! because your lungs cells vacuum and mop the surface area which is same as roughly half of a tennis court and keep it clean not only for every day but every minute!

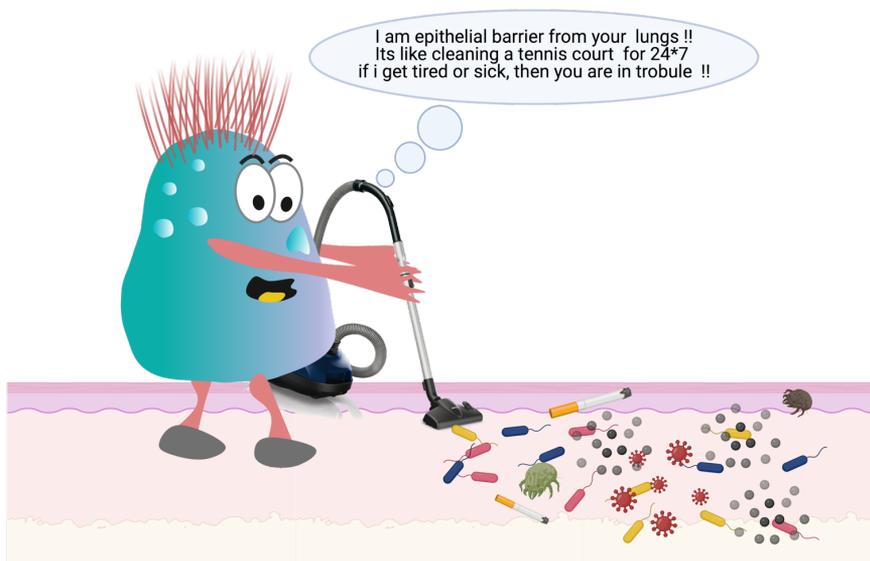


Image by Premkumar Siddhuraj

While most of the dangerous microbes are cleared by lung cells, some manage to stay and they can be removed by immune cells. Immune cells? yes, they exist in many different types and they communicate with each other and are constantly moving within our lungs and look for threats. Immune cells can be compared with police and fire departments in our society, they rush to the particular violence-hit areas and clear the threat. Although immune cells are guardians of our body, they also have bad and ugly side. When there is a problem in the communication among immune cells, they can potentially damage our lungs! and this is the main cause of

many mild (allergy) to life-threatening lung diseases (asthma, smoke and pollution-induced Chronic Obstructive Pulmonary Disease also known as COPD).

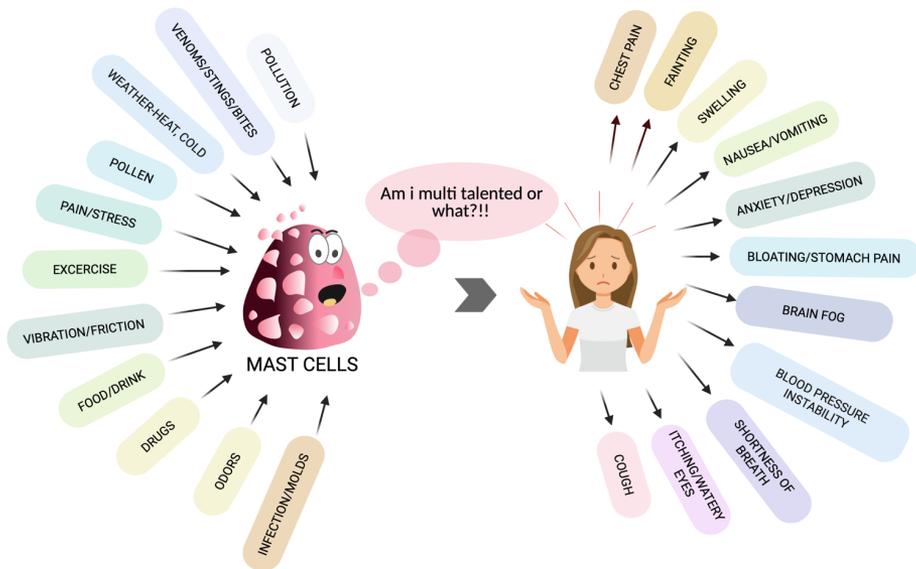


Image by Premkumar Siddhuraj

Have you ever wondered, why the beautiful spring and summer holidays are ruined by itchy eyes, cough and runny noses and who is responsible? well, their name is "Mast cells" and they are one of the immune cell types. These mast cells can be triggered by anything, any time, and anywhere literally!

In our study, we have found that mast cells are sneaky! In a study we have seen that they can change their identity depending upon the area of our lung. Also, we have observed that they may release large amounts of enzymes which may change the shape of the lung in such a way that breathing can be very difficult, for example in COPD and fibrotic diseases! Do you know this horrible disease (COPD) is not curable? and mainly caused by smoking cigarettes including e-cigarettes!

The immune cells fight against virus in your lungs

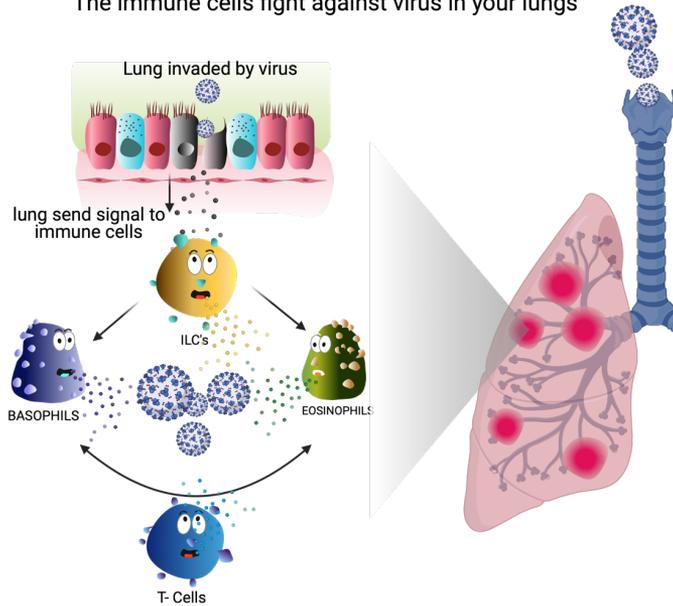


Image by Premkumar Siddhuraj

When your lung cells are damaged by dust or microbes, they release signalling molecules which alarm the surrounding immune cells. Similar to a criminal investigation, we have carefully observed the virus-infected particular areas of the patient's (COPD) lung and collected shreds of evidence. The evidence indicates that immune cells such as basophils, eosinophils, ILC cells accumulate (patchy appearance) at the site of infection and carry out the fight against virus. This observation is important because the greater number of these patchy sites in lung the sicker the patient become. In future, we will carry out a similar investigation to look out for other immune cell suspects which are making people sick.

Selected abbreviations

MCs	Mast cells
MCt	Mast cell (tryptase)
MCtc	Mast cell (tryptase+chymase)
COPD	Chronic Obstructive Pulmonary Disease
CF	Cystic Fibrosis
IPF	Idiopathic Pulmonary Fibrosis
BAL	Bronchial alveolar lavage
BALT	Bronchus associated lymphoid tissue
IgE	Immunoglobulin E
FCεRIα	Fc epsilon RI alpha
SCF	Stem cell factor
CPA3	Carboxypeptidase A3
MRGPRX2	Mas-related G-protein coupled receptor member X2
CTMS	Connective tissue mast cell
MMS	Mucosal mast cell
FEV	Forced expiratory volume
FVC	Forced vital capacity
CTFR	Cystic fibrosis transmembrane conductance regulator
GvHD	Graft versus host disease
ILC	Innate lymphoid cell
AM	Alveolar macrophages
MCAS	Mast cell activating syndrome

Introduction

The Global Impact of Lung Diseases

Topographically the lungs are exterior to our body and every day our lungs are most vulnerable to infection and injury because they are exposed constantly to particles, chemicals and infectious organisms in ambient air. Globally, half of humanity is regularly exposed to toxic air from both indoor and outdoor environments. In fact, according to the US environmental protection agency (EPA), the levels of indoor air pollutants are up to five times higher than outdoor levels, including one billion people exposed tobacco smoke.

Globally 65 million people have moderate to severe chronic obstructive pulmonary disease (COPD) and about 3 million die every year [1]. However, Lancet's Global Burden of Disease report (2017) indicates COPD is already the second leading cause of mortality globally, accounting for 5.36% of total deaths followed just after ischaemic heart disease [2]. Compared to the year 2000, the toxicity of inhaled air is projected to increase up to 90% by 2050 [3], this will further fuel the crisis and COPD may turn into the leading cause of mortality within two decades. Notably, COPD causes non-reversible damage to our lungs, in other words currently it is not a curable disease. An estimated 334 million people suffer from asthma, affecting 14% of children globally and one of the common causes of childhood chronic disease [4]. In recent years the prevalence of asthma in children is rising [5]. Lower respiratory tract infections are common triggers of exacerbations in asthmatic and COPD patients, especially viral infections in children [6].

The world's most common and deadly neoplastic disease is lung cancer, which kills roughly 1.6 million people each year and numbers are increasing at an alarming rate [7]. Smoking, toxic gas pollution and genetic factors are common risk factors for lung cancer. In addition, lung fibrosis diseases such as Idiopathic Pulmonary Fibrosis (IPF) kills roughly 10 people diagnosed per every 100,000 (general population) with a mean survival of 2-5 years from the diagnosis and Cystic Fibrosis (CF) affects around 70,000 to 100,000 globally [8-10].

Quantifying the global burden of acute lower respiratory tract infections remains challenging and likely causes 4 million deaths yearly, besides it is a leading cause of death among children under 5 years old. Children who suffer frequent lower respiratory tract infections are predisposed to chronic lung diseases (COPD and

asthma) in adulthood [11]. Infections are also a major culprit in other diseases affecting the lung, such as cystic fibrosis.

Ever rising pollution, deforestation (exposure to deadly viral species from wild animals), global warming-induced ice melting in polar regions (revival of deadly pathogens in permafrost) and antibiotic abuse (leading to more multi-drug resistant pathogens) are the 21st century's greatest challenges for human health. SARS-COVID-19 pandemic prefigures the medical disasters in coming decades, already COVID19 killed roughly 3 million people (grossly underestimated figure) [12] and with economic loss of 16 trillion dollars [13] the virus has brought the world to its knees! In a nutshell, the lung diseases are the leading cause of severe illness, disability and death worldwide. So, the significance of a better understanding of lung medicine is more profound than ever!

The Lung

The respiratory system provides for the exchange of oxygen and carbon dioxide to and from the blood. The system includes the lungs and a branching system of bronchial tubes connected to the gas exchange site. The system can be anatomically divided into upper (sinus, nasal cavity and pharynx) and lower respiratory tracts (larynx, trachea and lungs). It is customary to divide the system functionally into two principal components, the conducting and respiratory zones.

The conducting zone includes the nasal cavities, pharynx, trachea, bronchi, bronchioles and terminal bronchioles. The zone serves two main functions, conditions the inspired air and conduits conditioned air to and from alveoli.

The respiratory zone includes the respiratory bronchioles, alveolar ducts, and alveoli. The zone facilitates the gas exchange and is predominantly occupied by alveoli as a sack-like structure [14]. An average pair of human lungs composes roughly 480 million alveoli structures [15], each encircled by veins, arteries and lymphatic vessels.

Anatomical and Histological Features of the Respiratory Tract

In recent years with the help of advanced technologies sorting, purification, culturing, and phenotyping of cells has become more robust, resulting in the identification of 58 types of resident cellular components in the human lung. The 58 molecular types include 15 epithelial, 9 endothelial, 9 stromal and 25 immune populations. The number of cells is greater than previously thought in each lung compartment [16]. Human lungs consist of multiple compartments made of a complex mixture of cell types, each with a specialized role such as gas exchange, forming a barrier from external insults, secretion of mucus to clear contaminants and surfactants for airspace lubrication (Figure 1). The above-mentioned characteristics make the lung one of the complex organs in our body [17].

The trachea is a thin-walled tube lined with typical mucosa in which lamina propria consists of densely populated seromucous glands that produce watery mucus. A dozen C-shaped hyaline cartilage rings reinforce the tracheal wall which ensures uninterrupted airflow. The bronchial mucosa is highly similar to that of the tracheal wall except for the organization of smooth muscle bundles and the cartilage. Numerous mucus and serous glands are present with duct opening into the bronchial lumen. The smooth muscle bundles are crisscrossed across the entire lamina propria. Lymphatic nodules are located along the bronchial tree and mucosa-associated-lymphoid tissue (MALT) becomes more prominent as cartilage and connective tissue are reduced.

The bronchioles lack both the mucosal glands and cartilages. The epithelial lining at the larger bronchioles still presents with ciliated pseudostratified columnar cells but as they further branch into terminal bronchioles, the complexity of the epithelium decreases and it becomes ciliated simple cuboidal epithelium. The cuboidal epithelium is largely occupied with non-ciliated club cells. The club cells functions are secretion of surfactants (lipoproteins and mucins), detoxification of xenobiotic compounds, antimicrobial peptides and cytokines for local immune defence. Along with club cells, the terminal bronchioles also presented with chemosensory brush cells and DNES small granule cells. The bronchiolar lamina propria contains elastic fibres and smooth muscle bundles while mucosal contraction is regulated by the nerve cells.

