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Studies of monocytes and macrophages in the respiratory tract with focus on airway allergy

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ABBREVIATIONS

AhR aryl hydrocarbon receptor

AIT allergen-specific immunotherapy

AM alveolar macrophage

ANOVA analysis of variance

APC antigen presenting cell

AR allergic rhinitis

ATP adenosine triphosphate

BALT bronchus-associated lymphoid tissue

Batf3 basic leucine zipper transcriptional factor ATF-like 3

BCR B cell receptor

BDCA blood dendritic cell antigen

CCR chemokine receptor

cDC conventional dendritic cell

CDP common dendritic cell progenitor

CLEC C-type lectin domain family

CLR C-type lectin receptor

CRP C-reactive protein

DC dendritic cell

DC-SIGN dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

ECP eosinophil cationic protein

EDN eosinophil-derived neurotoxin

EPX eosinophil peroxidase

Fab antigen-binding fragment

Fc crystallizable fragment

FC flow cytometry

FcεRI high-affinity receptor for IgE

FISH fluorescence in situ hybridization

Flt3L FMS-like tyrosine kinase 3 ligand

FoxP3 forkhead box P3

GM-CSF granulocyte macrophage-colony stimulating factor

HLA human leukocyte antigen

HSC hematopoietic stem cell

HSCT hematopoietic stem cell transplantation

IFS immunofluorescence

IHC immunohistochemistry

hPAP hereditary pulmonary alveolar proteinosis

ICOS inducible co-stimulator

IDO indoleamine 2,3-dioxygenase

IFN interferon

IFS immunofluorescence staining

Ig immunoglobulin

IL interleukin

ILC innate lymphoid cell

iNKT invariant natural killer T

IPEX immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IRF interferon regulatory factor

ITAM immunoreceptor tyrosine-based activation motif

LC Langerhans cell

LT leukotriene

LPS lipopolysaccharide

mAb monoclonal antibody

MALT mucosa-associated lymphoid tissue

MBP major basic protein

MBL mannose-binding lectin

MC mast cell

MCP mast cell precursor

MCP-1 monocyte chemoattractant protein

MHC major histocompatibility complex

MLP multilymphoid precursor

MMP matrix metalloproteinase

moDC monocyte-derived DC

MPS mononuclear phagocyte system

NET neutrophil extracellular trap

NK natural killer

NLR NOD-like receptor

NOD nucleotide-binding oligomerization domain

NSG NOD/SCID/IL-2Rγ-null

PAMP pathogen-associated molecular pattern

PAP pulmonary alveolar proteinosis

pDC plasmacytoid dendritic cell

PD-L programmed death ligand

PG prostaglandin

pIgR polymeric immunoglobulin receptor

PMT photomultiplier tube

PRR pattern recognition receptors

PG prostaglandin

RA retinoic acid

ROS reactive oxygen species

ROR retinoic acid-related orphan receptor

S1P sphingosine-1-phosphate

S1PR sphingosine-1-phosphate receptor

SCID severe combined immunodeficiency

SIgA secretory IgA

sIgE specific IgE

SIRP signal regulatory protein

SLO secondary lymphoid organ

SPT skin prick test

STAT signal transducer and activator of transcription

t-bet T-box expressed in T cells

Tcm central memory T cell

TCR T cell receptor

Tem effector memory T cell

Tfh T follicular helper

Th T helper

TGF transforming growth factor

TLR toll-like receptor

TNF tumor necrosis factor

Treg regulatory T cell

TREM triggering receptor expressed on myeloid cells

Trm resident memory T cell

TSLP thymic stromal lymphopoietin

PAPERS INCLUDED

This thesis is based on the following papers

I. Rapid recruitment of CD14+ monocytes in experimentally-induced allergic rhinitis in human subjects.

Journal of Allergy and Clinical Immunology 2016 Feb 3. On-line available.

II. Monocytes accumulate in the airways of children with fatal asthma attacks.

Manuscript.

III. Long-term persistence of human alveolar macrophages in vivo.

Accepted in Thorax pending revision, 2016.

INTRODUCTION

1. Background

Allergic rhinitis (AR) and allergic asthma are chronic inflammatory disorders of the respiratory mucosa caused by sensitization to environmental allergens. AR is characterized by rhinorrhea, sneezing and nasal itching and blockage [1], whereas allergic asthma is characterized by reversible airflow obstruction and bronchospasm inducing symptoms like wheezing, coughing, chest tightness and shortness of breath [2].

The term “asthma” comes from the Greek verb aazein, meaning to pant (i.e. to exhale with the mouth open). The Corpus Hippocraticum by Hippocrates (460-370 BD) is the earliest text where “asthma” is used as a medical term, even though the word had already appeared in the Greek epic poem the Iliad (attributed to Homer). The ancient Greek physician Galen (130-200 AD) wrote several texts about “asthma” and defined it as bronchial obstruction. In the 12th century the Jewish-Andalusian philosopher and physician Moses Maimonides wrote Teatrise of Asthma linking asthma exacerbations to common cold. The first clinical description of AR was made in the 10th century by the Persian philosopher and physician Rhazes. In Europe, AR was first reported by John Bostock in 1828 with the name of “catarrhus æstivus” and regarded as an infrequent disorder affecting wealthy people [3]. AR was considered the consequence of the exposure to new hay in the summer (and subsequently termed “hay fever”) until Charles Backley identified the causative role of pollen in 1859. In 1886 Bosworth theorized a connection between asthma and hay fever [4]. The first insights into the mechanisms of allergy were provided 20 years later by the Austrian pediatrician Clemens von Pirquet who coined and defined the term “Allergy” as a “specifically altered reactivity of the organism”[5]. Nevertheless, asthma was largely

regarded as a psychosomatic illness during the first half of 20th century, until the 1960s when anti-inflammatory medications started to be successfully administered to asthma patients. Finally, IgE was simultaneously discovered in 1967 by Kimishige and Teruko Ishizaka in the US [6] and Gunnar Johansson and Hans Bennich in Sweden [7] further explaining the relationship between respiratory allergy and allergen exposure.

In the second half of the 20th century the number of primary-care consultations due to AR dramatically increased in the Western World [8]. This trend was also followed by other allergic diseases like asthma and atopic eczema [9]. Nowadays respiratory allergy is a global health care problem, and AR alone affects more than 400 million people worldwide, especially in the western societies [10], but the incidence in developing countries also increases as they acquire a westernized lifestyle. Asthma can affect close to 20% of the population with considerable variability among countries [2]. Since the extension of the use of inhaled corticosteroids, the natural history of asthma dramatically improved [11] even though many patients still remain poorly controlled and account for significant economic burden to society [2]. Additionally, severe and fatal asthma attacks still persists and are more frequent in certain geographical areas [9]. AR adversely affects many aspects of social and professional life, as well as school performance in children [12, 13]. AR is also strongly associated to other inflammatory disorders of the mucous membranes like conjunctivitis and represents a major risk factor for the development of asthma in adults [14, 15].

To better understand the immunopathological mechanisms of allergy we have focused our research on the role of monocytes in AR and asthma. Additionally, we investigated the origin of alveolar macrophages (AMs) in lung transplanted patients. AMs are key immune-regulatory cells with many functions in lung inflammatory diseases like asthma [16], and

knowledge about their precursors and longevity might pave the way to novel therapeutic strategies for patients with immune-mediated respiratory diseases.

2. Anatomy of the Respiratory Tract

The human respiratory tract is comprised of the conducting airways and the lung parenchyma. The nasal cavity constitutes the upper edge of the conducting airways and is the place where the air is warmed up before inhalation. The nasal cavity also hosts the nervous fibers responsible of olfaction. The central and lower airways are formed by the trachea, bronchi and bronchioles and are lined with the respiratory mucosa surrounded by concentric layers of submucosa, smooth muscle fibers and cartilage rings. Both the nasal and respiratory mucosae are covered by a pseudo-stratified epithelium composed by a major population of ciliated cells co-mingled with goblet cells. The epithelial layer is separated from the underlying lamina propia by a basement membrane. The lamina propia consists of fibroblasts, collagenous fibers, blood vessels and sero-mucous glands. Goblet cells and the glands in the lamina propia produce and secrete mucus that coats and protects the luminal surface of the respiratory tract. The cilia in the epithelium are constantly beating upwards to expel the particulates that reach the airways.

Pulmonary alveoli are found distal to the terminal bronchioles and constitute the lung parenchyma. The alveoli are formed by alveolar spaces separated by thin alveolar walls but communication is facilitated through the connecting pores of Kohn. Alveolar walls host numerous alveolar capillaries to permit the gas exchange. The luminal surface of alveoli is coated by a thin layer of surfactant, a lipid compound lowering the surface tension and preventing alveolar collapse during ventilation. The alveolar epithelium is formed by type I flat-shaped pneumocytes intermingled with type II round-shaped pneumocytes. Both types of epithelial cells are equipped with immune receptors to perform immune-regulatory functions, whereas type II pneumocytes synthesize and release surfactant (**Figure 1**).