Each terminal bronchiole branches into multiple respiratory bronchioles, with similar mucosal lining properties except for having several openings for alveoli. The respiratory bronchioles further branch into tubes called alveolar ducts and they are lined with tight squamous cells. The alveolar ducts, consisting of thin lamina propria, include smooth muscle cells, elastic fibres and collagen fibres that support both the ducts and alveoli.

The alveoli are responsible for the sponge-like structure of the lungs. It is in these structures the gas exchange occurs with the blood that circulates in the alveolar capillaries. The alveoli are separated by interalveolar septa consisting of scattered fibroblasts, and elastic and reticular fibres enable the alveoli to expand (during inspiration) and contract (during expiration).

Type 1 alveolar cells or type 1 pneumocytes make up the alveolar lumen side of the blood-air wall and roughly 95% of the alveolar epithelium. Type 1 epithelial cells have tight junctions that prevent tissue fluid leakage into alveolar space. Type 2 alveolar cells or type 2 pneumocytes are cuboidal cells that protrude into the air space, resting on the basal lamina. Type 2 cells continually release many lamellar granules over the inner surface of alveolar structures. These granules contain lipids, phospholipids and proteins. These secreted granules act as a pulmonary surfactant which reduces the surface tension of the air-epithelium interface. The granules contain surfactant proteins and notably, SP-D (surfactant protein -D) is important for the innate immune protection within alveoli. Alveolar macrophages are also present in the alveoli and the interalveolar septum. These monocytes migrate from microvasculature into the lung tissue and phagocytose erythrocytes (lost from damaged capillaries) and airborne dust particles. These filled macrophages have various fates: they can enter into bronchioles and be removed into the esophagus, while others enter lymphatic drainage and some stay in interalveolar septa for years.

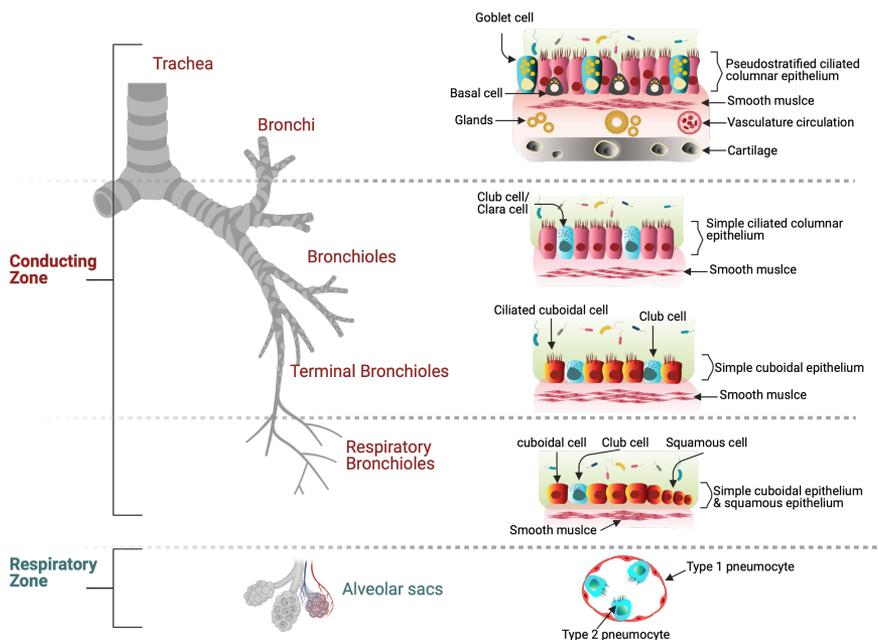


Figure 1: A schematic overview of the different lung compartments and the stromal cellular composition. *Image by Premkumar Siddhuraj*

The Clinical Relevance of Lung Compartments

Topographically lungs are external to the human body resulting in constant insults by specks of dust, microbes and chemical agents. The respiratory tract muco-ciliary clearance and the phagocytosis by alveolar macrophages provide the first line of defence.

The leukocyte reactions in the lung microenvironments vary depending upon the type of the insults and their reactions and interplay are regulated by different cytokines and mediators. Most of the cytokines released by a leukocyte seem to affect the target cell in close proximity (paracrine signalling). Thus, spatially mapping the distribution of the immune cells in specific microenvironments can offer a better understanding of immune reactions in health and disease. The human lungs can be functionally divided into several cellular compartments, such as the bronchoalveolar space (lumen), bronchus-associated lymphoid tissue (BALT), bronchial epithelium and lamina propria, interstitial tissue, perivascular space and intravascular leukocyte pool [18] and more.

The Bronchoalveolar Space

Apart from alveolar macrophages several other immune cell populations enter the lumen of conducting airways and alveoli. The bronchoalveolar lavage method is a widely used diagnostic technique to analyse the leukocytes, secretions and epithelial cells from airway mucosa and alveoli in lung diseases such as asthma, infections, sarcoidosis and tumours [19]. Not only cells jump from interstitium to lumen but the other direction has also documented, for example in rodents the lymphocytes and dendritic cells can migrate from lumen to interstitium and reach the regional lymph nodes [20].

Although BAL and sputum techniques have been proven to be helpful in clinical diagnosis, many unresolved issues remain regarding the leukocytes in bronchoalveolar space.

- Is the influx of leukocytes in diseases functional or just a spill over?
- To what extent do the BAL leukocytes reflect the immune cell composition of the actual lung tissue?
- Are the leukocytes that reenter from the lumen to the interstitium maintaining the disease? [21].

Bronchus-Associated Lymphoid Tissue

Under healthy conditions in adults, BALT is absent but can be found in 40% of children. However, BALT and other lung ectopic lymphoid tissues can return in adults under certain disease states, such as most inflammatory lung diseases like CF, asthma and COPD ([22]). Surprisingly, despite the clear expansion in many diseases the role of BALT in the disease remains poorly understood [23]. Remaining important questions include:

- What is the stimulus for the BALT formation "*de novo*" in certain clinical conditions?
- Is newly formed BALT (*i.e.*, ectopic lymphoid tissue) protecting or aggravating the disease?

Conducting Airways

The intraepithelial (IEL) and subepithelial leukocytes are believed to play a critical role in airway inflammatory and infectious diseases. The bronchial and bronchiolar epithelium is only one of several compartments of the conducting airways (bronchial lamina propria, interstitium or pulmonary vasculature) with a substantial population of lymphocytes. The lymphocytes population in IEL markedly differs in many respects from the other compartments and migration of lymphocytes between the compartments has also been shown, for instance from epithelium to lamina propria and then to bronchoalveolar space or vice versa [24]. In addition, mucosal and epithelial sites of the respiratory system are greatly enriched with tissue-resident $\gamma\delta$ T cells, roughly making up 8–20% of resident pulmonary lymphocytes in the lung, maintaining lung tissue homeostasis [25].

Bronchial lamina propria is also populated with scarce and scattered innate lymphoid cells ILC1s, ILC2s, and ILC3s which are long lived resident cells primarily eliciting a non-antigen specific response. For example, upon stimulation by IL-33 (secreted by airway epithelial cells) ILCs initiate robust type 2 immunity [26, 27]. As part of the innate immunity, the mast cells [28] and dendritic cells both [29] are strategically located in the airway epithelium. Basophils are virtually absent in healthy steady-state lung mucosa [30].

An adequate bronchial vascularization is needed for the recruitment of leukocytes to the airway wall. As a side note, since the mouse lacks well-vascularized mucosa, it is important to consider this species difference when interpreting the results from animal disease models, for example chronic bronchitis [21].

Pulmonary Draining Lymph Nodes

Lymphatic vessels are made of loosely connected single-layer lymphatic endothelial cells [31] and they lack pericytes or smooth muscle [32]. The pulmonary lymphatic system has two major roles: clearing the excess fluid from the lung interstitium and to act as a conduit for antigens and antigen-presenting cells to the lymph nodes.

Lymph nodes provide an ideal location for immune cells to communicate and get educated to combat against constantly presented external threats. Migratory DCs and alveolar macrophages (AM) migrate from the lungs to the draining lymph nodes and present antigens to T cells and initiate the effector T cell functions. Intriguingly, along with DCs and AMs, recent studies provide evidence that eosinophils [33] basophils [34] and mast cells [35] may also carry out an efficient antigen presentation and initiate the robust T cell effector functions. As these cells are commonly found in the draining lymph nodes, this indicates important roles regarding their overall contribution to antigen presentation as well as other immune modulatory actions.

Intravascular Leukocyte Pool

The leukocyte pool within capillaries and venules is believed to be critical for the extended immune reactions in the lung. However, the mechanism of leukocyte trafficking between capillaries and parenchyma and whether intravascular leukocytes are indispensable for lung immune reactions remains unclear [21].

Lung Parenchyma

Lung alveolar interstitial tissue is composed of alveolar epithelium, arterioles, venules, initial lymph vessels, dendritic cells, macrophages, fibroblasts and monocytes. The migration of dendritic cells and macrophages into the lymph node through the interstitium is critical for the systemic activation of T cells. Recent discoveries indicate the alveolar parenchyma is occupied by a large number of MCs, and although MCs are proven to be potential effector cells against host defence and lung homeostasis very little is known about their exact functions in alveolar region both in health and disease [36-38]. In a steady-state, pulmonary vasculature is occupied with resident neutrophils and studies have shown that they respond immediately upon any inflammatory challenges [21, 39].

Pulmonary Immune Homeostasis

Critical Checkpoints Dictate Pulmonary Immune Maturation

An antigen rich postnatal environment is critical for the maturation of the pulmonary immune system throughout childhood. The status of pulmonary immune homeostasis is very dynamic across the life course (Figure 2) and primarily dictated by environmental changes and circumstances. Several recent studies indicate that early life microbiota in children has both beneficial and detrimental consequences [40]. It has been shown recently that maternal microbiota can be transferred to the offspring [41]. This indicates that maternal environments such as nutrition, smoking, genetics and stress can have a huge implication on shaping also the offspring immune system. Early life microbiota development can be altered due to the mode of delivery (caesarean-section) [42], early life antibiotic exposure [43], nutrition [44], microbial and dust exposure from farms [45].

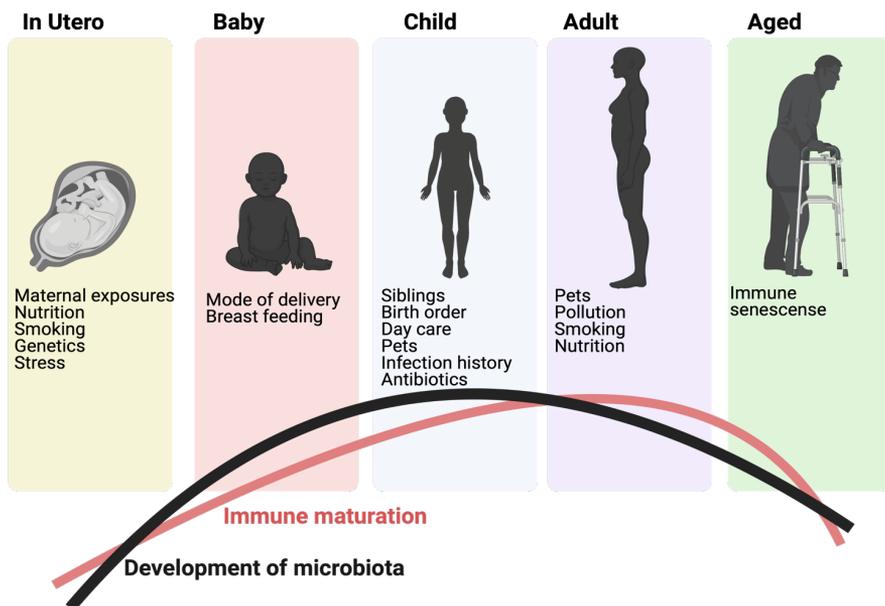


Figure 2: The check-points for human immune maturation at different stages of life. *Image by Premkumar Siddhuraj, redrawn from Clare M.Lloyd et al 2017*

The elderly is especially vulnerable to respiratory infections due to lifelong accumulation of environmental exposures which leads to elevated ROS (Reactive Oxygen Species) and a pro-inflammatory milieu that reduces the capacity to fight infections [46]. In addition to external exposures, lung physical changes such as

smaller tidal volume, decreased respiratory rate and muco-ciliary clearance can also have an impact on immune maturation [40].

The Adapted Island Model of Lung Biogeography

Topographically the airways are exterior to our body with a surface area which equals half of a tennis court (72 sq m), and they are constantly exposed to microbes and dust-laden air [40]. The surface area can be compared with human skin, which is on average 2 sq m [47]. Advancements in microbial identification techniques helped us to debunk the long-held dogma that lungs are sterile. Earlier, the lung microbial composition was considered a "binary phenomenon" with no microbial presence in healthy conditions and an overwhelming presence in disease (for instance in pneumonia). Like any community, microbial balance in the lung is determined by three critical factors: microbe immigration, elimination and local reproduction rates. The local reproduction rate of lung microbes is determined by lung physiological parameters such as local oxygen tension, pH, relative blood perfusion, relative alveolar ventilation and temperature and composition of host inflammatory cells (Figure 3).