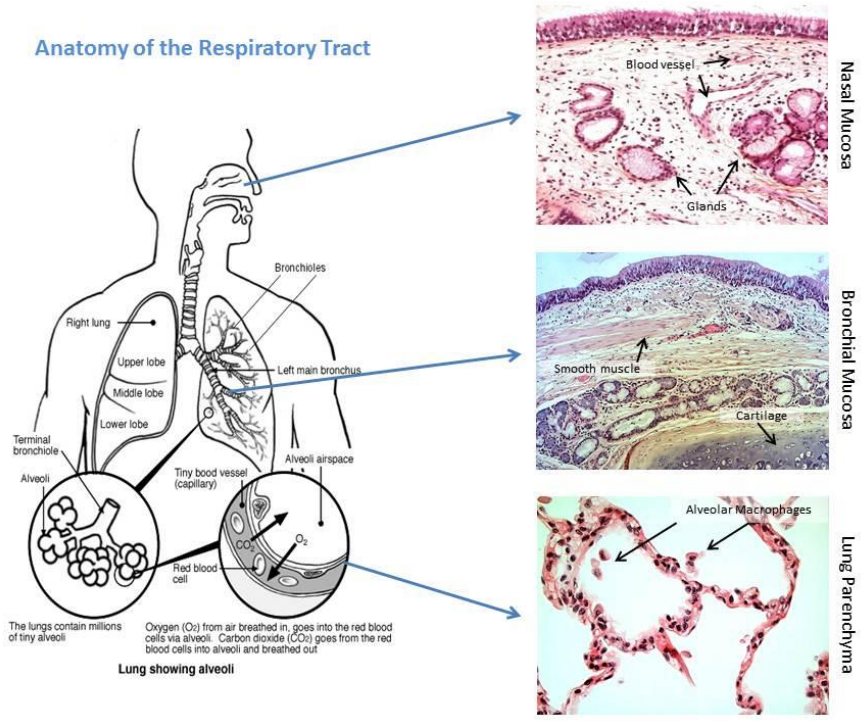

Figure 1: Anatomy of the Respiratory Tract

The respiratory mucosa is composed by an epithelial layer with ciliated epithelium, a basement membrane separating the epithelial layer from the lamina propia, and the lamina propia with vessels, glands and immune cells. The bronchial mucosa is surrounded by layers of smooth muscle and cartilage. The lung parenchyma is formed by the pulmonary alveoli where alveolar macrophages reside. *Adapted from www.humanpath.com-Human pathology.*

3. The Mucosal Immune System in the Airways

The main function of the human immune system is to protect the individual against pathogens. The immune system is also responsible to eliminate genetically damaged cells thus protecting the organism from neoplastic disorders. The human immune system can be divided into an innate and adaptive arm. The innate immune system appeared earlier in the evolutionary development and is characterized by a fast but less diverse response. It comprises different cell types including macrophages, dendritic cells (DCs), mast cells,

basophils, eosinophils, neutrophils, innate lymphoid cells and natural killer T cells together with humoral components like the complement system. The adaptive immune system provides an antigen-specific response that requires some days to develop but persists in the form of immunological memory. The adaptive immune system comprises T and B lymphocytes; the latter differentiate into antibody producing cells (plasma cells). The two arms of the immune system usually act together, with an initial innate response that shapes the ensuing adaptive response.

The mucosal immune system in the airways is comprised of many different immune cell types (as described above) that reside or traffic through the respiratory mucosa and lung parenchyma and the correspondent secondary lymphoid organs (SLO). The latter can be divided in the loco-regional (cervical, para-tracheal, para-bronchial and hiliar) draining lymph nodes and the mucosa-associated lymphoid tissue (MALT), that, despite being less prominent than in the gut, is also present in the airways. MALT in the respiratory tract is comprised by the lymphoid tissue associated to Waldeyer's ring (pharyngeal, tubal, palatine and lingual tonsils) and the bronchus-associated lymphoid tissue (BALT). These structures are clusters of immune cells (T cells, B cells, macrophages, DCs) and similarly to lymph nodes are the sites of induction of adaptive immune responses. BALT is consistently found in children but tends to regress from adolescence [17], whereas Waldeyer's ring persists throughout adult life. Both lymph nodes and MALT possess efferent lymphatic vessels but MALT lack afferent lymphatics, and this difference influences the arrival of antigens to the tissue [18]. Both the gut-associated lymphoid tissue and the BALT are populated with microfold (M) cells that can take up the antigens from the lumen and deliver them via transcytosis to BALT-resident DCs and lymphocytes [19].

The Innate Immune System

a. The Mononuclear Phagocyte System (MPS)

The MPS consists of professional antigen presenting cells (APCs) comprising monocytes, DCs and macrophages. Cells of the MPS express MHC class II (MHC-II; HLA class II in humans) molecules and can take up, process and present antigens to T cells, thus having a pivotal role in the generation and maintenance of immune responses. Moreover, MPS components secrete many different cytokines and chemokines to efficiently regulate and shape inflammatory reactions. Monocytes constitute a large population of blood leukocytes, whereas macrophages are stationary tissue-residing cells. DCs are thought to be dynamic cells that traffic between the blood stream, peripheral tissues and SLOs [20].

Cells of the MPS express Pattern Recognition Receptors

Cells within the MPS express pattern recognition receptors (PRRs) that recognize highly preserved pathogen-associated molecular patterns (PAMPs) and allow them to constantly survey the airways in search of potential threats [21]. PRRs comprise a heterogeneous family of innate immune receptors that can be divided in membrane-bound, cytoplasmatic or secreted receptors. Membrane-bound PRRs include different families such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs). TLRs are expressed in either external or internal membranes and their ligation induces the expression of pro-inflammatory cytokines and co-stimulatory molecules [22]. CLRs can be further divided into group I mannose receptors (with the ability to activate the complement system) and group II asialoglycoproteins receptors such as the dendritic cell-specific intercellular adhesion molecule-3-grabbin non integrin (DC-SIGN/CD209) [23]. Cytoplasmatic receptors include nucleotide-binding oligomerization domain receptors (NOD-like receptors or NLRs) which are

involved in the stimulation of the inflammasome, the intracellular machinery leading to the activation of interleukin (IL)-1β in DCs and macrophages [24]. Secreted PRRs are formed by a diverse family of molecules including C-reactive protein (CRP) or mannose-binding lectin (MBL), which is able to activate the complement system [25].

The Monocyte-Macrophage Axis in the Airways

Monocytes

Monocytes differentiate in the bone marrow from common granulocyte/macrophage progenitors (myeloblasts) and monoblasts. They are found in the circulation were they express CD11c, CD68 and calprotectin (S100A8/A9) and can be divided into three main subsets based on their surface level of CD14 and CD16 [26, 27]. Classical CD14++CD16- monocytes (referred from now as CD14+monocytes) can migrate to tissues where they differentiate into macrophages or DCs [28]. On the other hand, non-classical CD14+CD16++monocytes exert their functions primarily within the circulation where they patrol the luminal surface of the endothelium and sense viruses and immune complexes via TLR7 and TLR8, thus contributing to tissue immune surveillance [29]. Intermediate CD14++CD16+monocytes seem to be involved in reparative processes (they express growth factors and chemokine receptors) and are specifically enriched in the bone marrow [26].

Mucosal Macrophages

a. **Ontogeny of Mucosal Macrophages**: The classical "Mononuclear Phagocyte System" theory was postulated in 1968 by the Dutch researchers van Furth and Cohn whom proposed that CD14+monocytes maintain the pool of resident tissue macrophages by constantly migrating into peripheral tissues where they completed their differentiation [30, 31]. Nevertheless, this theory has been challenge by different studies reporting a negligible contribution of blood monocytes to many types of

resident macrophages including microglia in the brain [32] and Kupffer cells in the liver of mice [33], and Langerhans cells (LCs) both in mice and humans [34, 35]. However, recent mouse studies have demonstrated that the intestine there is a constant replenishment of blood monocytes to maintain the pool of resident macrophages [36]. In line with this, examining patients undergoing pancreas-duodenal transplantation, we find that resident macrophages in the human duodenum are constantly replaced by circulating monocytes (Bujko et al, unpublished). Nowadays mouse resident macrophages are regarded as cells with a dual origin. On the one hand, macrophage precursors generated during embryogenesis in the yolk sac or in the fetal liver migrate to the developing tissues and differentiate into resident macrophages [37]. On the other hand, the definitive hematopoiesis in the bone marrow gives rise to circulating monocytes that under certain circumstances can also enter the tissues and mature to resident macrophages; but the relative contribution of these blood precursors to the population of tissue-resident macrophages significantly varies among the different organs, being minimal in tissues with immune privilege like the brain, whereas in anatomical locations heavily exposed to the environment like the mucosae, monocyte contribution seems very important [38] (**Figure 2**). In this regard, the macrophages populating both the upper and lower respiratory mucosa [20] are believed to arise from CD14+ CD68+ calprotectin+ circulating monocytes which after migration into the airways would remain CD14 and CD68+, gain expression of DC-SIGN and become calprotectin- [36, 39].

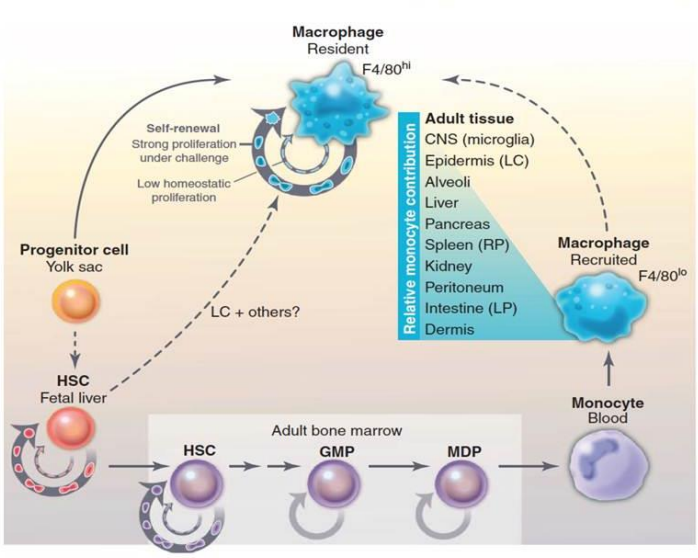

Figure 2: Ontogeny of Tissue-resident Macrophages

Macrophages can develop from yolk sac monocytes during embryogenesis and self-perpetuate throughout adult life. Definitive hematopoiesis in the bone marrow gives rise to circulating monocytes that under certain circumstances can differentiate into resident macrophages after migration to peripheral tissues. Monocyte contribution is highly tissue-dependent and varies from no contribution for brain microglia to complete contribution for intestinal lamina propia macrophages. LC: Langerhans cells, RP: red pulp, LP: lamina propia. *Reprinted under the terms of the Creative Commons Attribution License (CC-BY); Sieweke & Allen. Beyond stem cells: self-renewal of differentiated macrophages. Science 2013, 342(6161):1242974. © 2013 by the American Association for the Advancement of Science.*

b. Functions of Mucosal Macrophages: Mucosal macrophages are essential for local homeostasis and in keeping a balance between airway microbiota and the host. During steady-state the local microenvironment conditions incoming CD14+monocytes towards a regulatory phenotype with reduced potential to initiate inflammation. During homeostasis, mucosal macrophages display a high phagocytic capacity to ensure clearance of apoptotic cells. Moreover, upon TLR-ligation they do not express co-stimulatory molecules or pro-inflammatory mediators, but release the

regulatory cytokine IL-10 instead. Of note, they are able to take up and kill luminal microorganisms without starting adaptive immune responses [40]. On the other hand, during inflammation they can act as powerful aggressors to protect the host. However, it is not clear whether they promote or prevent respiratory allergy, as some studies support a pro-inflammatory role for mucosal macrophages, whereas other animal models assign them a tolerogenic function through the inhibition of type 2 immune responses [20, 41].

Monocytes may differentiate into Dendritic Cells in the Airways

Like in atopic dermatitis [42, 43] or psoriasis [44], during respiratory infections high numbers of CD14+monocytes are recruited to the airways where they differentiate into inflammatory DCs [28] able to mount potent immune responses and fight pathogens. These cells express CD14, the high affinity receptor for IgE (FcεRI) and signal regulatory protein α (SIRPα) but are negative for CD16. They display relatively low migratory capacity but are potent recruiters and re-activators of memory T cells and other immune cells [45]. Their specific roles in respiratory allergy will be discussed in detail later.

Moreover, it has been recently demonstrated that CD14+monocytes also contribute to tissue DCs during homeostasis. Fate-mapping systems in animal models have suggested that ~50% of CD11b+DCs in the lung are not derived from DC-committed precursors during the steady state [46]. In the human intestinal mucosa during homeostasis, monocyte-derived CD103-SIRPα+DCs have been described and unlike inflammatory DCs or interstitial macrophages, do not retain expression of CD14 [39]. Nevertheless their function and relationship with other monocyte-derived APCs are still far to be understood.

Alveolar Macrophages

The lumen of the bronchioles and the alveolar spaces in the lung parenchyma are populated with bronchial and alveolar macrophages (AMs) respectively. It is not clear whether these two populations represent the same functional specialization, and the majority of published studies use models of either broncho-alveolar lavage specimens or digests from lung biopsies, which impede the discrimination between these two populations of luminal macrophages [47]. AMs in both mice and humans are highly auto-fluorescent CD11c+ CD68+ DC-SIGN+ luminal cells that display weak expression of CD14 and are calprotectin- [48, 49]. The mouse lung harbors one-two millions of AMs [50] that can travel through the connecting pores of Kohn [51] to cover the 3-times more abundant pulmonary alveoli.

Ontogeny of Alveolar Macrophages

In an early article using a human model to assess the origin of AMs the presence of Y chromosome was examined in AMs populating the lung parenchyma of patients with hematological malignancies undergoing hematopoietic stem cell transplantation (HSCT) from sex mismatched donors. In lung biopsies from these patients, the investigators found that the vast majority of AMs in male patients receiving a female bone-marrow did not have a Y chromosome 80 days after HSCT, thus favoring the existence of bone marrow precursors for human AMs [52]. Nevertheless shortly after the publication of this study several articles based on rodent models showed opposite results [53-55]. Furthermore, Guilliams and colleagues demonstrated that in experimental mice with reduced numbers of circulating monocytes, AMs are not dependent on replenishment from blood monocytes within two weeks of parabiosis with a wild type animal [56]. In this study the authors show that AMs arise from fetal monocytes that seed the lung during embryological development to shortly after birth differentiate into mature AMs in a process fully dependent on granulocyte

macrophage-colony stimulating factor (GM-CSF) signaling. The pool of mouse AMs can be self-maintained throughout adult life with minimal recruitment of blood precursors because these fetal-derived cells display a high ability to in situ proliferate [57]. On the other hand, the recent finding of mouse and human intestinal macrophages being rapidly replenished by blood monocytes after birth [36] and Bujko et al, in prep] has raised the question of whether other embryonic-derived macrophages in mucosal tissue can survive until adulthood. Even though the population of mouse AMs seems to have a low dependence on blood monocytes [38], in humans the origin of AMs has not been investigated in depth and this was the aim of the third article included in this thesis.

Immune-regulation by Alveolar Macrophages during Lung Homeostasis

As professional phagocytes, the primary function of AMs during homeostasis is to clear pathogens in the alveolar spaces and to eliminate damaged cells or cell debris. AMs are mainly stationary cells but some animal studies have found them in the regional lymph nodes where they might contribute to T-cell priming [58]. AMs regulate tissue immune homeostasis and are equipped with a broad range of immune-receptors [59] including both activatory (TLR2, TLR4, TLR6 and IL-1, TNF-α and IFN-γ receptors) and inhibitory (TREM2 and TGF-β, SIRPα, IL-10 and mannose receptors) receptors [60, 61]. During steady state, the alveolar epithelium provides anti-inflammatory signals (IL-10, CD200) that activate the inhibitory receptors on AMs [47]. These inhibitory signals render AMs in an anti-inflammatory state and they can interact in an antigen-specific manner with the rare population of luminal T cells to promote a regulatory phenotype. Among AM-products transforming growth factor (TGF)-β and prostaglandins (PGs) oppose T cell activation. TGF-β together with retinoic acid (RA) also promotes FoxP3 (forkhead box P3) expression in luminal T cells [62]. By these mechanisms AMs prevent over-reaction to harmless antigens during

homeostasis which represents another way to protect the lung parenchyma from excessive inflammation (**Figure 3**).

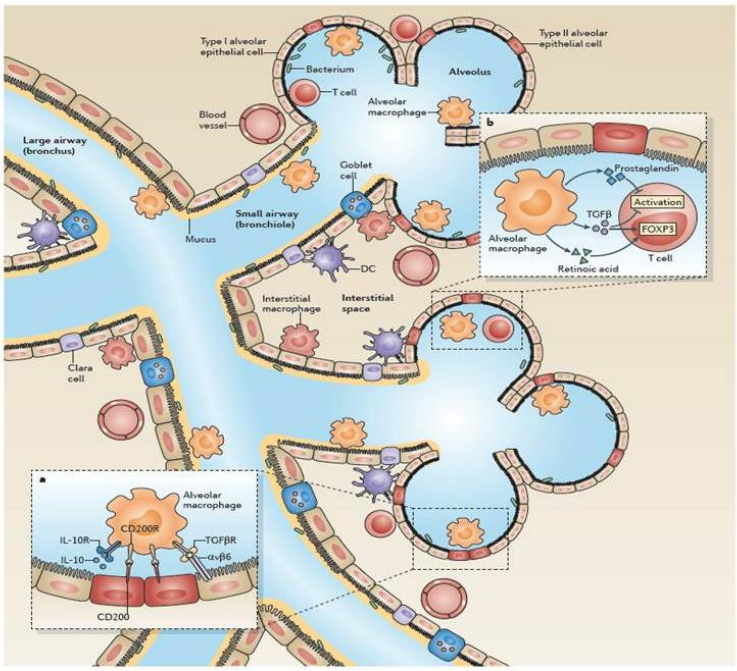

Figure 3: Immune Regulation by Alveolar Macrophages during Lung Homeostasis

The inhibitory input that alveolar macrophages receive from the alveolar epithelium during the steady state (a) renders them into an anti-inflammatory state. They can therefore communicate with the rare population of luminal T cells (b) to promote on them a regulatory phenotype thus protecting the lung parenchyma from immune over-reaction. *Reprinted under the terms of the Creative Commons Attribution License (CC-BY); Hussell &Bell. Alveolar macrophages: plasticity in a tissue-specific context. Nat Rev Immunol 2014 14(2):81-93. © 2014 Macmillan Publishers Limited.*

Alveolar Macrophages and Surfactant Homeostasis

In addition to phagocytosis and immune-regulation AMs play a pivotal role in surfactant homeostasis [63]. Surfactant is comprised primarily of phospholipids contributing to the formation of the surface tension-lowering lipid layer that prevents alveolar collapse during ventilation [64]. The compound is synthetized by type II pneumocytes which also contribute partially to its catabolism [65]. Nevertheless, AMs are the main cells eliminating damaged

and oxidized surfactant. As efficient phagocytes they take up the surfactant and degrade it in a process fully dependent on GM-CSF signaling and expression of the myeloid master transcription factor PU.1 [66]. Interestingly, GM-CSF and PU.1 have been also reported to be crucial for the terminal differentiation of AMs in both humans and mice [56] and importantly AMs from GM-CSF-deficient mice display multiple abnormalities including altered cellular morphology, impaired cell adhesion and phagocytosis and defective expression of differentiation markers and TLRs [67]. Patients with pulmonary alveolar proteinosis (PAP) have defects on GM-CSF signaling leading to the accumulation of surfactant in lung parenchyma, respiratory insufficiency and impaired microbicidal activity by neutrophils [66], because GM-CSF is also a crucial regulator of myeloid cell host defense functions [68]. The respiratory insufficiency observed in PAP patients often produces a radiological pattern termed “crazy paving” and requires repeated whole lung lavages performed under general anesthesia [69]. Among the different types of the disorder, autoimmune PAP is more frequent and is caused by IgG auto-antibodies directed to circulating GM-CSF [70], whereas hereditary PAP (hPAP) is due to mutations in the chains of GM-CSF receptor on the surface of AMs [71]. HSCT has been shown to ameliorate experimental models of hPAP in mice [72]. However, the morbidity and mortality related to the myeloablative conditioning and HSCT have significantly limited the usefulness of this approach in humans.

Classical Dendritic Cells

DCs comprise a heterogeneous group of cells that can be defined by their high ability to migrate to the draining lymph nodes and their unique capacity to activate naïve T cells [29, 73]. DCs subsets in the airways have been extensively studied in mouse models, whereas knowledge of human subsets is much more restricted. Tissue DCs are derived from circulating precursors, and in humans, blood DCs can be divided into three main subsets

according to the expression of surface markers: CD1c+ (blood dendritic cell antigen, BDCA-1) DCs, CD141+ (BDCA-3) DCs and CD303+ (BDCA-2/CD123+) plasmacytoid DCs (pDCs) [74]. CD1c+ and CD141+ DCs are usually classified together as myeloid or conventional DCs (cDCs), because they express typical myeloid antigens such as CD11c but lack monocyte markers such as CD14 or CD16. CD1c+cDCs and CD141+cDCs share homology with mouse CD11b+cDCs and CD103+cDCs respectively. pDCs typically lack CD11c and retain subtle lymphoid features and unique secretory properties. Homologues are recognized in many species, including mouse pDCs [75]. Blood DCs display an immature phenotype, and during homeostasis they are believed to migrate at a low but constant rate to the airways where they complete their differentiation [76].