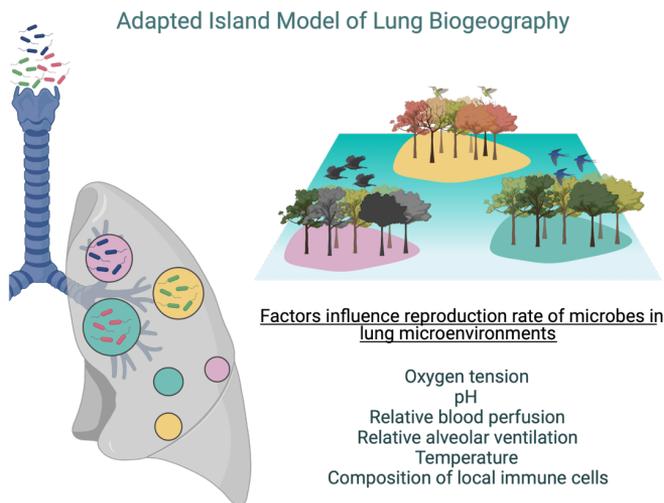


Figure 3: A drawing depicts the adapted island model of the human lung biogeography. *Image by Premkumar Siddhuraj*

Despite if lungs present with distinct local parameters in health, the lung microbiome has surprisingly little spatial variation [48]. However, in disease (*e.g.*, COPD, CF and IPF) the alterations in microbe immigration and elimination mechanisms lead to increased local reproduction and substantial spatial variation in microbiota [49].

The ecological determinants of the lung microbiome change when a healthy individual (with balanced immigration and elimination state) turn into a diseased patient (*i.e.*, a local reproduction state) [48] (Figure 4).

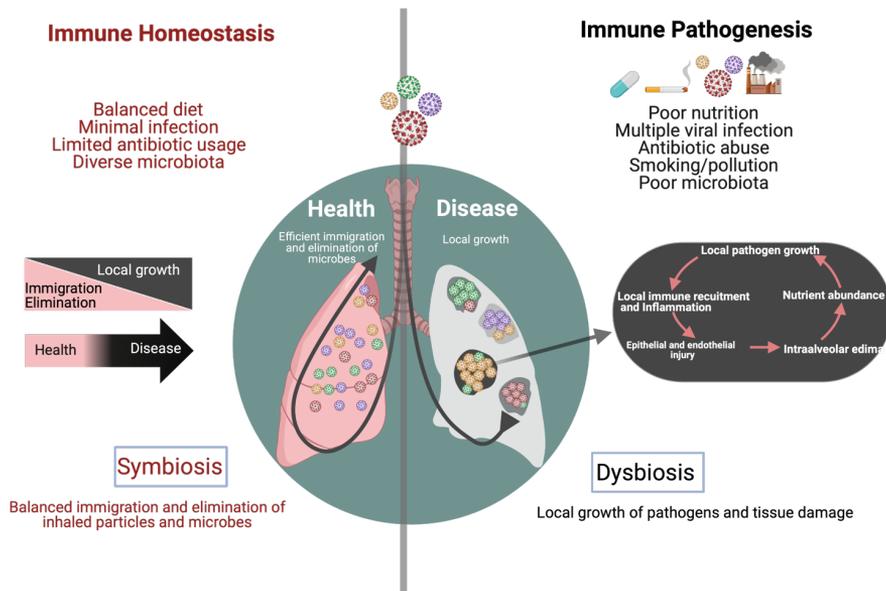


Figure 4: A simplistic overview of balance between the lung immune homeostasis and immune pathogenesis. Left side depicts the efficient immigration and elimination of microbes in healthy steady state of lung, whereas the right side has increased local growth of microbes in lung disease states. *Image by Premkumar Siddhurar*

An increased local reproduction of microbes in diseased lungs depends on a local nutritional level, inter signalling between microbial communities and resident and immune cell pathways [40, 48]. A healthy airway is a nutrient-poor environment for most microbes. Yet, in conditions of lung diseases such as CF, chronic bronchitis and asthma, upper lung compartments are filled with glycoprotein rich growth medium from mucus and in conditions such as ARDS and pneumonia, parenchymal parts flooded with nutrients result from injured alveolar stromal cells [50]. In many cases of ARDS (acute respiratory distress syndrome) and pneumonia, secondary infections pose a grave threat to the patients. This is primarily due to the presence of so called pathobionts which are a residential community that is harmless until the

introduction of a new community. For instance, when COPD individuals with a low level of *H. influenzae* infected with rhinovirus, the *H. influenzae* community starts to bloom [51]. This forward loop mechanism of local microbial growth, inflammation, tissue injury, nutrient abundance is believed to be critical in ARDS and pneumonia [48] (Figure 4, right side in the dark circle).

Lung Microenvironment Influence on the type2 Immune Landscape

Eosinophils, Basophils and ILC2 in Pulmonary Infection

Eosinophils and basophils belong to the innate immune system. These are granulocytes densely packed with a plethora of inflammatory mediators. They originate from bone marrow-derived CD34⁺ cells and their maturation are regulated by the granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5 cytokines. Eosinophils and basophils complete their maturation in the bone marrow and then enter the systemic blood circulation. Next, the matured circulating cells migrate into peripheral tissues in various pathological settings including allergy and infections. The major characteristics of eosinophils are their high content of granular proteins in their granules, including major basic proteins (MBP-1 & MBP-2) and eosinophil cationic protein (ECP). The matured granules are also packed with pro-inflammatory cytokines, chemokines and growth factors like IFN- γ , IL-4, IL-6, IL-10, tumour necrosis factor alpha (TNF- α), IL-12 (p70), and IL-13, which can be rapidly released in response to specific stimuli [52-54]. Similar to mast cells, basophils are also known for their pre-stored granular contents of heparin, histamine, tryptases (alpha and beta) and carboxypeptidase A3 (CPA3). Basophils are one of the important sources of IL-4 and IL-13 cytokines and are believed to amplify local type2 immune response [54, 55].

Innate lymphoid cells (ILCs) are a newly added family of lymphocytes that reside at barrier surfaces such as the lungs, skin and intestine. Unlike traditional classical lymphocytes, ILCs do not express antigen-specific receptors but can instead get activated through a wide range of local tissue milieu without any adaptive immunity processes. ILC2s are one of the subgroups of the ILC family. They depend on GATA3, a key activator of type-2 cytokine gene expression. Upon activation by alarmin cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), ILC2s elicit robust type-2 associated immunity. Besides, ILC2s are activated by other cytokines (IL-2, IL-4, IL-7 and IL-9) and lipid mediators (PGD₂, LTD₄ and LXA₄). Some of the effector molecules produced by ILC2s are IL-5, IL-13, methionine-enkephalin (Met-Enk) and amphiregulin. Despite ILCs having been

intensively investigated in recent years, the development, activation, and turnover of these cells in tissues remain largely unknown [27, 56, 57].

It is a well-established fact that bacterial and viral infections induce exacerbations in asthma and COPD patients. Nearly 85% of asthma exacerbations in children, and nearly 50% in adults, are a result of viral infections [58]. High doses of inhaled corticosteroids (ICS) associated with elevated airway bacterial load and increased risk of pneumonia [59]. In addition, elevated eosinophils and basophils levels have been observed in severe COPD patients receiving higher doses of inhaled corticosteroids treatment and this aligns with the fact that non-allergic eosinophilic patients are resistant to steroid treatments. Type 2 immune response may cause non-allergic eosinophilia in COPD and steroid-resistant asthmatic patients. Type 2 immunity is orchestrated by T helper 2 (Th2) cells, ILC2 cells, basophils and eosinophils through secretion of IL-4, IL-5, IL-9 and IL-13 cytokines [27].

Very little is known regarding tissue infiltration of eosinophils, basophils and ILCs in different anatomical compartments in inflammatory lung diseases, especially regarding non-allergic asthma and COPD. The severity of airway infection can be different among lung compartments, as the regional growth of infectious pathogens can be influenced by their microenvironments. As stated in the previous section, lung microenvironments can be altered by local oxygen tension, pH, relative blood perfusion, relative alveolar ventilation and temperature, dysbiosis due to pathobionts and composition of host inflammatory cells [48]. Hence, decoding the spatial correlation between type 2 effector cells and infectious agents is crucial to better understand the mechanisms behind the tissue eosinophilia in airway inflammatory diseases.

The Mast Cell

Mast cells (MCs) are part of the innate immune system. In general, they are tissue-resident, multi-functional effector leukocytes [60] and were originally described by Paul Erlich in 1879 based on their large granules and unique dye-staining properties [61]. Nearly a century later, their origin and development from CD34⁺/CD117⁺ hematopoietic stem cells was convincingly demonstrated [62]. These MC progenitors translocate from the bone marrow (BM) to peripheral blood and mature upon arrival into various organs. A recent study supports the possibility that BM-independent self-renewed stem cells can also give rise to progenitors which subsequently may develop into tissue resident matured tissue mast cells [63].

Tissue-resident matured mast cells contain large granules (roughly 50-200 per cell) filled with pro-inflammatory and immune and tissue-modulating properties. They are positioned in mucosal tissue and virtually all vascularized tissues throughout the body (including the gastrointestinal tract, skin and respiratory system). These highly

specialized leukocytes contribute to innate and adaptive immunity [64], revascularization and tissue repair [65, 66] by releasing preformed or de novo synthesized pleiotropic mediators or both. MCs are also capable of sensing their environment by membranous projections into vessel lumens, and these sensitised cells orchestrate an antigen-specific response [67]. Their strategic location in tissues facing the external milieu allows them to respond within seconds to any foreign insult and they play a critical role in host defence [68, 69]. Tissue-resident mast cells may survive for years and unlike other innate immune cells, MCs do not clear away when the inflammation halts. Rather after episodes of degranulation the granules are restored within days [70]. MC functions in allergic inflammation was revealed by two key clues: pre-stored large quantities of histamine and immunoglobulin E (IgE) dependent degranulation [71, 72]. Through both IgE-dependent and IgE-independent pathways, MCs can release preformed or de novo synthesized mediators into lung microenvironments. The released MC mediators have a profound effect on pulmonary resident cells (stromal cells and immune effector cells) and contribute to pulmonary pathologies including asthma, COPD, pulmonary fibrotic diseases and airway infections [69, 73].

Origin, Homing and Maturation

The MC progenitor cells originate from bone marrow and enter into peripheral blood circulation. The progenitors can be identified as negative for lineage markers and expressing CD34, the SCF receptor KIT (CD117), and the IgE receptor FcεRI [75]. These mast cell committed progenitors turn into mature MCs upon arrival into tissues. Thus, unlike other innate immune cells, matured MCs are virtually undetectable in the circulatory system under steady-state situations [76]. Recent studies show transcription factor GATA binding protein 2 (GATA2) and Microphthalmia-associated transcription factor (MITF) to be critical for the differentiation of MC progenitor cells into the MC lineage [77, 78]. Mast cell progenitors express alpha4beta1 and alpha4beta7 integrins which bind to VCAM1 on the endothelial line, a process that is needed for the diapedesis and subsequent recruitment into the lung tissue [79]. Earlier it was believed that stem cell factor (SCF)-KIT signalling is indispensable for MC maturation, but recently it was shown that human progenitors are able to survive and proliferate without SCF-KIT signalling [80]. Hence, although SCF seems important for the development and maturation of MCs, other mediators may also contribute. Human interleukins such as SCF, IL-3, IL-4 and IL-6 have effects on the proliferation and SCF, and IL-4 facilitate the MC maturation under certain conditions [60].

Human recombinant SCF induces the development of MCs from their progenitor form (CD34+KIT+) [81]. With the administration of recombinant SCF to humans, the number of MCs increases significantly at the injection sites [82]. Human stromal cells secrete two forms of SCF: membrane-bound and soluble. Membrane-bound

SCF regulates MC adhesion and homing for progenitors and matured cells, and later acts as an MC chemoattractant [83, 84]. Thus, SCF plays a central role in MC transmigration, for their local development into tissue-resident MCs and for their survival.

Mediators and Other Components Produced by Tissue Mast Cells

Human MCs store abundant functionally defined and clinically relevant constituents in their secretory granules. Among these are amines like histamine and serotonin (less abundant in humans), a large amount of lysosomal hydrolases, various cytokines and growth factors such as tumour necrosis factor (TNF) and vascular endothelial growth factor (VEGF). Other predominant constituents of the granules are proteases such as tryptases, chymase, carboxypeptidase A3 (CPA3) and proteoglycans of serglycin species. Also, mast cells are packed with prostaglandins, leukotriene C4, platelet-activating factor and plasminogen activator (tPA) [38, 60, 73]. Highly negatively charged heparin or chondroitin sulphate proteoglycans provides a base for the storage of positively charged bioactive monoamines and different mast cell-specific proteases. Notably, the high anionic properties of densely packed proteoglycans are responsible for the strong metachromatic staining properties of mast cells [85].