Dendritic Cells residing in the Airways during Homeostasis

The different DC subsets express unique patterns of surface markers and display different functional capacities, thus arguing for a division of labor between DC subsets in human respiratory mucosa. [77, 78]

a. **CD1c+ cDCs** are the major population of human cDCs in blood [79] and in the tissues [73]. In vitro differentiation and gene expression analysis has supported the precursor-progeny relationship between blood and tissue CD1c+ cDCs [80], yet the tissue counterparts are more activated in terms of lymph node migratory capacity and expression of co-stimulatory molecules [81-84]. Human CD1c+ cDCs are equipped with a wide range of PRRs including lectins and TLRs allowing them to take up, transport and present the antigens. Through TLRs 1-8 they respond to many different PAMPs including lipopolysaccharide (LPS) or flagellin [85]. Subsets of CD1c+ cDCs co-express CD1a and are efficient in presenting lipid antigens to T cells [86] and have been implicated in the

generation of CD4+ T cell immunity to the glycolipids of the wall of mycobacteria. C-type lectin domain (CLEC)6A/7A is also highly expressed by CD1c+ DCS, suggesting a role in fungal recognition [87, 88]. In general they are very good stimulators of CD4+ T cells, but do not excel in driving external antigens into the MHC-I pathway (cross-presentation), thus being less potent activators of CD8+ T cells [39, 89]. CD1c+cDCs secrete tumor necrosis factor (TNF)α, IL-8, IL-10, IL-12 and IL-23 depending on the type of stimulation [90-92], thus being potentially able to drive CD4+ T cell polarization to different effector classes. Even though CD1c+cDCs do not produce IL-4, their mouse equivalent CD11b+cDCs seem crucial for the generation of type 2 immunity. [45, 93, 94] Moreover, in the human nasal mucosa they are responsive to the Th2-polarizing cytokine thymic stromal lymphopoietin (TSLP) [84]. Such DCs have also been implicated in the generation of tolerance to commensal bacteria through the production of IL-10 and the regulatory molecule indoelamine 2,3 deoxygenase (IDO)[95].

b. CD141+ cDCs represent ~10% of human blood cDCs [74, 75] and can also be found at low numbers in the respiratory mucosa. They are the counterparts of mouse CD103+cDCs which are located above the basement membrane in the respiratory epithelium [76]. CD141+cDCs are currently regarded as the “cross-presenting” DCs and appear specialized in the defense against viral infections [96]. Viruses are primarily fought by cell-contact dependent killing of infected cells and this task is mainly performed by cytotoxic CD8+ T cells with the help of Th1 cytokines [97]. Because viruses preferentially infect epithelial cells, the initiation of antiviral cytotoxic responses is dependent on the cross-presentation of antigens from apoptotic stromal cells in the context of MHC-I molecules expressed on DCs. In this regard, CD141+ cDCs excel in driving extracellular antigens to the MHC-I pathway [98]. The group II CLR CLEC9A is able to take up dead or necrotic cells

[99] and in humans is uniquely expressed by CD141+cDCs [100], thus being useful as an identification marker. CD141+ DCs can also sense viral nucleic acids through TLR3 and TLR8 and efficiently secrete TNFα and interferon (IFN)-γ [80] which associate CD141+cDCs to the generation and maintenance of Th1 responses.

c. pDCs are the most abundant blood DCs, lack myeloid antigens and express CD45RA, CD123, CD303 and CD304 [101]. They may also harbor T-cell receptor and immunoglobulin rearrangements. pDCs display abundant secretory capacity and are rare in the airways during homeostasis [102]. They express very high levels of TLR7 and TLR9 whose ligation induces the release of type I (anti-viral) IFNs [103]. Blood pDCs are not able to prime efficiently T cells [104], but the ability of their tissue counterparts to polarize CD4+ T cells to Th1 or Th2 responses appears to be variable and context-dependent [105]. In other systems, pDCs have been implicated in the generation of regulatory T cells or tolerance to antigens [106]. Depletion of pDCs in mouse models of asthma has been associated with allergen sensitization and Th2 lung inflammation [107]. Moreover, pDCs isolated from human tonsils can induce FoxP3+ Tregs with the ability to suppress in vitro allergen-specific T cells in patients with respiratory allergy [108]. On the other hand, aberrant chronic pDC stimulation with associated secretion of type I IFNs has been related to the development of autoimmune and inflammatory disorders, such as systemic lupus erythematosus or psoriasis [103, 109].

Ontogeny of Human Dendritic Cells

In mice, DCs arise from a common DC precursor (CDP) that can differentiate into the different subsets of immature DCs in response to lineage restricted differentiation factors [110, 111]. In humans, there is no definitive evidence for the existence of committed DC precursors in the bone marrow and in vitro both granulocyte-macrophage precursor and

multilymphoid progenitors (MLP) can differentiate to DCs [112]. Human blood DCs appear to serve as precursors of tissue DCs as indicated by HSCT studies and human DC deficiency states [113, 114]. The differentiation program of all human DC subsets requires the growth factor FMS-like tyrosine kinase 3 ligand (Flt3L) and its receptor Flt3 [29], as illustrated by the massive increase in the number of blood DC subsets following Flt3L injection [115]. Patients suffering from several hereditary disorders with deficiency of DCs have been described to date. The autosomal dominant syndrome associated with monocytopenia, B and NK cell lymphopenia and mycobacterial, fungal and viral infections (abbreviated DCML) is caused by mutations in GATA2 [116, 117]. This abnormality results in the complete loss of MLP and in the increase of serum levels of Flt3L further underscoring the role of this mediator in the generation of human DCs. Autosomal dominant mutations in the transcriptional regulator interferon regulatory factor (IRF)8 cause reduction in the number of monocytes and CD1c+cDCs, but not CD141+cDCs, pDCs or macrophages in humans [114]. Moreover, DC subsets have been proposed to depend on specific master transcription factors. In this regard IRF4, Batf3 and E2-2 have been related to the development of CD1c+ cDCs, CD141+ cDCs and pDCs respectively [92] [118] [101, 119] .

Immune-regulation by Dendritic Cells during Homeostasis

The task of immune regulation in tissues requires a complex balance between tolerance and immunity. For lung DCs, the rapid generation of immune responses upon recognition of danger signals is a function as important as avoiding immune over-reaction to harmless antigens. This role is especially important in the lung parenchyma where gas exchange takes place and excessive inflammation can damage the delicate architecture of alveoli. To ensure this tolerance, both lung and lymphoid DCs are constantly presenting self- and external non-pathogenic antigens to T cells during homeostasis in a process inducing the generation of

suppressive T cells rather than effector T cells [120, 121]. This tolerogenic capacity of DCs is basically achieved though the regulation of their activation status and through the control of the tissue cytokine milieu [122]. Antigen presentation by immature DCs typically results in immune tolerance due to the lack of co-stimulatory molecules, whose expression is only induced after activation of PRRs [123]. On the other hand, epithelial and other stromal cells in the airways are able to provide the cytokine milieu necessary for the development of tolerance. During homeostasis, respiratory epithelial cells in mice produce TGF-β and RA, which are master drivers of regulatory T cell differentiation [124, 125]. Furthermore, such epithelial-derived mediators can shape DCs to a tolerogenic state. Human in vitro-generated DCs acquire tolerizing capacity under the influence of anti-inflammatory factors such as vitamin A, PG-E2, IDO, IL-10 and TGF-β [123]. The enzyme IDO degrades the essential amino acid tryptophan by catalyzing its catabolism. Depletion of tryptophan provides many immune-regulatory effects such as the promotion of regulatory T cell activity and the inhibition of the effector responses driven by both CD4+ and CD8+ T cells [126, 127].

A summary of the MPS subsets and progenitors in the human respiratory tract can be seen in **Figure 4**.

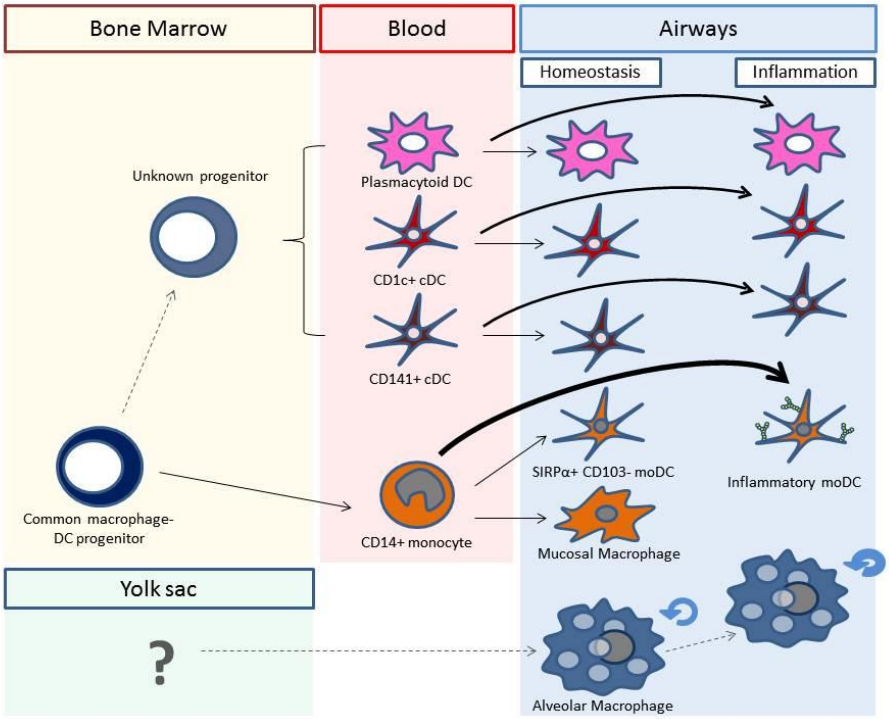

Figure 4: The Mononuclear Phagocyte System in the Human Airways

Human blood dendritic cells (DCs) arise from unknown bone marrow progenitors and migrate at a low but constant rate to the airways to maintain the pool of resident DCs during homeostasis. During inflammation blood DCs experience increased migration to the airways, yet the recruitment of specific subsets depends on the type of inflammatory response. Blood monocytes are the source of CD103- SIRPα+ DCs and mucosa-resident macrophages during homeostasis, and during inflammation they are massively recruited to the airways to give rise to inflammatory DCs. The ontogeny of human alveolar macrophages is obscure, and it is unclear whether, similarly to mice, they derive from yolk sac progenitors that self-perpetuate during adult life. cDC: conventional dendritic cell, moDC: monocyte-derived dendritic cell.

b. Granulocytes: Pro-granulocytes in the bone marrow give rise to the three types of blood granulocytes: eosinophils, basophils and neutrophils. Such cells are recruited to the airways during inflammation but are scarce during homeostasis. Conversely, mast cells are not present in circulation, but it is thought that their bone marrow precursors migrate through blood to peripheral tissues where they complete their differentiation.

Mast Cells

Mast cells (MCs) arise from unknown bone marrow progenitors, yet are found in the mucosal tissues where they play a central role in protection against helminth infections [128]. Both in mice and humans MC precursors (MCPs) migrate from blood to the mucosae where they complete their differentiation [129]. Mature MCs contain multiple granules where they store preformed mediators such as serine proteases (tryptase), vasoactive amines (histamine) and proteoglycans (heparin). In the airways MCs are scarce during homeostasis. On the other hand, the number of MCs present in the bronchial epithelium of patients with asthma is markedly increased compared to that in biopsy specimens from non-asthmatic control subjects [130]. Moreover, the number of circulating MCPs is increased by several folds in the blood of patients with asthma compared with the blood of healthy control subjects [131]. MCs express surface receptors for different immunoglobulin isotypes and prostaglandin. They respond to antigenic stimulation through cross-linking of IgE bound to FcεRI expressed on their surface. Upon activation MCs release vast amounts of pre-stored mediators from the granules that promote vascular dilatation and permeability. Within minutes they start synthetizing and releasing lipid mediators (eicosanoids) with potent broncho-constrictor activity and within hours they also produce cytokines with different inflammatory functions [132]. In addition to their central role in allergic inflammation and defense against helminth infection, MCs are recognized as effector cells in various types of clonal, auto-immune and cardiovascular disorders [133, 134] and also contribute to wound healing and blood-brain barrier function [135].

Eosinophils

These granulocytes differentiate in the bone marrow from myeloid precursors under the influence of IL-3, IL-5 and GM-CSF. They enter the circulation in a mature state and during homeostasis populate some organs like the lower gastrointestinal tract but are not present in the lung or the esophagus [136]. They have been largely associated with the initiation and maintenance of Th2 immune responses in particular allergic inflammation and the defense against parasitic infections. In this regard, the infiltration of the airways by eosinophils is one of the hallmarks of allergic asthma, even though non-allergic eosinophilic asthma also exits [137]. Their migration to the airways during inflammation occurs via specific interactions between chemokine receptors (CCRs) such as CCR3 and different eotaxins like CCL11, CCL24 or CCL5 [138]. Upon activation by Th2 cytokines, eosinophils immediately release pre-formed mediators stored in their granules, including proteolytic enzymes like elastase and cytotoxic proteins such as eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPX) and eosinophil-derived neurotoxin (EDN). Within minutes they start releasing reactive oxygen species (ROS) and de novo generated eicosanoids. Most of these mediators are toxic to both parasites and host cells and largely contribute to the tissue damage and remodeling observed during chronic Th2 inflammation [139]. Activated eosinophils can also release different growth factors and cytokines, and importantly they were shown to be a major source for IL-4, the cytokine required for Th2 polarization [140].

Basophils

These bone marrow-derived circulating granulocytes are very rare in the airways during homeostasis but can be recruited during inflammation [141]. They are importantly involved in Th2 immune responses like the defense against parasitic helminthes and allergic reactions. Basophils are equipped with surface receptors for complement and different

immunoglobulin (Ig) isotypes including IgE, IgG and probably IgD [142]. Upon activation, they release the stored content of their granules including histamine and heparin. Unlike MC they release very low amounts of serine proteases (tryptase). Basophils also produce lipid mediators (eicosanoids and platelet activating factor) and different cytokines. They have been also regarded as a source of the Th2-polarizing factor IL-4 [143]. The degranulation of basophils can be investigated in vitro by using flow cytometry and the so-called basophil-activation test (BAT). This test might have important clinical implications for the diagnosis of food allergy and drug reactions [144]. Basophils could be also involved in the pathogenesis of other immune mediated diseases such as chronic spontaneous urticaria [145].

Neutrophils

These granulocytes are polymorphonuclear phagocytes which constitute the most abundant population of leukocytes in the blood stream. They are professional phagocytes equipped with a broad spectrum of receptors which allow them to rapidly engulf damaged cells or cell debris as well as pathogens coated with antibodies or complement. Moreover, upon activation their granules release vast amounts of bactericidal molecules such as cathepsin G, lysozyme and myeloperoxidase and other compounds involved in the formation of ROS [146]. They are also able to form neutrophil extracellular traps (NETs) which are composed by fibers of chromatin and serine proteases that trap and kill microorganisms extracellulary [147]. Neutrophils are massively recruited to the airways under the influence of IL-8 or IFN-γ during bacterial infections and other disorders with Th1 or Th17 inflammatory patterns [148]. In this regard, some asthma phenotypes with absent or non-predominant sensitization to aeroallergens are characterized by a Th17-driven neutrophilic infiltration of the airways [137].

c. Innate Lymphoid Cells (ILCs) are a group of innate immune cells that belong to the lymphoid lineage but do not respond in an antigen-specific manner, as they lack T/B cell receptors [149]. ILCs were first described as playing important functions in the development of lymphoid tissues, but more recently they were shown to play complex roles in tissue immune homeostasis, especially in the transition from innate to adaptive immunity [150]. In addition, dysregulation of ILCs might result in chronic inflammatory pathologies. ILCs develop in the bone marrow through ID2+ lineage- precursors [151] and can be divided into three main subsets. Groups 1 ILCs (ILC1s) predominantly express interferon-γ and include cytotoxic natural killer (NK) cells. They are involved in tumor surveillance and in the defense against viruses and intracellular bacteria [152]. ILC3s secrete lymphotoxin, IL-22 and IL-17 and play roles in homeostasis of epithelia and immunity against extracellular pathogens. A subset of ILC3s is also crucial for the development of lymphoid tissues [153]. Dysregulation of ILC1 and ILC3 may lead to inflammatory conditions including inflammatory bowel disease [150]. ILC2s produce IL-5, IL-9 and IL-13 and have important functions in protective immunity against parasitic worms [154], but also promote allergic diseases [155] and obesity [156]. Stimuli such as allergens or parasitic worms lead to the release of ILC2-inducing factors (TSLP, IL-25, IL-33) from the epithelium and infiltrating inflammatory cells at mucosal surfaces [157]. These cytokines cause ILC2s to proliferate and produce IL-5, IL-13 and IL-9 which will activate in turn type 2 effector pathways and drive the transition from innate to adaptive immunity [158]. Nevertheless, the stimulation of ILC2s can also lead to type 2 inflammation and bronchial hyper-responsiveness without the involvement of adaptive immunity as has been shown in experimental models of asthma [159]. ILC1s, ILC2s, and ILC3s have been proposed to represent the innate counterparts of Th1, Th2 and Th17 cells respectively, whereas NK cells are regarded as the innate equivalents of CD8+ cytotoxic T

cells. This idea is supported by the fact of innate and adaptive lymphoid cells sharing many of their phenotype-driving transcription factors [150] (**Figure 5**).

d. Natural Killer T (NKT) Cells are a heterogeneous group of T cells that share properties of both T cells and NK cells. Type I NKT cells are the most studied subset and often termed invariant (i)NKT cells due to the expression of a very restricted repertoire of T cell receptors (TCRs) (Vα24-Jα18 and Vβ11 in humans) [160]. The most potent ligand for their TCR is the glycolipid α-galactosylceramide which is exclusively presented by the MHC-I-like molecule CD1d [161]. Lung iNKT cells can be activated by environmental agents or epithelium-derived factors. The stimulation of iNKT cells in the respiratory mucosa induces the release of high amounts of cytokines including IFN-γ, IL-4, IL-13 and IL-17 which can promote both eosinophilic and neutrophilic inflammation [162, 163]. They have also been proposed as a potential source of IL-4 during Th2 polarization.

The Adaptive Immune System

a. T cells

T cells are defined by their surface expression of the T cell receptor (TCR) which in 98% of the T cells is comprised by one α chain and one β chain equipped with the highly variable antigen-binding sites which determine T cell specificity [164, 165]. T cells originate in the bone marrow from common lymphoid progenitors and mature in the thymus in a process involving two consecutive steps. During the positive selection, only T cells that have successfully rearranged the α and β chains of their TCRs and are thus capable to recognize peptide-MHC complexes with appropriate affinity are allowed to continue their maturation. During the ensuing negative selection, T cells that bind too strongly to self-antigens

expressed on MHC molecules are eliminated [166]. Mature naïve T cells surviving clonal selection in the thymus express either CD8 or CD4 depending on whether they bound their cognate antigen on MHC class I (MHC-I) or MHC-II respectively. Naïve T cells exiting the thymus migrate to SLO where they may become activated by peptides presented on the surface of APCs [167].

Priming of T cells

The TCR is a transmembrane heterodimeric protein that binds peptides (T epitopes) from processed antigens displayed by APCs in the context of MHC molecules. Activation of T cells in the SLO is usually called "immunological synapse" and involves three simultaneous signals. The first signal is provided by the presentation of the specific peptide by professionals APCs. The TCR of CD4+ T cells recognizes peptides presented in the context of MHC-II molecules (CD4+ T cells are MHC-II restricted), whereas CD8+ T cells requires presentation in the context of MHC-I molecules (MHC-I restriction). The immunological synapse also requires the expression of co-stimulatory molecules (second signal) such as CD28 on T cells and CD80 or CD86 on APCs. The ligation of the former molecules induces the upregulation of CD40 ligands on T cells which bind to CD40 on APCs further strengthening the activation. The third signal is provided by the mediators or cytokines present in the environment where the synapse occurs and will determine the effector class of the activated T cell. APCs often act as sources of these "third signal" mediators but sometimes they are produced by other stromal or immune cells [168, 169] (**Figure 6**).

T Effector Subsets

A summary of the main CD4+ T cell effector subsets and their innate counterparts can be seen in **Figure 5**.