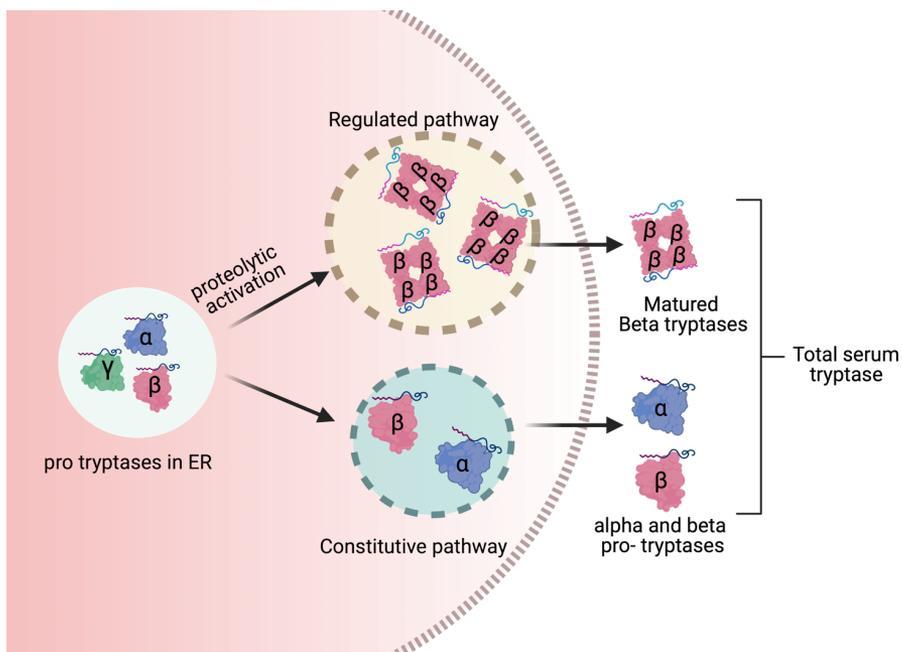


Figure 4: Maturation of pro-tryptases (alpha, beta and gamma) and their secretion pathways (ER= Endoplasmic reticulum). *Image by Premkumar Siddhuraj*

Tryptase, a serine protease, is the most abundant pre-stored enzyme (10-35 pg/cell) and accounts for around 25% of total protein in human mast cells [86]. Human tryptase genes (5 loci) are located on chromosome 16p13.3, TPSAB1 (alpha-tryptase and beta I-tryptase), TPSB2 (beta II and beta III-tryptase), TPSD1 (delta-tryptase) and TPSE1 (epsilon-tryptase). In humans, delta tryptase seems to be inactive and epsilon-tryptase is functionally very distinct from alpha and beta tryptases. Among all these tryptases, only TPSAB1 and TPSB2 encode the secreted isoforms of tryptase that are measurable and clinically relevant [87] (Figure 4). Proteolytic activation leads beta-monomers to assemble into a mature tetrameric form. Some portion of beta monomers are redirected from the tetrameric process and released as monomers along with alpha monomers. The monomeric gamma tryptase is found to be membrane-bound. The active sites of beta tetrameric tryptases face a narrow central pore that is virtually inaccessible to all endogenous protease inhibitors [88, 89].

In recent years, tryptase polymorphism is increasingly considered clinically relevant as direct correlations were found between tryptase gene copies and multi-organ failure, irritable bowel syndrome, cutaneous complaints, connective tissue abnormalities and dysautonomia [90]. In one study the alpha-tryptase deletion was reported in 30% of the study population [91] and even up to 57% of the UK population [92]. Another study revealed that in a healthy population, the serum tryptase was correlated with increased single allele TPSAB1 copy numbers (mainly alpha tryptase) [93].

Mast Cells in Tissue Homeostasis

MCs play critical roles in many physiological and pathological process. Given their close contact with blood vessels, lymphatics, mucosal (epithelium) and smooth muscles, it is not surprising that their constituents regulate blood flow, vascular permeability, gland secretion and smooth muscle contraction in many tissue sites [94]. MCs also regulate the key events in the wound healing process: initiating the inflammatory stage, induce the proliferation of connective cellular elements and remodelling of the newly formed connective tissue matrix [95]. During the proliferative stage after tissue damage, re-epithelialization and angiogenesis are key features and MCs promote revascularization through their angiogenic mediators. For instance, heparin stimulates the migration of endothelial cells to form new blood vessels [96], tryptase promotes the proliferation of microvascular endothelial cells [97], whereas chymase regulates angiogenesis through the angiotensin II pathway [98]. MCs induce proliferation of epithelial cells and fibroblasts through their mediators such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF). As a result of fibroblast proliferation, collagen and other extracellular matrix proteins deposit into scar tissue [99].

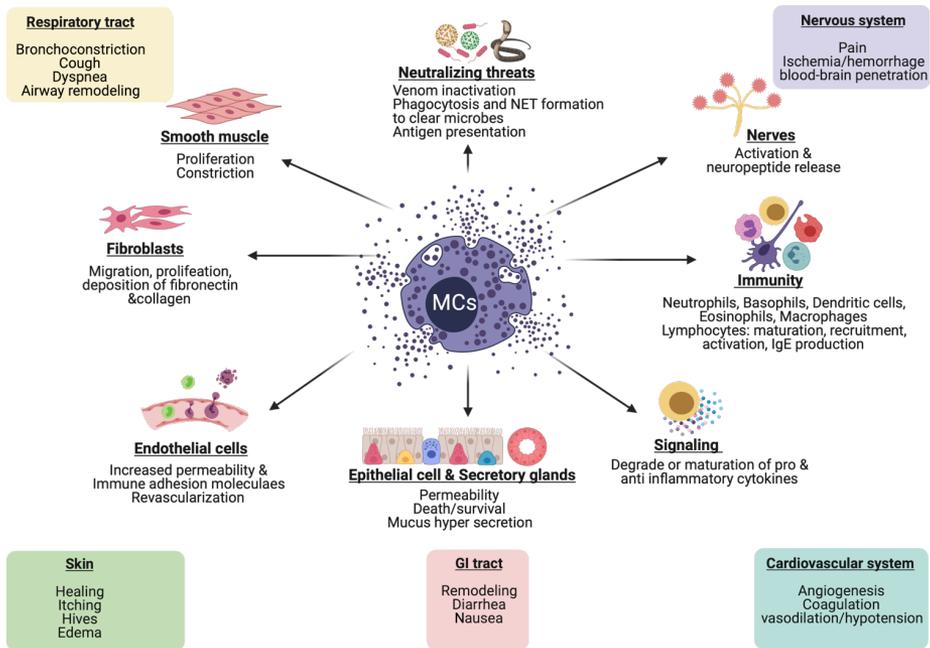


Figure 5: A plethora of human mast cell mediators and their contribution to homeostasis and diseases. *Image by Premkumar Siddhuraj*

Given the strategic locations of MCs in tissues, they are able to detect and quickly initiate a response against external insults. MCs surveil their environment using MRGPRX2 receptors [100], toll-like receptors, complement receptors and Fc receptors. Upon encountering a threat, they release a set of mediators which recruit neutrophils (to clear pathogens) and contribute to adaptive immunity by activating dendritic cells and T lymphocytes [60, 101].

The balance between the pro and anti-inflammatory mediators defines the MC role as protective (*e.g.*, counteracting bacterial infections, venom defence, kidney fibrosis and post-traumatic brain inflammation) or detrimental (*e.g.*, in colitis, asthma, burn injury, pulmonary fibrosis and arthritis). Some of the protective MC effects depend on TNF-alpha availability, which augments influx of phagocytes, leukocyte rolling and adhesion to the target site and efficiently clears the infection. Besides, chymase has been shown to play a critical role as an anti-inflammatory mediator, as it degrades various pro-inflammatory cytokines such as IL-6, IL-13, IL-33, CC-Chemokine, CCL3, CCL5 and alarmins such as IL33 [38, 60, 101].

MC Secretory Pathways and Selective Release

With mast cell granule components being important in a multitude of immunological and homeostatic processes, the mechanisms of degranulation and mediator release is under intense exploration. Recent studies show that particular conditions of stimulation can trigger a selective release of granule content: *i.e.*, there can be complete emptying of the so-called cargo or just a portion [102]. For instance, IgE- Fc receptor (FcεRI) mediated stimulation generally leads to a full response (anaphylactic degranulation with complete release) whereas Toll-like receptor 2 and 4 may induce differential release without complete degranulation. The strength of the stimulus influences the release characteristics. For example, a weak IgE based stimulation favours chemokines over cytokine release [103]. It has been reported that compared to FcεRI mediated response, MRGPRX2 activation releases more tryptase and less monoamines. This indicates that MRGPRX2 mediated activation excites non-histaminergic itch sensory neurons [104]. In addition to the G protein coupled receptors, the MCs can be activated via complement receptors, TLRs, cytokine receptors and many more. Depending upon the mode of activation, MCs selectively or preferentially release through different secretion modes (Figure 5).

Recent evidence indicates a selective release of proteases as lung mechanical stretch induces MCs to release only tryptase but not chymase, which leads to the MCc (only chymase and no tryptase) phenotype [105]. Apart from MC release of a variety of important mediators through regulated exocytosis (degranulation), other pathways depend on *de novo* production including vesicle trafficking, exosomal and endosomal pathways and constitutive secretion. Regulated exocytosis can be the result of changes in the extracellular environment by for instance temperature, pH, radiation, osmolarity or ligation of a surface receptor. It is possible that constitutive exocytosis occurs even without particular stimuli and this may happen throughout the lifetime of a cell. MCs can initiate the rapid release of pre-stored granule contents in their cytoplasm (amines and proteases) by anaphylactic or piecemeal degranulation. Pro-granules and lysosomal proteins are released through exosome secretory pathways.

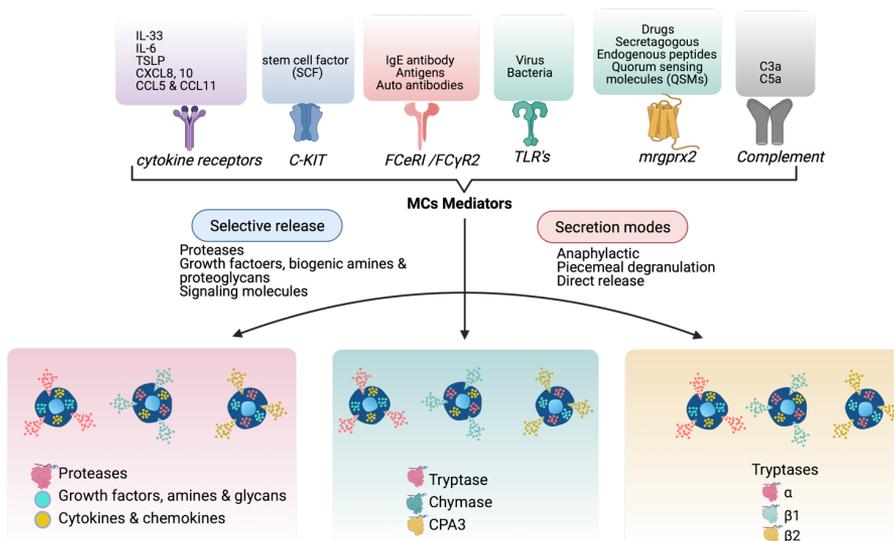


Figure 5: A schematic representation of different modes of mast cell activation and secretion of their mediators. Image by Premkumar Siddhuraj

De novo synthesized lipid bodies (prostaglandins and leukotrienes) are released through active transporters and various chemokines and cytokines are released through constitutive exocytosis [106, 107]. Importantly, there is a growing number of evidences suggesting that proteases (*e.g.*, tryptases) are also de novo synthesized and secreted spontaneously [87, 90, 93, 108]. However, the exact mechanism of this type of release is yet to be revealed.

Human Lung Microenvironment Influence on MC Heterogeneity and Plasticity

The local tissue milieu determines the MC maturation and their versatile response. As a result, MCs from various tissue sites display marked differences in sensitivity to various stimuli, the composition of their mediators (amines, proteases, chemokines and cytokines) and tissue-specific receptor expression profiles. It is well known that MCs release their granules differentially or selectively based on the type and strength of the stimuli (immunological and non-immunological) [109, 110].

MC heterogeneity is further compounded by the fact that, unlike other innate immune cells, they are long lived cells and capable of re-granulating and refilling

with new mediators. It is plausible that the composition of MC granules highly depends on the degree of local tissue antigenicity and local tissue milieu, (Figure 8). In any case, as evidenced by many studies, human pulmonary MC granule composition and receptor expression (proteases, IgE receptor, MRGPRX2, growth factors, lipid mediators and cytokines) display a remarkable variation based on their anatomical locations (*e.g.*, central and small airways, parenchyma and vessels) and type and severity of disease [37, 111-115].

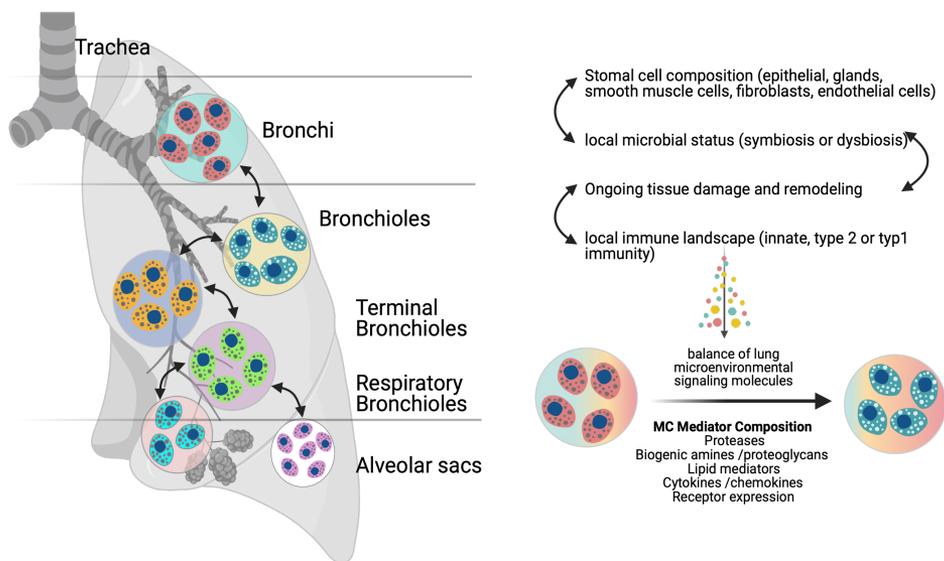


Figure 8: An over-simplified drawing depicts how the lung local tissue milieu in different compartments may influence the mast cell plasticity. *Image by Premkumar Siddhuraj*

Lennart Enerbäck, was first to classify rodent MCs as mucosal MCs (MMS) and connective tissue MCs (CTMS) based on their locations, size, dye-binding and metachromatic properties [116]. Subsequently, numerous studies have revealed the rodent MC heterogeneity based on their functional and morphometric aspects [76]. On the other hand, human MCs are classically characterized based on their neutral protease composition with MCT positive only for tryptases and MCtc mast cells positive for tryptase, chymase and carboxypeptidase A3 (CPA3) [117]. The MCT type is mostly found in the mucosal lining. Attempts have been made to relate human MCT with mouse MMC and human MCtc with murine CTMC in respiratory and intestinal locations. However, this correlation does not seem to be strict since extracutaneous tissues contain both the MCT and MCtc types [60]. In addition to MCT and MCtc, human MCs without tryptase but positive for only chymase (*i.e.*, MCC) has also been reported [113].

Under certain pathophysiological conditions, human MCs display a unique protease composition, e.g., MCs positive for tryptase and CPA3 but not chymase [114]. It has been shown that the MC phenotype can be altered by various stimuli. For example, when human umbilical cord blood-derived MCs are cocultured with human airway epithelial cells (HAEC's), they changed from MCtc to a MCT phenotype [118]. Conversely, when the connective tissue MCs (CTMC) were injected into the mouse stomach mucosa they converted into a mucosal mast cells (MMC) phenotype [119]. In addition, the conditioned medium from human mastocytosis cell strain can induce both bone marrow-derived and umbilical cord blood-derived MCs expressing only chymase (MCc) but not tryptase [120]. Although multiple studies have described the plasticity of cultured human MCs and MCs in animal models, this plastic capacity has yet to be proven for matured tissue-resident human MCs.

Mast Cell and Effector Cell Interactions in Pulmonary Diseases

Mast cells are recognized as quintessential elements in pulmonary immune responses because of their strategic positioning in different lung anatomical sites and their ability to release a plethora of inflammatory constituents upon various immunological and non-immunological responses. Decades of work have showed MC mediators in complex interactions with several types of pulmonary effector cells including epithelial cells, innate immune cells (eosinophils, macrophages and neutrophils), blood vessels, nerves, smooth muscle cells, and mucus-producing glands [74]. However, most of these interactions have been revealed in animal or in *in vitro* test systems. Hence, which of the many potential roles of mast cell mediators that are actually operating in diseased human tissues of real-life patients remain largely unknown. To explore this further it seems important to define the mast cell phenotypes and their activation status in relation to the distinct anatomical structures and spatial regions affected by disease.

Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterised by persistent airflow limitation and is related to a chronic inflammatory response in airways and lungs to toxic particles or gases. The chronic inflammation results from long term exposure to inhaled irritants and can lead to bronchitis, bronchiolitis and emphysema.

Symptoms

The most important symptoms of chronic obstructive pulmonary disease are breathlessness on exertion and chronic cough with or without phlegm. Fatigue, anorexia and weight loss can arise as the disease progresses [121].

Risk Factors

Although COPD is generally associated with smoking tobacco, increasing pieces of evidence indicate non-smokers may account for between one fourth and one-third of all COPD cases [122]. The risk factors for non-smoking COPD include occupational airborne exposure, outdoor and indoor pollution, socioeconomic status, early-life environmental factors and genetic factors. The prevalence of non-smoking COPD patients varies across nations and the pathological features and underlying molecular mechanisms of the syndrome are very poorly known [121, 123].

Clinical Features

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) graded COPD into four stages of severity based on spirometry measurements.

- *Mild (stage I)- FEV1/FVC <0.70 and FEV1 >80% predicted,*
- *Moderate (stage II)- FEV1/FVC <0.70 and 50% ≤ FEV1 <80% predicted,*
- *Severe (stage III)- FEV1/FVC <0.70 and 30% ≤ FEV1 <50% predicted,*
- *Very severe (stage IV)- FEV1/FVC <0.70 and FEV1 <30% predicted [121].*

Fletcher and Peto's model demonstrated that accelerated smoking-associated decline of lung function starts from 25 years of age [124]. Measuring lung function starting from age 25 was proven to be inefficient since the study showed that only half of the study cohort followed Fletcher and Peto model and the other half never displayed a normal lung function peak at age 25 [125]. Early loss of lung function is associated with a number of comorbidities and is one of the critical risk factors for development of COPD [126, 127].

Pathological Features

The severity of airflow limitation in COPD is correlated with tissue infiltration of neutrophils, macrophages and lymphocytes. Development of an adaptive immune response in severe COPD is indicated by the presence of tertiary lymphoid organs. It is well established in the scientific literature that in COPD the small conducting airways are less than 2 mm in diameter due to airway wall remodelling of epithelium, lamina propria, smooth muscle, and adventitia [126]. Using micro-CT authors have shown that when compared with smoking controls the number of terminal bronchioles decreased 40% already in mild (GOLD 1) COPD, transitional bronchioles decreased by 56% and alveolar surface area decreased by 33%. The

remaining small airways presented with thickened walls and narrowed lumens [128]. Along with airway and alveolar destruction, pulmonary vascular inflammation and endothelial dysfunction have also been shown in milder COPD patients [129].

“Loss of nearly half of the terminal bronchioles and alveolar structures already at GOLD 1&2 COPD raises the argument that, are we losing critical time to treat the patients by adhering to COPD GOLD standards? Perhaps additional efforts should be made for the identification of better biomarkers for early intervention of milder COPD patients.”

Following tissue injury, the repair process begins with activation of anticoagulation pathways before infiltration of neutrophils and macrophages to clear the external insults and damaged resident stromal cells. The terminal bronchioles and alveolar tissues from COPD patients present with increased infiltration of T and B-lymphocytes. This adaptive immune response against both self-antigens (autoimmunity) or foreign antigens (virus, bacteria and fungi) can contribute to the pathogenesis of COPD [126]. A cohort of COPD patients with emphysema had distinct B-cell transcriptomic signature and circulating antibodies against elastin and pulmonary epithelium and endothelium cells [130]. The subsequent arrival of fibroblasts, myofibroblasts, and endothelial precursor cells form a microvascular network to support the restoration of the epithelial surface. Repetitive injury due to smoking and other risk factors results in tissue repair that combines tissue destruction and scar formation [126].

The Heterogeneity of COPD

The results of multiple observations indicate ample heterogeneity in its clinical presentation and pathogenic mechanisms; hence, it is appropriate to consider COPD as a syndrome rather than a disease [131, 132]. For this reason, a precision medicine strategy is now proposed for airway diseases (including COPD), which is named label-free. This label-free approach is the opposite of the traditional diagnostic labelling of COPD, asthma, or bronchiectasis [133]. It is based on identifying the so-called treatable traits in individual patients through recognizing each patient's phenotype or endotypes (using biomarkers) [127]. For example, circulating eosinophils is now considered as a promising biomarker for inhaled corticosteroids-based treatments for COPD patients [134].

Mast Cell Infiltration in Different Lung Compartments of COPD Patients

Studies are accumulating on the characterization of MC phenotypes and decoding their spatial distribution in COPD lungs and their potential role in disease

pathogenesis. Although the role of MCs in COPD is poorly explored, some studies have examined MCs in lung anatomical sites of COPD patients. The total density of MCs in late-stage COPD lungs was significantly lower than healthy counterparts. Interestingly, in a study exploring surgical resections and explanted COPD lung samples the lung compartments (small airways, pulmonary vessels and alveoli) displayed a dramatic shift in MC subtypes compared to non-COPD controls. The MCt subtype was significantly lower in all the compartments of late-stage COPD than healthy. On the other hand, the MCtc subtype was significantly increased in small airways and alveoli of late-stage COPD compared to healthy controls. Authors have also shown that mast cells displayed increased expression of C5a (CD88) and TGF-beta. Besides, an increased infiltration of the MCtc subtype in smooth muscle bundles of distal airways was positively correlated with better lung functions in COPD patients [36]. Further, increased infiltration of the MCtc subtype in smooth muscle bundles of distal airways was positively correlated with better lung functions in COPD patients [135].

The MC infiltration level in bronchial mucosal compartments has also been compared between asymptomatic smokers and healthy individuals. This comparison revealed that the smoking group displayed a higher MC density in the epithelium, lamina propria and smooth muscle and this correlated with various lung function tests measuring hyperinflation (FRC, TLC, RV) [136]. The MC number was increased significantly in the sputum of active smokers compared to ex-smokers. In another study, the airways of active smokers displayed higher CXCL-10 expression, which is interesting since this chemokine has been implicated in MC migration to airway smooth muscle bundles [137]. The sputum tryptase level was 1.9 times higher in severe compared to mild COPD patients. In separate investigations, the tryptase enzyme activity correlated with the disease severity [138]. In addition, elevated histamine and tryptase levels have been observed in BAL fluid from smoking individuals as compared to BAL from non-smokers, indicating that MC mediators may be involved in alveolar destruction [139]. Taken together, there is ample data to suggest an MC involvement in the COPD and IPF pathogenesis. However, more data is needed to understand the magnitude and dynamics of the mast cell phenotypes, both in COPD and other inflammatory diseases like asthma and fibrotic lung disease.

Pulmonary Fibrotic Diseases

Cystic fibrosis

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes the CFTR membrane protein. It is an anion channel present in different epithelia and conducts chloride and bicarbonate. Mutations in CFTR are associated with altered fluid and electrolyte homeostasis of epithelial cells and leads to thick and viscose mucus that clogs airways.

Cystic fibrosis (CF) is the most prevalent autosomal recessive disease in the Caucasian population. It affects approximately 1/3500 births and globally about 70,000 children and adults are carrying this disease. Most patients show signs of symptoms already soon after birth and frequent respiratory infections and poor weight gain are the most common symptoms. CF is the most common reason for lung transplantation in early adult life. CF manifests as chronic infections with pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*) and excessive inflammation that leads to bronchiectasis and subsequent decline in lung function. In addition to the lung, complications can occur in almost every organ including liver, CF-associated diabetes, nasal polyps, intestinal obstruction and many more.

Activation of TLRs (Toll Like Receptors) promotes the epithelial cells to secrete abundant pro-inflammatory cytokines such as IL-8 which contributes to the epithelial and smooth muscle remodelling and increased smooth muscle contraction. At the sites of chronic infection, increased neutrophils and phagocytic macrophages are evident. Furthermore, defective cilia function, hypoxia and availability of free nutrients and decreased antimicrobials are hallmarks of CF [140, 141].

Idiopathic Pulmonary Fibrosis

Unlike cystic fibrosis (CF), the idiopathic pulmonary fibrosis (IPF) etiology is poorly known. IPF is a progressive and fatal disorder that targets mostly the elderly population and immune senescence may play a critical role.

The histological features of IPF are excessive deposition of cellular matrix proteins, presence of fibroblast foci and spatially heterogenous fibrosis. Frequent epithelial injury leading to chronic inflammation, exaggerated wound repair and tissue remodelling may contribute to the development of fibrosis. The basis of excessive innate and adaptive inflammatory responses in IPF is thought to be multi-factorial and transforming growth factor- β (TGF- β) is believed to play a central role.

Although increasing evidence points out that the neutrophils, macrophages, fibrocytes, monocytes, type 2 innate lymphoid cells (ILC2) and mast cells are the major driving forces behind the inflammation, the treatments interfering with inflammatory machinery (*e.g.*, steroids) have failed to contain IPF symptoms [142].