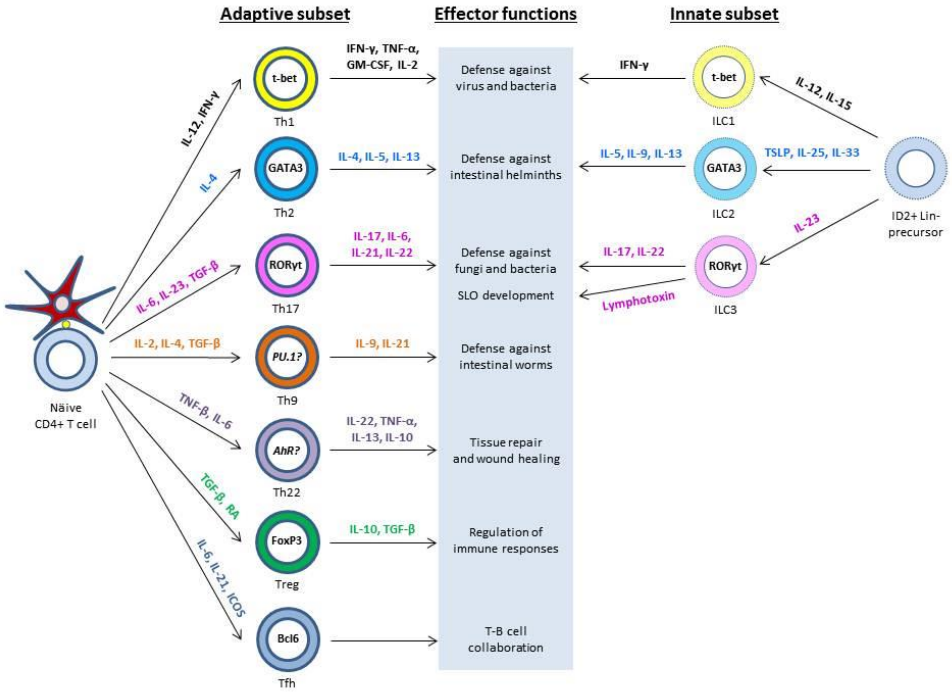

Figure 5: CD4+ T-Cell Effector Classes and their Innate Counterparts

Naïve CD4+ T cells polarize into different effector classes in response to class-specific mediators and transcription factors during activation and priming. Each class is specialized in specific reparative or immune processes, such as the protection against a certain group of pathogens. Th1, Th2 and Th17 cells have innate counterparts that differentiate in response to similar class-specific mediators and transcription factors. Innate lymphoid cells (ILCs) secrete different cytokines and collaborate with their adaptive counterparts in the correspondent immune processes. RA: retinoic acid; Tfh: T follicular helper

In the blood and SLOs, CD4+ T cells account for 60-70% of T cells, while 30-40% are CD8+ cells. After activation CD8+ T cells acquire the capacity to directly kill other cells and are therefore called cytotoxic T cells. On the other hand CD4+ T cells primarily regulate the

cellular and humoral immune responses by producing cytokines and are therefore called T helper (Th) cells. Naïve CD4+ T cells may be polarized into several different effector classes of which the best characterized are Th1, Th2, Th17 and T-regulatory cells (Tregs). The polarization to a specific effector class mainly depends on the network of cytokines present during the immunological synapse (third signal), which will determine the transcription factors whose expression will be up-regulated in the activated T cell. For the polarization of each T helper effector class, more than one cytokine is usually required, and cytokines involved in the differentiation of one lineage may suppress the differentiation in other subsets, thus driving the immune response in specific directions [170-172].

Th1 Cells: IL-12 and IFN-γ promote the expression of the transcription factors T-box expressed in T cells (t-bet) and Signal Transducer and Activator of Transcription (STAT)4 in naïve CD4+ T cells, which mediate their polarization towards a Th1 phenotype. Th1 cells are important for the defense against viruses, microbes that persist in macrophage vesicles (e.g. mycobacteria, Listeria, Leishmania donovani, Pneumocystis carinii) and extracellular bacteria. They produce high amounts of IFN-γ that stimulate the phagocytic ability of macrophages, suppress IL-4 mediated effects and further enhance Th1 polarization. Th1 cells also secrete TNF-α, GM-CSF and IL-2 which is critical for the maturation of both effector and memory T cells and for the maintenance of CD8+ T cell immune responses. Dysregulated Th1 cells also contribute to several inflammatory diseases including multiple autoimmune disorders (rheumatoid arthritis, type I diabetes), graft-versus-host disease, and delayed hypersensitivity reactions [168, 171-176].

Th2 Cells: IL-4 is the main cytokine driving Th2 polarization by upregulating the transcription factors GATA3 and STAT6 in naïve CD4+ T cells. Other epithelium-derived mediators such as TSLP, IL-25 and IL-33 can also contribute to Th2 polarization, but cannot replace the pivotal role of IL-4. Most professional APCs do not produce IL-4 which determines their inability to provide the third signal required for Th2 polarization. Several innate immune cells have been postulated as sources of IL-4 as has been discussed before. Th2 cells are effective in the defense against parasitic helminthes and the main effector cytokines they produce are IL-4, IL-5, and IL-13. IL-4 and IL-13 provides help to B cells for antibody production, especially switching to IgE and stimulate the expression of pro-inflammatory molecules like MHC-II. IL-13 acts as the bridge between Th2 immune cells and stromal cells in contact with them and is largely responsible for the tissue remodeling associated to chronic Th2 inflammation [177]. IL-5 is the most important cytokine regulating eosinophil biology by stimulating the maturation and egression of eosinophils from the bone marrow, and acting as a chemotactic molecule for them. Dysregulation of Th2 cells has been largely associated with immediate hypersensitivity reactions and other allergic disorders like AR, asthma or atopic dermatitis. Some autoimmune diseases like Systemic Sclerosis or Churg-Strauss syndrome also display a Th2 inflammatory pattern [172, 178-181].

Th17 Cells: co-exposure to several different cytokines including IL-6, IL-23 and TGF-β promote the expression of retinoid acid-related organ receptor γt (RORγt) and STAT3 on naïve CD4+ T cells, inducing their polarization towards a Th17 phenotype. The presence in the immunological synapse of either IFN-γ or IL-4 blocks Th17 polarization. This effector class is effective in the defense against extracellular bacteria like Klebsiella pneumoniae and fungi like Candida albicans. Th17 cells secrete different cytokines including IL-17, IL-6, IL-21 and IL-22, of which IL-6 and IL-21 exert an autocrine positive feed-back loop. Th17 cytokines

enhance neutrophil response and promote barrier integrity in the skin and the intestine. Dysregulated Th17 cells have been related to a number of autoimmune conditions, particularly when accompanying Th1 inflammation. In asthma, Th17 cells promote neutrophilic lung inflammation with associated increase of disease severity and poorer response to corticosteroids than eosinophilic asthma. Autosomal dominant hyper-IgE syndrome is a primary immunodeficiency caused by a mutation in STAT3, resulting in a deficiency in Th17 cells. These patients develop multiple staphylococcal and candidal infections, skin eczema and bronchiectasias, and display characteristic facial features with delay of shedding in primary teeth. [168, 171, 173, 175, 176, 182-184]

Th9 Cells: These cells arise from reprogrammed Th2 cells upon stimulation with TGF-β. To date no transcription factor driving Th9 polarization has been identified in humans, but in mice the myeloid master regulator PU.1 has been suggested to exert this role. Th9 cells produce IL-9 and IL-21 and have been involved in mucus production. IL-9 also contributes to mast cell growth and eosinophil survival during allergic inflammation in the airways. More recently they have been proposed to feature potent anticancer properties [185-189].

Th22 Cells: TNF-β and IL-6 provided by DCs represent the third signal necessary for Th22 polarization. The aryl-hydrocarbon-receptor (AhR) has been suggested as the transcription factor driving their polarization but it is not exclusively expressed in Th22 cells and does not explain all the complexity of the Th22 phenotype. These cells produce IL-22, TNF-α, IL-13 and IL-10 but not IFN-γ, IL-4 or IL-17 and express CCR4, CCR6 and CCR10. Chemokines that bind to these receptors are strongly expressed in the skin which explains the abundance of Th22 cells in this organ and their initial involvement in the immunopathology of skin diseases. Th22-cells protect epithelial barrier organs such as skin and lung by favoring wound healing

through the induction of epithelial cell proliferation and production of antimicrobial peptides. In addition, they have been implicated in the pathogenesis of atopic dermatitis by promoting the switch from an acute Th2 predominant disorder to a more complex Th1/Th17 chronic inflammation. [190-192]

FoxP3+ Regulatory T Cells: Regulatory T cells generated in the thymus during T cell maturation are called natural Tregs (nTregs) and are important to maintain tolerance to self-antigens. The regulatory T cells induced in the SLO are usually termed inducible Tregs (iTregs) or peripheral (p)Tregs and are responsible for the achievement of tolerance to external antigens and the downregulation of ongoing immune responses. The third signals driving the polarization to Treg phenotype are TGF-β and retinoid acid and both natural and inducible Tregs express the transcription factor forkhead box P3 (FoxP3). Tregs execute their regulatory activities through different mechanisms, including the production of anti-inflammatory cytokines such as IL-10 and TGF-β, and by cell-contact dependent suppression of effector T cells. Tregs play a vital role in fine-tuning the balance between effector and tolerogenic responses, and their dysregulation may cause autoimmunity or allergy. Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome is a rare immunodeficiency caused by mutations in FoxP3 gene and deficient generation of Tregs. These patients develop multiple autoimmune disorders and eczematous skin together with nail dystrophy and severe food allergy [193-199].

Tr1 Cells: These FoxP3- regulatory T cells are generated in the peripheral tissues and secrete high levels of IL-10. They also synthetize lower levels of TGF-β, IFN-γ and IL-5 and can exert cell-contact dependent suppression of immune cells. It is unresolved which mediators and transcription factors drive Tr1 differentiation but they can be generated after constant

exposure to peripheral antigens in the presence of IL-10 or the active form of vitamin D among other factors [200]. They are involved in the maintenance of healthy immune responses preventing allergy and other immune disorders. In this regard, allergen-specific immunotherapy, which is currently regarded as the only disease-modifying therapy for airway allergy and hymenoptera venom hypersensitivity, is believed to act through the generation of allergen-specific T regulatory cells, which possibly belong to the Tr1 phenotype [201].

Tfh Cells: IL-6, IL-21 and inducible costimulator (ICOS) induce the expression of the transcription factor Bcl6 in naïve CD4+ T cells and drive their differentiation into follicular helper T (Tfh) cells. Such cells are essential for the formation of germinal centers in SLO, and collaborate with B cells in isotype class switching and affinity maturation of antibodies. They also contribute to the generation of memory B cells. Some authors consider that Tfh cells represent a particular state of the main T helper phenotypes rather than a permanent terminal differentiation. [202]

Plasticity of T Cells

The existence of some CD4+ T cell effector classes is still controversial and sometimes Th9 or Tr1 are proposed to represent subsets of Th2 and Treg phenotypes respectively, rather than separate lineages. Other authors suggest that a certain degree of plasticity could exist for some effector classes during early differentiation states, where CD4+ T cells can be redirected to other phenotypes if the signals in the environment are strong enough [203-205].