Mast Cells in Pulmonary Fibrosis

In pulmonary fibrotic diseases, increased infiltration of MCs correlate with the degree of severity of the disease [143]. The MCs found in fibroblast-rich connective tissue in IPF patients have been reported to contain unusually few granules. Furthermore, the BALF of IPF patients display elevated histamine, tryptase and other MC mediators [74, 144, 145]. This elevation in mediator release may be the reason for MCs with fewer granules. Other similar studies have also shown that the plasma tryptase level is elevated in IPF patients and that tryptase level is negatively correlated with lung function [105]. It has also been shown that in IPF lungs, MC density is significantly elevated in the fibrotic parenchymal region. In addition, MCs in fibrotic areas display markedly increased TGF-beta expression [146].

In IPF patients, myofibroblasts are key cells that play an intermediary role between fibroblasts and smooth muscle. Extensive literature indicates that mast cell mediators such as tryptase, fibroblast growth factor (FGF), chymase and TGF-beta have been implicated in advancing pulmonary fibrotic conditions. For example, tryptase from MCs has been shown to promote myofibroblast activation, migration and proliferation, and collagen synthesis through protease-activated receptor 2 (PAR-2) [147, 148]. Similarly, studies have shown that fibroblast growth factor (FGF) also plays a crucial role in excessive collagen deposition [145]. In CF patients, which apart from having ongoing infections also develop lung fibrosis, total MC density has been demonstrated to be decreased in small airways and pulmonary vessels, whereas the MC infiltration increased significantly in fibrotic and inflamed areas of the parenchymal region [146]. Notably, upon activation by IL-9, MCs in CF lungs produce IL-2, and this in turn may lead to the expansion of type 2 innate lymphoid cells and activation of inflammatory Th9 cells [149]. Of potential clinical importance, MC modulation may also be beneficial to CF patients in the fights against *Aspergillus* [150], *Candida* [151] and *P. aeruginosa* [152] infections. Taken together, there is ample of data to suggest an MC involvement in the COPD and IPF pathogenesis. However, more data is needed to understand the magnitude and dynamics of the mast cell phenotypes, both in COPD and other inflammatory diseases like asthma and fibrotic lung disease.

SARS- COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the coronavirus disease 2019. It is a rather heterogeneous disease. The disease course may vary from asymptomatic to mild, severe and to critical. Among symptomatic patients, 10-20% are at the risk of fatality with multi-organ failure. Swiftly after the COVID19 outbreak, it became clear that angiotensin-converting enzyme 2 (ACE2) is a vital entry point for the virus. Besides, recent studies indicate TMPRSS2 and neuropilin 1 also play crucial roles in viral entry. The signalling mechanisms of pattern recognition receptors (PRRs) and SARS-CoV-2 are not well understood. However, studies show TLR3, TLR7 and retinoic acid-inducible gene 1 (RIG-I) play a critical role in other SARS virus recognition events. Upon recognition by PPRs of viral substances, they evoke a strong type 1 IFN response [153]. A longitudinal study shows an early spike of type 1 IFN and temporal decline of IFN α and IFN λ in mild to moderate COVID-19, whereas levels rise in late severe stage of the disease [154].

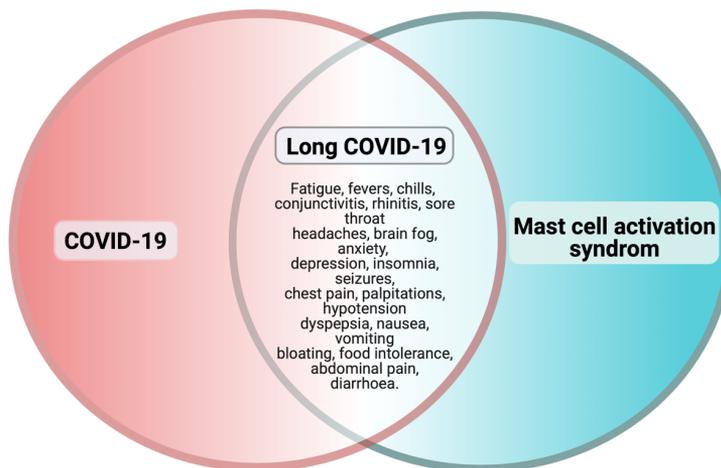


Figure 9: Overlap of symptoms between long COVID-19 patients and mast cell activation syndrome (MCAS).

Mounting evidence indicates as an evasion strategy SARS-CoV-2 antagonizes the INF system with clinical consequence [155]. SARS-CoV-2 enters type 2 pneumocytes and triggers the release of multiple proinflammatory cytokines (IL-1, IL-6 and CXCL8) and provoke NF- κ B-dependent proinflammatory pathways and

accumulation of neutrophils and macrophages [153, 154]. These events are prominent during the second week of the infection. Similar to INF, IL-6 showed a dynamic change in the concentration [154]. Elevated neutrophils and NK cell numbers, and less frequency of dendritic cells and T lymphocytes in sputum are features of severe patients [156]. The ratio of circulating neutrophils-to-lymphocytes is newly introduced as a biomarker for disease severity [157]. In addition, an increased number of NET (Neutrophil Extra cellular Traps) forming neutrophils [158] and impaired functions of plasmacytoid DCs (pDCs) are other hallmarks of severe COVID-19 [159]. Eotaxin-2 and eosinophils are found to increase in severe patients, in addition to type 2 cytokines IL-5 and IL-13 which increase from moderate to severe phases. Accumulating evidence suggests the COVID-19 disease course continues even after the homeostasis phase to potentially prolonged chronification of the disease (long COVID-19) [154]. An unknown fraction of patients suffers from long COVID-19, which is a heterogeneous chronic illness with symptoms including chronic fatigue and psychiatric disorders [160]. Since the long covid symptoms significantly overlap with mast cell activation syndrome (MCAS), there is a strong rationale to suspect that mast cells may play a critical role in long COVID 19 [161] (Figure 9).

Why Histology?

In recent years, the ability to map the immune landscapes in tumour microenvironments has proven to be a powerful way to understand and diagnose various cancers [162]. Recent mounting evidence points out that tissue microenvironments play a critical role for MC immune homeostasis, dendritic cell phenotypes, macrophage phenotypes and their plasticity in health and disease [37, 163, 164].

To understand or find out the ultimate truth of any given human disease, it is indispensable to freeze or stop the clock of ongoing immune reactions or signalling mechanisms in tissues by both spatial and temporal aspects. In the human lungs, both the structural cells and the immune cells are designed to react profoundly to any mechanical and chemical insults. Patients with respiratory diseases under mechanical ventilation often develop gross damages in lung structural cells as exemplified by epithelial transformations (*e.g.*, reduced surfactant levels), fibroblast proliferation, lung edema and initiation of immune-inflammatory machinery [165]. In addition, upon mechanical injury mast cells display a profoundly altered protease phenotype and degranulate various compositions of inflammatory mediators [105, 166]. Hence, mast cells lose their steady-state molecular mechanisms and their original phenotype identity. Immune cells isolated from human lung, skin and intestine have been used in many studies. Unfortunately, the effects of the isolation and enrichment procedures on their phenotypes and functions have not often been carefully analysed. For these reasons, we rely heavily on immunohistochemistry and in-situ hybridization techniques to capture the actual state of immune mechanisms directly in diseased human tissues and spatially decode their distribution and disclose their clinical relevance in pulmonary inflammatory diseases.

Primarily our goal is to perform an observational study using histology to draw clinically relevant conclusions and to discover biomarkers for airway inflammatory diseases. In search of a clinically relevant biomarker for any disease, it is crucial to minimize the artefacts during sample preparation. The FACS technique is a well-standardized and mainstream technique used to characterize cells with a wide range of markers. However, as the nature of the technique (FACS) is to isolate cells from the tissues, information regarding the spatial context is unattainable. We have also closely watched developments in spatial transcriptomics. The method allows measuring thousands of genes in tissue sections but the spatial resolution remains very poor.

In recent years, intense investigations are underway to develop a single-cell level spatially resolved analysis using the spatial transcriptomic method. For these reasons, we had to adapt and combine immunohistochemistry, in-situ hybridization and computational image analysis platforms to characterize the cells in a dynamic (like fluorescence-based FACS measurement) single-cell based measurement of target protein + mRNA, and notably without losing the critical spatial information with the tissue sections. In recent years the integration of computational image analysis platforms into immunohistochemistry workflows was a breakthrough in the high-throughput qualitative and quantitative measuring aspects of multiple parameters in tissue sections.

In our improvised workflow (Refer Figure 10) the following steps are involved: sample collection, sample processing, staining markers of interest, image analysis and data interpretations. Upon collection of the patient lung tissue the tissue was immediately transferred into formalin and fixed for an optimal time period. A similar fixation time was strictly maintained across all samples in order to quantify target protein and mRNA levels across different samples. Formalin forms additive compounds and cross-links with proteins without precipitation. The cross-linking of formalin with specimen protein molecules often leads to a significant change in three-dimensional structures of proteins which can hinder the binding of antibodies to the target protein. However, this change can be reversed by heat or enzymatic based antigen retrieval protocols. The major advantages of formalin are that the penetration into tissue specimen is rapid and it is economically viable. This rapid penetration of formalin in tissue prevents the unwanted changes in patient samples during processing which is very critical to draw clinically relevant conclusions.

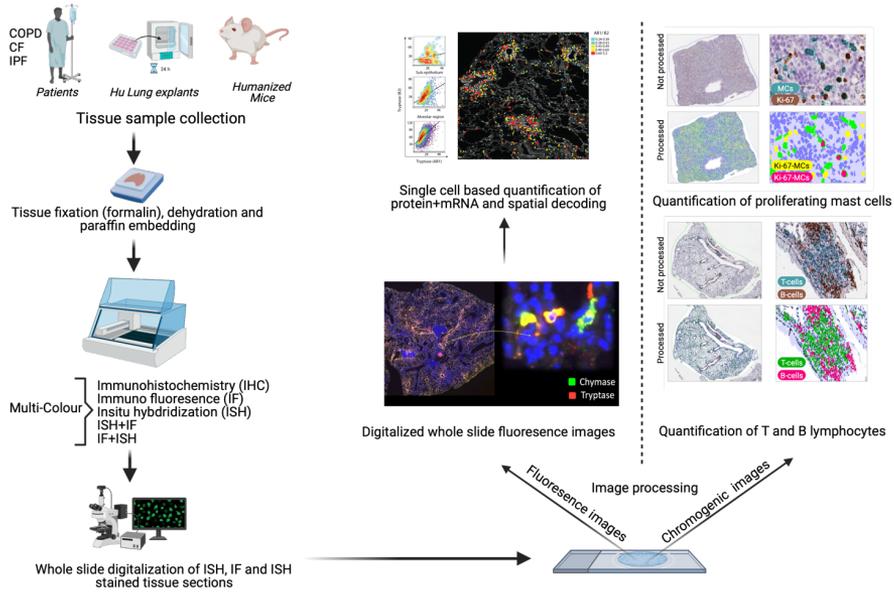


Figure 10: An improvised workflow for the histology based single cell analysis.

To visualize and quantify the target protein and mRNA in the tissue sections we have adapted multi-colour immunohistochemistry (IHC; chromogen based), immunofluorescence (IF) and in-situ hybridization (ISH; chromogen + fluorescence based). To quantify the ratio between protein and mRNA at a single-cell level, we have combined IF+ISH methods. As we have heavily relied on computer-based platforms to quantify a large number of tissue samples, it is crucial to consider the quality of the applied antibodies. We evaluated the antibody quality by finding out the cellular sources for the target proteins and visually confirming the staining pattern, for instance, and then omitting the antibody in case the antibody recognizes the non-immune targets. Please see the manuscripts for the detailed protocol descriptions.

Aim

The general aim of this thesis is to study the immune contribution to airway inflammatory diseases such as COPD (Chronic Obstructive Pulmonary Disease), IPF (Idiopathic Pulmonary Fibrosis) and CF (Cystic Fibrosis) and in addition, to discover histology-based biomarkers for early intervention of the above-mentioned diseases.

The study goals in this thesis stem from our believe that to draw clinically relevant conclusions and discover disease-relevant biomarkers for any airway disease, it is vital to understand the following nature of the immune system in health and disease:

- What are the phenotypes of tissue-resident immune cells in healthy and diseased lungs?
- What is the magnitude of plasticity among the tissue-resident immune cell phenotypes?
- What is the composition of immune and non-immunological factors and their spatial context in the health and diseased lung microenvironments?

Hence, to address the above-mentioned questions, our objective was also to develop a high throughput histology-based single-cell level quantitative workflow.

The biological questions that are addressed in the thesis are focused around

- The true nature and dynamics of mast cell protease phenotypes in diseased lung tissues (papers 1 and 2)
- The tissue infiltration patterns of eosinophils, basophils and markers of type 2 immunity in COPD (paper 3)
- The formation of spontaneous and immune stimulation-induced complex human immune cell patterns in a cutting-edge humanized immune system mouse (paper 4).

Synopsis of the Original Work

Paper 1

Lung Mast Cells Have a High Constitutive Expression of Carboxypeptidase A3 mRNA That Is Independent from Granule-Stored CPA3

Background and study rationale:

The mast cell metalloprotease CPA3 is proposed to have critical role in the lung homeostasis and traditionally, in a steady-state, the granule storage of CPA3 is linked with mast cell subpopulation MCtc. However, our knowledge is very limited in relation to the magnitude of baseline expression of CPA3 by human tissue mast cells subtypes.