T-Cell Homing

Naïve T cells migrate from peripheral blood to SLO, explore the lymphatic network in search of foreign antigens and return to blood in case they do not find their cognate ligands. If they recognize their specific antigens, naïve T cells become activated, proliferate and acquire effector properties before trafficking to the peripheral tissue where their cognate antigens were first encountered. This process is called T-cell homing and is fully dependent on specific tissue-homing receptors whose expression is imprinted during T cell priming in SLO. T cell migration to peripheral tissues occurs at a low rate during homeostasis but is higher during inflammation. The process is regulated by T cell interactions with the vascular endothelium and requires three sequential steps before cell extravasation: tethering-rolling (mediated by selectins and their ligands), activation of integrins (and their attachment to vascular ligands) and firm arrest of T cells (driven by chemokines and their receptors) [206]. In this regard, a role for CCR3-CCL28 interactions has been recently reported for the homing of T cells to the upper airways both during homeostasis and inflammation [207]

Memory T-Cell Subsets

During the resolution phase of inflammation, most effector CD8+ and CD4+ T cells die by apoptosis and only a minor population survives and differentiates into memory T cells. These memory CD8+ and CD4+ T cells can be further divided into central memory (Tcm) and effector memory (Tem) T cells [208] according to their surface expression of homing receptors. Similarly to naïve T cells, Tcm cells patrol SLO and after recognizing their cognate antigen undergo rapid and robust proliferation and migrate back to the tissues where the antigen was first encountered [209]. Akin to recently stimulated effector T cells, upon antigen recognition Tem cells rapidly execute effector functions such as cytolysis of infected

host cells but display a limited proliferative capacity. Moreover, Tem cells do not express the SLO homing receptor L-selectin/CD62L but are rather armed with homing receptors for peripheral tissues [208]. More recently a new subset of memory T cells lacking CD62L and CCR7 expression has been described and termed tissue-resident memory T cells (Trm cells) [210]. Trm cells differentiate from KLRG1- T cells that enter peripheral tissues during the acute phase of immune responses and adapt to the local environment in order to permanently reside in the tissue [211]. Trm cells are able to ignore tissue-egress signals and when located at mucosal surfaces, can resist shedding into the lumen. For instance, lung CD8+ Trm cells upregulate CD103 (integrin subunit αE of the αEβ7 dimer) expression that attach them to E-cadherin on respiratory epithelial cells [212]. Lymphocyte egress from SLO and thymus is dependent on sphingosine-1-phosphate (S1P) receptor [213], and it has been proposed that inhibiting S1P responsiveness might represent and important checkpoint for the generation of Trm cells [211]. In support of this hypothesis, the S1P receptor 1 (S1PR1)-antagonizing molecule CD69 is induced on Trm cells after their migration into sites of permanent residence [214]. Trm cells usually occupy the major barrier tissues of the body that are heavily exposed to environmental threats. CD8+ Trm cells maintain constitutive expression of granzyme B and upon viral re-infection they display a cytotoxic capacity similar to recently activated CD8+ T cells [215]. Moreover, Trm cells seem also involved in the maintenance of airway allergic responses. In this regard, resident Th2 memory cells were reported to be sufficient for the induction of airway hyper-responsiveness in animal models of allergic asthma [216] and allergen-stimulation of mucosal explants from AR patients induced IL-5 release by resident T cells [217].

b. B Cells and Immunoglobulins

The main role of B cells is to produce antibodies (also termed immunoglobulins), which can be either soluble or membrane-bound molecules. Membrane antibodies are usually called B cell receptors (BCRs) and determine the specificity of the B cells. An antibody molecule is composed of two identical heavy chains and two identical light chains. Soluble antibodies can be also divided into two functionally different fragments: the Fab (antigen-binding) fragment and the Fc (crystallizable region) fragment. The Fab fragment possesses the highly variable regions of the antibody that specifically bind to the antigen, whereas the Fc fragment contains the region attaching to immune receptors and determining the effector properties of the antibody [218]. Differences in the Fc fragment of the antibodies determine the various isotypes of immunoglobulins: in humans IgM, IgA, IgG, IgE and IgD [219].

B cells are generated in the bone marrow from lymphoid precursors that differentiate into pro-B cells. The next maturation steps involve the recombination of the genes for the V (variable), D (diversity) and J (joint) segments of the heavy chains followed by a similar V-J recombination of the light chains. These phenomena determine the antigen specificity of the B cell and give rise to IgM+ immature B cells which will finally differentiate into IgM+ IgD+ mature B cells. The combination of the previous processes is called antigen-independent B cell maturation and is followed by the migration of naïve B cells to the germinal centers of SLO where they reside [220]. The germinal centers are also populated by follicular DC cells that retain unprocessed antigens for long periods of time [18]. Mature B cells on germinal centers may take up these antigens and present them to Tfh cells in the context of MHC-II molecules. When a Tfh cell encounters its cognate antigen, it starts providing help to the presenting B cell to initiate its antigen-dependent maturation (germinal center reaction). Stimulated B cells originally produce antigen-specific IgM, but with the help of T cells most B

cells rapidly undergo isotype class switching [221]. The effector class of the CD4+ T cell has the potential to influence the terminal isotype of the antibody (mainly IgA, IgG or IgE). The antibodies will further increase their antigen-specificity in a process called somatic hyper-mutation that also requires T cell help [222]. After completing their differentiation, de novo generated memory B cells enter the circulation to differentiate into plasmablasts that after migration to the bone marrow or peripheral tissues differentiate into long-lived plasma cells. Similarly to T cells, the migration is dependent on the expression of specific tissue-homing receptors. When the antigen driving the stimulation was first encountered at the Waldeyer's ring, B cells are induced to express α4β1 and CCR10 which allow them to migrate to the upper airways [223].

The production of high-affinity IgE is tightly regulated at the molecular and cellular level, resulting in serum concentrations that are several orders of magnitude lower than that of either IgG or IgA. Unlike B cells producing IgG, recently switched IgE+ B cells often rapidly differentiate into plasma cells [224] and B cells in IgE-producing germinal centers undergo high levels of apoptosis [225]. However, the generation of high-affinity IgE can be maintained by plasma cells through a low level switching from IgG to IgE [225].

Immunoglobulins at the Respiratory Mucosa

The most abundant immunoglobulin isotype at mucosal surfaces is IgA. A complex of two IgA molecules attached to a J (joining) chain is produced by plasma cells in the lamina propia of the mucosae [226]. This dimeric IgA is taken up by epithelial cells and transported by transcytosis to the luminal surface using the polymeric immunoglobulin receptor (pIgR). A complex formed by parts of pIgR and dimeric IgA, jointly called secretory IgA (SIgA), is cleaved from the membrane of epithelial cells and released into the lumen of the airways [227] . SIgA acts as a neutralizing antibody coating luminal microbes and limiting their

contact with stromal and immune cells. Respiratory and intestinal mucosae host the largest populations of IgE-producing plasma cells in humans [228]. Importantly, class switching to IgE can occur in the respiratory mucosa of patients with AR [229], nasal polyposis [230] or asthma [231]. Even though it is not clear whether mucosal IgE switching is necessarily associated to germinal center-like structures, lymphoid aggregates were detected in the nasal mucosa of patients with nasal polyps [230]. Mucosal IgE can undergo luminal transcytosis through CD23 expressed on respiratory epithelial cells [232]. Moreover, allergen-specific IgE constitutes a much higher proportion of total IgE in the respiratory secretions than in the blood of patients with AR, implying that local IgE production is important for mast cell sensitization and local allergic reactions [233]. IgD is also produced by some plasma cells in the nasal mucosa, and even though the nature of its receptor remains elusive, IgD is able to activate basophils in response to microorganisms commonly found in the nasal cavity [234].

A summary of the generation of adaptive immune responses in the respiratory mucosa can be seen in **Figure 6**.

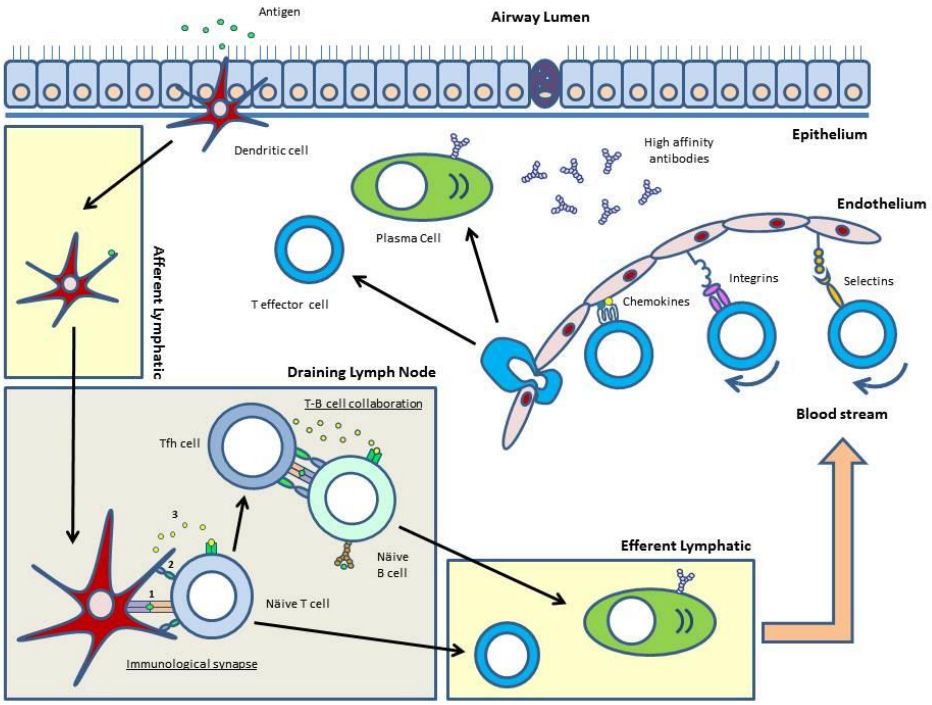

Figure 6: The Generation of Adaptive Immune Responses in the Respiratory Mucosa

Mucosal dendritic cells (DCs) catch up luminal antigens and transport them via afferent lymphatics to the T-cell rich para-cortical area of the draining lymph nodes. Processed antigens are presented in the context of MHC-II molecules to the TCR of CD4+ T cells (1). The priming of CD4+ T cells also requires the ligation of co-stimulatory molecules (2) between CD4+ T cells (CD28, CD40L) and DCs (after TLR stimulation APCs express CD80, CD86, CD40), and the presence of one or various cytokines (3) that polarize the activating CD4+ T cell into an specific effector class. After priming, CD4+ T cells migrate to the B-cell rich germinal centers where they may recognize their cognate antigen presented by B cells. B cells can catch up their cognate antigen by the BCR, and also express MHC-II and co-stimulatory molecules. CD4+ T cells produce cytokines that help B cells to perform isotype class switching and somatic hypermutation of antibodies. The resulting effector T cell and plasma cell exit the lymph node through the efferent lymphatics and travel back to the respiratory mucosa. T cell and plasma cell extravasation in peripheral tissues depends on specific homing receptors whose expression is imprinted during T/B cell activation in the lymph nodes. Lymphocyte extravasation consists on three consecutive steps mediated by selectins, integrins and chemokine receptors. Tfh cell: T follicular helper cell.