Aim:

To investigate the true nature of baseline expression of carboxypeptidase A3 protein and mRNA by mast cell phenotypes (MCt and MCtc) in non-inflamed human lung tissues.

Results:

Granule stored CPA3 protein content was predominantly linked with MCtc population:

For this study we applied a novel histology based single-cell quantitative analysis of CPA3 protein and mRNA content in tissue residing mast cells in human lung, gut and skin. Our approach demonstrated that, during baseline conditions, granule-stored CPA3 protein content was primarily restricted to the MCtc population in lung MCs. Traditionally, CPA3 protein has been linked with MCtc population. Hence, our finding aligned with the previous reports by others. However, our novel and sensitive technique further revealed that the granule storage level of CPA3 protein in MCtc cells displayed a remarkable discrepancy among organs. Skin MCs had a dramatically high CPA3 protein intensity compared to lung and gut tissues. In addition, we also observed rare MCs with unique protease combinations; CHYM-negative, CPA3-positived & CHYM-positive, and CPA3-negative. These latter combinations may reflect atypical but potentially important MC phenotypes.

CPA3 mRNA expression was not restricted to any distinct MCs subpopulations:

Next, we adopted a combined ISH-IF approach for single cell quantification of CPA3 mRNA expression among MCt and MCtc subpopulations in lung, gut and skin. Our approach revealed that CPA3 mRNA expression was, surprisingly, not restricted foremost to the MCtc population, as was the case for CPA3 protein. Rather, the highest CPA3 level was found within the MCt population in the lung. Intriguingly, this inverse correlation between CPA3 mRNA and protein storage in the granules was evident in lung, gut and skin MCs.

Next, we sought to determine, if MCt and MCtc populations differ in terms of CPA3 mRNA expression profile between lung anatomical sites (small airways, pulmonary vessels and alveolar region). Both MCt and MCtc cells in all the lung anatomical sites displayed roughly equal level of CPA3 mRNA expression. Strikingly, the highest CPA3 mRNA intensity was found in alveolar MCt cells, notably, meanwhile, granule stored CPA3 protein was virtually absent in these cells. In addition, our findings based on in-situ hybridization and GTEX data analysis indicates the human lung MCs presented with the highest level of discrepancy between CMA1 and CPA3 mRNA expression.

Discussion & Future Perspective

The present detailed histology-based single-cell mapping uncovers a series of novel observations in relation to CPA3 expression profile in human MCs subpopulations. Among these, the markedly disparate patterns of stored CPA3 granule protein and mRNA production in the lung stands out as seemingly surprising. CPA3 is a zinc-containing exopeptidase of the metalloproteinase family and traditionally associated with MCtc subtype of MCs [38, 60]. Previous studies have shown that CPA3 and chymase share a same storage mechanism and they are co-dependent for granule storage, for example, deletion of mMCP-5/CMA1 leads to loss of mCPA3 [167]. However, this association is not entirely strict with human lung MCs, for instance, the epithelium of steroid-resistant asthma patients displayed MCt type with high CPA3 mRNA [168].

Importantly, the presently revealed high baseline CPA3 mRNA expression in lung MCs lacking granule CPA3 may indicate a spontaneous release of this protease, similar to what has been shown possible for alpha tryptase [169]. A baseline production of CPA3 could have an impact on the lung and the systemic circulation in several ways. One effect could be related to the complex relationship between endothelin-1 and mast cells [170], where mast cells have been shown to have important protecting roles by limiting a toxic effect of endothelin-1 [171], possibly through CPA3-mediated endothelin-1 inactivation. As further support for a protective role, decreased serum levels of CPA3 are associated with risk factors of blood vessel disease and cardiovascular damage [172]. Although more research is needed, the proteolytical cleavage of endothelin-1 and other known CPA3 targets, such as neurotensin, angiotensin-1 and apolipoprotein B, further implies potentially

broad roles of CPA3 in vascular homeostasis [173]. Our observations of lung MCt cells with no apparent granule CPA3 protein despite very high CPA3 mRNA levels may reflect a hitherto underestimated baseline of CPA3 production by mucosal MCt cells.

In comparison with other MCs proteases (tryptase and chymase), there is to date very limited insight into the substrate cleavage profile of CPA3. In any case, considering the high turnover of baseline CPA3 mRNA in lungs, future studies investigating the complex dynamics of CPA3 in health and disease seem highly warranted.

Paper 2

Environmental-Induced Mast Cell Plasticity Creates Infinite Tryptase and Chymase Profiles and Altered Protease Phenotypes in Chronic Lung Diseases and COVID-19

Background and study rationale:

Traditionally, the human mast cells are characterized based on their granule stored protease content, MCt (only tryptase) and MCtc (tryptase+ chymase + CPA3), this division is largely based on "binary" classification. Although MCs are known for their tissue-specific "tunable" characteristics, still very little is known in relation to the true dynamics of protease expression in tissue-resident human MCs in health and disease.

Aim:

To explore human lung microenvironments influence on mast cell protease phenotypes and their plasticity in health and airway diseases such as COPD, pulmonary fibrosis (IPF and CF) and COVID-19.

Results:

MCs display tissue site-specific gradient storage pattern of tryptase and chymase which results in a lack of distinct MCt and MCtc phenotype clusters:

To explore MCt and MCtc MC clusters in human lungs, we have developed histology based high-throughput workflow to measure the intensity of MCs proteases per cell basis in the tissue sections. Strikingly, the lung MCs displayed a non-clustered gradient storage pattern of chymase, hence no distinct MCtc cluster was observed in tissue sections. In addition to our histology approach, the fluorescence-activated cell sorting (FACS) technique also corroborated a similar non-clustered gradient storage pattern of chymase in MCs (isolated from human lung tissues). Surprisingly, tryptases also displayed greater variation in granule storage intensity, similar to chymase.

Next, we have explored to what degree the lung anatomical sites influence the MCs protease expression profiles. Our approach enabled for the first time to pinpoint the MCs in their exact tissue location and characterize based on their protease expression. A combination of spatial data and single-cell protease profiling enabled us to observe for the first time, a substantial site-specific alteration in chymase and tryptases (AB1 and B2) profiles in the bronchial (epithelial, sub-epithelial, glands and smooth muscle) and distal (small airways, pulmonary vessels and alveolar) lung tissue. Tissue site-specific highly pronounced difference in the ratio of AB1/B2 tryptases was also evident. For example, a significant portion of MCs in small airways presented with the very low-intensity AB1 tryptase.

Site-specific alteration of MCs protease (protein and mRNA) profiles in COPD, pulmonary fibrosis and COVID-19:

The impact of disease-induced tissue environmental alterations on MCs protease patterns was examined by spatial analysis. Tissue area-selective regulation of tryptases (AB1 & B2) and chymase was observed in all disease conditions (COPD, IPF, CF and COVID19). In comparison with non-inflamed healthy lung tissues, the granule-stored chymase protein level was elevated in COPD, fibrotic diseases (IPF&CF) and COVID-19. However, our combined ISH-IF technique revealed that chymase mRNA remains unchanged from health to diseases condition.

Next, we explored the ratio of AB1/B2 tryptases in health and diseases. Notably, site-specific variation in this ratio was evident, for example small airways displayed a significant increase of the AB1/B2 tryptase ratio from health to diseases (COPD, IPF and CF), but no such change was found in distal lung regions (alveolar region). Besides, AB1/B2 tryptases ratio was clearly influenced by diseased microenvironments especially at the sites of fibrotic lesions. Interestingly, selective upregulation of AB1 tryptase was observed in lung tissues from fatal COVID19 patients. Tryptase mRNA was significantly elevated in lung fibrotic and fatal COVID-19 patients. Intriguingly, similarly as was reported for CPA3 in paper the reversed pattern of tryptase protein and mRNA was observed in diseases lung tissues; *i.e.*, protein and mRNA were inversely correlated.

Preferential release of MCs proteases in human lung ex-vivo and humanized mice settings:

In the human lung explant model, upon stimulation by compound 48-80 and stem cell factor (SCF), MCs selectively released tryptases (AB1 & B2) and retained the chymase and displayed MCc phenotypes. This MCc phenotype was also observed in humanized mice lungs. In addition, human MCs selectively upregulated AB1 tryptase when humanized mice were challenged intranasally with LPS and Poly I:C, *i.e.*, B2 tryptase and chymase level remained stable.

Discussion & Future Perspective

Based on the granule-stored protease content, MCs are typically classified into MCT (tryptase) and MCTc (tryptase + chymase), whereas a third phenotype has also been suggested; MCc (only chymase+) cells lacking tryptase. Through an unprecedented quantitative mapping of tissue mast cell proteases, our finding of a lack of chymase-high and chymase-low MCs clusters, this study questions the concept of “stable” and differentiated MCT and MCTc phenotypes.

Another important aspect of the present study is that it provides novel insights into the differential/preferential regulation of proteases. In addition, our data suggest that local environmental cues affect individual mast cells to constantly and gradually alter their granule proteases in a differentiated manner. This was for example evident from the change of ratio between AB1/B2 tryptases in different lung anatomical sites and different organs of humanized mice. Recent studies show that MCs either completely empty their “cargo” or just some portion based on the type of stimulant [102]. For instance, compared to classical FcεRI-mediated response, MRGPRX2 activation releases more tryptase and less monoamines. [104]. In addition, recent evidence further indicating a selective release of proteases, is an observation that lung mechanical stretch induces MCs to release only tryptase but not chymase which leads to MCc phenotype [105]. We have here shown the emergence of a similar MCc phenotype in our human lung ex-vivo model upon stimulation by compound 48-80 and stem cell factor (SCF). Speculatively, this MCc phenotype may result of activation of MRGPRX2 signalling mechanism. The MRGPRX2 signalling mechanism may contribute to the increased serum tryptase in a wide range of chronic lung illness and future studies into the pharmacological manipulation of MRGPRX2 signalling mechanisms seem highly warranted.

One of the important findings in this thesis is that the mRNA expression and actual granule protein content may be strikingly different, or in many cases even reversed. This phenomenon must be now be taken into an account when the tissue MCs are judged based on their granule stored proteases, otherwise the drawn conclusion can be very ambiguous. The tryptase mRNA level in our studies varied greatly among individuals. This could be perhaps partly be explained by the occurrence of tryptase gene haploids [93]. Besides, the degree of tryptase mRNA expression differed markedly within diseased lung microenvironments, especially the upregulation was more pronounced in the fibrotic areas in COPD, IPF, CF and COVID-19 lungs. The molecular mechanisms by which tryptase may causes fibrosis remains unknown but may include the capacity of tryptase to expand and activate lung fibroblasts via enzymatic cleavage of PAR-2 [148]. The inverse correlation of tryptase mRNA and protein indicates the possibility of a significant spontaneous, and potentially direct, release of tryptases into the microenvironments. Although the mechanism of spontaneous release of MCs proteases is very poorly studied, it has been reported that alpha tryptase can be directly released without granule storage [108]. The MCs in fibrotic diseases and COVID-19 presented with a significant increase in tryptase

mRNA but only containing few tryptase granules may contribute to the elevated serum and BALF tryptase level in these conditions. It has been shown that plasma tryptase level is elevated in IPF patients and that tryptase level is negatively correlated with lung functions [105] and in COVID-19 patients [174].

In summary, our reports on how the fine-tuned regulation of MCs proteases by the tissue microenvironments, the preferential/selective protease release, indication of spontaneous secretion, and phenotypic plasticity, together radically change the view on mast cell heterogeneity. These new insights have, apart from broad bearings to the mast cell biology field, also important implications for more applied approaches of pharmacological mast cell regulation.

Paper 3

Eosinophils, basophils and type 2 immune microenvironments in COPD-affected lung tissue

Background and study rationale:

Eosinophils are proven to be an efficient biomarker for type 2 associated inflammatory diseases. Although several studies show elevated blood and sputum eosinophils in a portion of COPD patients, we know very little in relation to what extent these cells infiltrate into the different lung anatomical sites.

Aim:

To spatially decode tissue infiltrating basophils, eosinophils and markers of type 2 immune-associated tissue microenvironments in COPD affected lungs.

Results:

The conducting mucosa of most of the COPD patients presented with scattered eosinophils and basophils, however, only a subset of the patients displayed a pronounced infiltration of these cells and a significant increase in density of eosinophils and basophils were observed only at advanced-stage COPD (GOLD 4). Notably, in controls and mild diseased patients, bronchial epithelium virtually lacked basophils but a marked infiltration was evident in COPD (GOLD 4). Similarly, the type 2 surrogate marker GATA3 were increased on advanced disease.

In distal lung compartments, the COPD GOLD 4 patients displayed a significant increase in infiltration of basophils, eosinophils compared to controls and patients with mild disease. The high eosinophil counts were associated with GATA3⁺ rich ectopic lymphoid follicles. Tissue eosinophilia and basophil infiltration were spatially linked to confined microenvironments within both the conducting mucosa and distal lung compartments. Both lung compartments displayed a highly patchy distribution of eosinophils where the eosinophilic clusters were spatially

accompanied by basophils and GATA+ positive cells. These “type 2 immunity pockets” of basophils, eosinophils and GATA3+ clusters were significantly increased in COPD (GOLD 4).

In addition, we demonstrated the presence of ILC 2 cells (*i.e.*, type 2 innate lymphoid cells) within eosinophil foci. To comprehend this patchy eosinophilic phenomenon in COPD GOLD 4 patients, we also explored a mouse model of influenza virus-induced exacerbation. Upon influenza infection the mouse lungs displayed patchy eosinophilia which was IL33 dependent since IL-33^{-/-} (or ST2^{-/} mice) did not mount a virus-induced eosinophilia. These data suggest that viral-induced eosinophilia may be regulated through the IL-33/ST2 axis, a phenomenon that now need to be confirmed in humans. Further, upon viral infection the mice elicited a strong localized CCL11 (eosinophilic chemoattractant) expression that was spatially linked to infected areas, and these viral infected areas were rich in eosinophils.

Discussion & Future Perspective

This study reveals several new aspects of eosinophils, basophils and Th2 immunity in non-allergic COPD. Earlier studies have reported eosinophilia to be a prominent feature in a subset of COPD patients [175] and treatments targeting eosinophilia are currently tested for COPD. In the present study, a statistical increase in eosinophilia was detected only in very severe GOLD 4 COPD, this may due to all severe patients quit smoking whereas roughly 40% of milder patients are current smokers and smoke exposure can counteract type 2 responses and contribute to lower eosinophilia. In addition, non-allergic eosinophilic asthmatic patients display steroid resistant phenotype which further supports the elevated eosinophil and basophil numbers in GOLD 4 COPD patients who are under a high dose of steroids. In any case, the primary aim of this study was never to show that COPD is characterized by eosinophilia. Rather, the goal was to investigate the nature and pattern of eosinophilia in those patients that do have a eosinophil and type 2 signature. Thus, a critical observation was that the spatial distribution of tissue eosinophils displayed distinct patchiness at a both a larger anatomical as well as microenvironmental level. This means that the underlying immunological responses manifesting eosinophilia are probably also highly localized. This was backed by our observation of a clear spatial relationship between eosinophils and potentially type 2 cytokine secreting cells such as GATA3+ ILC 2 and Th2 cells and basophils. Our exploratory mouse study further revealed one potential underlying mechanism of patchy eosinophilia, since upon influenza infection, these mice displayed a robust IL-33 dependent localized eotaxin response which strongly associated with patchy eosinophilia. To what extent this translates to virus-induced exacerbation in COPD patients remain to be investigated. In any case, it is known that viral infections are one of the common cause of exacerbations in COPD patients. Hence, one may propose patchy localized infections as one possible trigger of the present novel type of patchy type 2, eosinophil-rich pockets. Indeed, our data suggest that patchy

eosinophilia is more common in GOLD 4 COPD, severe COPD patients are prone to infections compared with earlier stages [176]. Our systematic mapping of tissue-infiltrating basophils and their strong spatial correlation with eosinophils is another critical finding in this study. Basophils are one of the potent type 2 immune modulators that, upon stimulation by IL-33 and thymic stromal lymphopoietin (TSLP), can release IL-4, IL-5 and IL-13, histamine and other proinflammatory cytokines to their microenvironments, events which may aid in the recruitment of eosinophils to combat the infection [27]. Dysregulation of type 2 immune response can be an important driver of a wide range of inflammatory diseases. Hence, in the future, spatially decoding these type 2 associated microenvironments in relation to infiltration of basophils, ILC's, eosinophils and in addition, mast cells and fibroblasts in severe asthma, inflammatory bowel syndrome (IBS) is highly warranted.

In summary, this study identifies basophils as having a potential role in COPD and demonstrates that tissue eosinophilia in COPD is anatomically widespread but commonly confined to distinct Th2-skewed and ILC2-containing microenvironments. This feature of tissue eosinophilia is likely to have clinical implications. Furthermore, our data suggest respiratory infections as a potential trigger of patchy eosinophilia in COPD.

Paper 4

Development of Highly Organized Human Leukocyte Infiltration Patterns and Human Lymphoid Tissue in Lungs of Humanized Human Immune System (HIS) Mice

Background and study rationale:

Humanized HIS mice have recently evolved as a powerful tool for mechanistic studies of human immune cells in vivo. Although there has been a rapid progress in optimizing engrafting conditions, basic questions remain regarding what constellations of human immune cells are established in major target tissues.

Aim:

To characterize the tissue infiltrating human immune cells in the humanized mice (NSG-SMG3) and spatially map their infiltration pattern in different organs.

Results:

Following the irradiation and the engraftment of human hematopoietic stem cells into humanized mice (NSG-SGM3), the mice organs (spleen, small intestine, lung, liver, heart and colon) were harvested at three different time points (week 8, 11 and

16). We have examined the overall engraftment levels of human leukocytes and their complex spatial distribution in NSG-SGM3 mice organs.

Among the sub aims was to evaluate the spatial and temporal dynamics of human myeloid (mast cells, basophils, eosinophils, basophils and macrophages) and lymphoid leukocytes (plasma cells, B cells, T lymphocyte subsets) across the organs. Organs such as lung, liver and spleen accounted for most of the human leukocyte infiltration and displayed substantial temporal dynamics. For instance, in lung tissue, a striking reduction in basophils and neutrophil cell density from week 8 to 16 was observed, whereas mast cells, macrophages, T and B lymphocytes density was increased from week 8 to 16. Further, we evaluated T cell subsets such as Th1, Th2, Tregs and GATA3 positive CD8 (“cytotoxic”) T cells. Intriguingly, GATA3+CD8+ cells were very abundant in lungs and liver.

In addition, we have examined if human lymphocyte engraftment at week 16 in the lung and liver could form more advanced lymphoid structures. Our data showed that at week 16 the lungs and liver displayed lymphoid-like aggregates with proliferating T and B lymphocytes. In these aggregates an array of immunoglobulin (IgE, IgG, IgA and IgM)-positive plasma cells were also evident, suggesting a local Ig class shift.

Temporal and spontaneous progress of inflammatory conditions in the mice organs were examined by quantification of tissue collagen accumulation and human cytokines. Trichrome staining revealed the spontaneous occurrence of enhanced collagen-deposition that was significantly elevated from week 8 to 16 in the heart, liver and lungs. Besides, a multiplex Luminex assay used to measure 27 human cytokines in murine organs. From week 8 to week 16, most of the human inflammatory cytokine levels were elevated in both lung and spleen (eg. IL-1A, IL-1B, IL-2, IL-6, IL-8, IL-9, IL-10, IP-10, MPI-1B, TNF-a, VEGF and GM-CSF).

Discussion and Future Perspective:

Our study illustrates human macrophages and mast cells were repopulated to a higher degree in most of the mice organs, surprisingly the density of human MCs in mice alveolar region was greater than to their human counterparts. So, we believe this can be an added advantage to explore MCs role in lung interstitial fibrosis using this mice model, since natural wildtype mice lack alveolar MCs.

We have also report signs of graft versus host disease (GvHD) in this mice models, for example proliferating MCs (in steady state, human MCs do not proliferate in tissue) and evidence for severe macrophage activation syndrome (MAS). The macrophages were localized close proximity to the subset of activated fibrogenic cells and collagen deposition. This may indicate the human macrophages may one of the driving forces of spontaneously occurring fibrotic lesions in humanised murine organs. Further, the human neutrophils also exhibited signs of activation via the formation of extracellular traps (NET's) mainly in lungs and liver. In humans,

increased formation of neutrophil extracellular traps in patients with graft versus host disease was reported [177] [178]. In human lungs, basophils are very few in numbers but in mice lungs, we observed greater presence of human basophils accompanied with patchy human eosinophils. This may have clinical implications, since recent works suggests the association of basophils and eosinophil's role in type2 immunity and influence of their spatial context in lung functions of COPD patients [30].

The lymphocyte aggregates we observed at week 16 in lung and liver were occurred (spontaneously) and presented with proliferating T cells and B cells. We also characterized the T cell subsets in these aggregates, most of these aggregates were dominated with Th2 cells (CD4+GATA3+) and surprisingly, a substantial number of cytotoxic T cells exhibited GATA3 expression. The recent studies suggest that GATA3 expressing cytotoxic T cells (CD8+GATA+) are a major source for type 2 cytokines in *e.g.*, severe asthma patients [179]. Another striking finding was spontaneously induced immunoglobulin class switching mechanisms in plasma cells, we have found immunoglobulin bound plasma cell infiltration into the lymphoid aggregates. Together, elevated levels of pro-inflammatory mediators and increased collagen accumulation in lung and liver indicates a strong ongoing GvHD.

In a nutshell, humanized mice model (NSG-SGM3) can serve as a powerful platform to explore human immune mechanism, however the spontaneously occurring sub-clinical developments must be taken into account while setting up future humanized mice models for human diseases.

Concluding Remarks of this Thesis

The central theme of this thesis revolves around one fundamental question - Do the human lung microenvironments contribute to the distinct spatial organization of immune cells and site-specific plasticity among the immune cell populations?

The long story short, the answer is, yes! the human lung microenvironments regulate immune homing and determines the immune phenotype.

The findings from our novel histology based single-cell protease profiling of human mast cells questions the long-standing dogma of the existence of distinct mast cell phenotypes based on their protease content (MCt & MCtc).

Our findings indicate that the lung local tissue milieu such as,

- Central airways - epithelium, sub epithelium, glands, smooth muscle
- Distal airways- small airways, pulmonary vessels and alveolar regions,

tunes the MCs protease contents such as tryptase (AB1 and B2), chymase and CPA3 in a gradient pattern that is not compatible with the existence of distinct MCt or MCtc phenotypes.

We believe the above-mentioned different lung compartments act as a distinct “test tubes” with unique molecular milieu which not only fine-tune the selective expression of certain mast cell proteases but also dictates the selective or preferential release of proteases as well. Apart from the chymase, CPA3 protein content was also used to classify MCs into MCtc phenotypes and it was believed that in a steady state MCt does not express CPA3. We have shown that both MCt and MCtc express an equal level of baseline CPA3 mRNA. This further strengthens the idea of considering MCs as highly plastic in nature rather than definite phenotypes like MCt, MCtc, MCt+CPA3+ and MCc.

In addition to the lung microenvironments induced MCs plasticity, we have shown a selective/preferential release of proteases such as chymase, AB1 & AB2 tryptases using human lung ex-vivo and humanized mice (NSG-SGM3) experimental models. For instance, upon induced by compound 4880 with the combination of SCF (stem cell factor) the MCs in human lung explants selectively release the tryptase and meanwhile retains granular storage of chymase and give rise to rare MCc like cells. This is contradictory to the previous belief that lung mast cells do not respond to the secretagogues molecules such as compound 4880. Notably, since previous attempts

to tune the protease content in matured mast cells were failure, most of the MCs plasticity studies were conducted using invitro MCs. This has a huge clinical relevance because we have observed this unique MCc type cells are dominating in the lungs from COPD (GOLD 4) and lung fibrosis (IPF & CF) diseased patients and we suspect they spontaneously release high level of tryptase since they express significantly higher tryptase mRNA.

Similarly, we have shown the MCs selectively upregulates AB1 tryptase (protein) but not B2 tryptase and chymase when the humanized mice (NSG-SGM3) challenged with LPS and Poly I: C. This unique phenomenon was also evident in fatal COVID-19 patients. In addition to the selective release, we have shown that temporal based gradient change in MCs proteases (tryptase and chymase). This indicates apart from the local tissue milieu; temporal dynamics may also play a role in MCs plasticity but this yet to be proven in humans. A strong association of human mast cell engraftment and collagen accumulation in humanized mice lungs offers an excellent platform to study human mast cell-induced pulmonary fibrosis which we suspect strongly the case based on the outcomes from paper 2.

Our extensive histology based spatial mapping of human leukocytes in humanized mice model revealed the unique and intriguing human immune behaviours. An early (week 8) tissue homing of a large number of basophils and subsequent exodus in the later stage (week 16), recently shown basophils plays a central role in orchestrating early lung immune homeostasis. The distribution of basophils and eosinophils and also an expression of type 2 cytokine such as Hu IL-13 were spatially clustered in humanized mice lungs which has a huge clinical significance since we have shown a similar phenomenon in COPD (GOLD 4) patients.

GOLD 4 COPD patients lung displayed a spatially confined basophil and eosinophil rich type 2 immune microenvironments. The spatial correlation of eosinophils and basophils was associated with the severity of the diseases. In general tissue basophils are scarce, hence, humanized mice (NSG-SGM3) model can be used to study the underlying mechanism of tissue homing of basophils and eosinophils into type 2 immune landscapes. Spontaneously formed lymphocyte aggregates that are rich in unconventional phenotypes such as GATA3 expressing cytotoxic T cells (GATA3 + CD8+). This offers a unique platform to study these rare cells since GATA3+ CD8+ cells are believed to be a major source of type2 cytokines in asthmatic patients.

In a nutshell, for the first time, we provide an explicit view of the true dynamics of mast cell plasticity and spatial coordination of immune cells in the world of lung microenvironments and its likelihood of clinical implications.

In addition, author would like to stress the significance of studying the immune cells in their original state (minimize the molecular changes from the immune cell enrichment process from tissues) in order to draw a clinically relevant conclusion.

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