Airway Microbiota

Until recently, the airways were thought to be sterile unless infected; however the new methods for the quantification and sequencing of microbial nucleic acids have revealed that the airways harbor a steady-state microbiota [235, 236]. In the respiratory tract bacteria represent >80% of the microorganisms during homeostasis. The most prevalent phyla are Bacteroides and Firmicutes, and to a lesser extent Proteobacteria and Actinobacteria [237]. The early colonization of the airways by microorganisms after birth has been related to the generation of mature tolerogenic DCs by inducing the expression of the suppressive transmembrane protein programmed death ligand (PD-L)1/CD274 [238]. This effect would represent one of the first steps in the generation of regulatory T cell responses to harmless external antigens. Moreover, the modification of the normal airway microbiota could promote inflammatory diseases. The exposure to cigarette smoke and pollutants damages the respiratory epithelium and promotes bacterial dysbiosis with associated outgrowth of pathogenic bacteria [239]. This alteration activates several inflammatory pathways leading to the accumulation of macrophages and neutrophils in the airways. Furthermore, the asymptomatic colonization of the nasopharynx with Streptococcus was has been related to asthma development in children [240], and the lung microbiota of patients with poorly controlled asthma is characterized by an outgrowth of Haemophilus influenza and Tropheryma [241]. Nevertheless whether these changes related to asthma represent a cause or a consequence of the disease remains to be elucidated.

4. Pathomechanisms of Allergic Inflammation in the Airways

One of the main tasks of the immune system in the airways is to discriminate between harmful threats and harmless antigens in order to both mount defensive responses against pathogens and induce tolerance to other antigens. The airways are constantly exposed to many environmental agents such as infectious bacteria and viruses, allergens or dust particles. The development of tolerance is crucial to prevent over-reaction in the lower airways and to preserve the delicate architecture of the lung parenchyma where gas exchange takes place [20]. Airway allergy represents an important failure of this function of the immune system [242]. Aeroallergens (e.g. pollen, house dust mites, dander from furry animals, fungi, cockroach…) are neither invasive nor life-threatening and tolerance is the natural response to them as occurs in healthy non-atopic individuals.

Mechanisms driving Allergen Sensitization

The mechanisms driving allergen sensitization are heterogeneous and not fully understood. Allergens can be inhaled concomitantly with microbes and the activation of PRRs by the later could lead to the misinterpretation of the former by the immune system [243]. Moreover, some allergens are enzymatically active molecules (proteases) [244] with the ability to cleave tight junctions between respiratory epithelial cells gaining thus access to deeper layers of the mucosa. Some of these proteases are able to directly activate PRRs on stromal or immune cells partially explaining the initiation of immune responses against allergens [245-247]. Tissue injury generated by protease allergens promote the release of danger signals such as uric acid or ATP by damaged cells that activate in turn the inflammasome machinery in DCs and macrophages further boosting the immune system [248].

Nevertheless, not every aeroallergen is a protease and healthy individuals are exposed to the same burden of antigens without developing symptoms. To further complicate the issue, not every individual with detectable allergen-specific IgE (sensitized) experienced rhinitis upon exposure to the relevant allergen (allergic) [249]. This fact possibly implies that the development of symptoms relies more on the balance between allergen-specific Th2 cells and tolerogenic Tregs than on the mere existence of allergen-reacting T cell clones. Of note, allergen-specific immunotherapy, which is still regarded as the only disease-modifying treatment for airway allergy, is believed to act earlier through the generation of regulatory T cells rather than the depletion of existing allergen-specific Th2 cells [174, 201]. There is a clear familiar association of allergic diseases, implying that genetic factors are also involved in the pathogenesis. A large meta-analysis of genome-wide association studies performed in asthma patients concluded that only 5 genes were significantly linked to the development of asthma, of which 3 of them were related to factors produced by stromal cells: TSLP, IL-33 and its receptor ST2 [250].

Moreover, the rapid increase in allergy over the last decades implies that environmental factors probably play a role. Concomitant exposure to allergens and pollutants is suggested to promote sensitization, maybe via the PRR-activating potential of the later agents [251]. Additionally, the hygiene hypothesis suggests that the decreased exposure to pathogens during infancy associated to a westernized lifestyle lead to a defective immune maturation resulting in increased risk to develop allergic diseases [252]. In this regard, it has been recently proposed that being raised in a farming environment is associated to LPS-mediated induction of the enzyme A20 on respiratory epithelial cells. This phenomenon could modify the communication of the epithelium with resident DCs and ultimately protect the individual from allergy and asthma [253].

Sensitization Phase of Allergic Inflammation

The first step in the allergy response involves the activation of mucosal-resident DCs. Allergen molecules can activate TLRs directly on professional APCs, or more often on epithelial cells or other stromal cells. Epithelial cells will release in turn pro-Th2 cytokines (TSLP, IL-25, IL-33) that will indirectly activate resident DCs to become immunogenic [84, 254]. ILC2s can also be triggered by epithelium-derived factors and release Th2 cytokines that will subsequently stimulate DCs [159]. Activated DCs will take up the allergen, process it intracellularly and migrate to the regional lymph nodes via lymphatic vessels. The activation of DCs induces the expression of lymph node-specific homing receptors such as L-selectin and CCR7 [76, 84]. During the migration to lymph nodes DCs up-regulate co-stimulatory molecules (second signal) to ensure a successful immunological synapse. In recent years important progress in the understanding of the functional specialization of mucosal DCs has been done in animal models of allergic asthma [157]. CD11b+cDCs (mouse counterpart of human CD1c+cDCs) were shown to be the main subset capturing the allergen and migrating to the lymph nodes (in other words, driving sensitization) whereas epithelial CD103+cDCs (mouse counterpart of human CD141+cDCs) were unable to exert these roles [45]. When mature allergen-bearing DCs arrive to the lymph nodes they can activate allergen-specific naïve T cells in the T-cell rich para-cortical area. If IL-4 is provided by basophils or other immune cells, the naïve T cells will acquire a Th2 phenotype and upon migration to the germinal centers they will activate the B cells that present the allergen in the context of MHC-II molecules [76]. Th2 cells secrete IL-4 that help allergen-specific IgM+ B cells to perform isotype class switching to IgE followed by somatic hyper-mutation and ultimately produce high affinity allergen-specific IgE.

A summary of the sensitization phase of allergic inflammation can be seen in **Figure 7**.

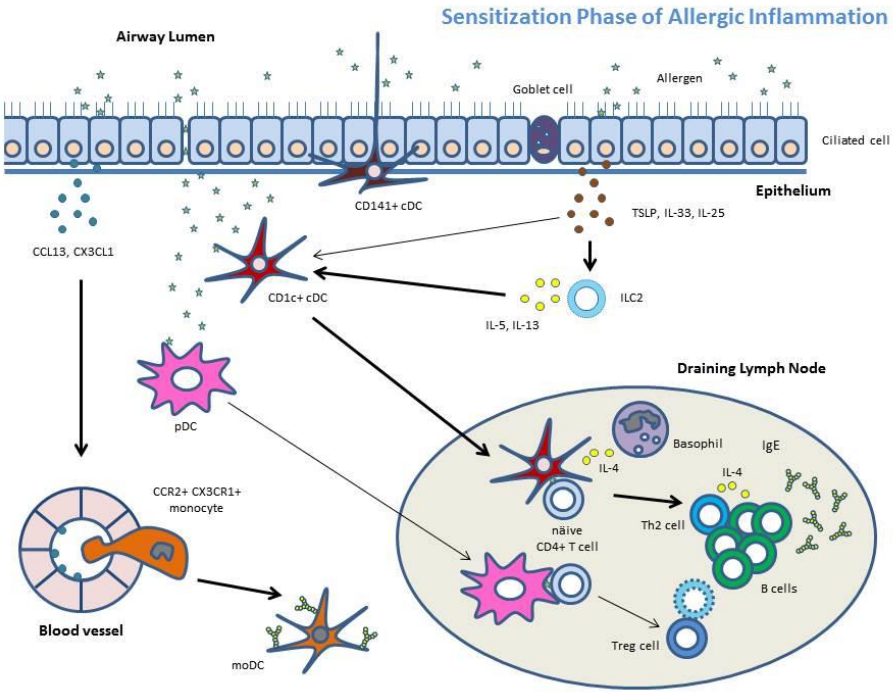

Figure 7: Sensitization phase of allergic inflammation

Protease allergens can disrupt the tight junctions between respiratory epithelial cells and directly activate mucosal dendritic cells (DCs) in the lamina propia, or more often, indirectly through the stimulation of epithelial cells and innate lymphoid cells 2 (ILC2s). Activated DCs catch up the allergen and acquire a mature phenotype while they migrate to the lymph nodes. CD1c+ conventional DCs (cDCs) are the main drivers of allergen sensitization by efficiently priming allergen-specific CD4+ T cells towards a Th2 phenotype. The IL-4 required for Th2 polarization is provided by basophils among other innate immune cells. Primed Th2 cells will subsequently collaborate with allergen-specific B cells to generate high affinity allergen-specific IgE. Plasmacytoid DCs (pDCs) might be involved in the generation of allergen-specific regulatory T cells (Tregs) but in allergic individuals they are not enough to compensate pro-inflammatory Th2 cells. The respiratory epithelium secretes chemokines in response to allergen stimulation. These chemoattractants will recruit in turn CCR2+ monocytes from the circulation which in inflammatory settings differentiate into inflammatory monocyte-derived DCs (moDCs).

Effector Phase of Allergic Inflammation

Allergen re-exposure in some sensitized individuals induces an inflammatory response that is temporally divided in an immediate and late phase reaction.

a. The Immediate Reaction

The IgE generated during the sensitization phase will bind to FcεRI on the surface of resident mast cells (MCs) [255]. This receptor on MCs and basophils is a tetrameric transmembrane complex comprised by one α chain with a single IgE-binding site, one β chain with various transmembrane domains and two γ chains with intracellular immune-receptor tyrosin-based activation motifs (ITAMs) to transmit the activatory signal [256]. Among all the different Fc receptors, FcεRI has the highest affinity (Kd: 10^{-10}) for its cognate ligand, meaning that during homeostasis FcεRI receptors are saturated by IgE molecules [256]. The cross-link of two or more IgE/FcεRI complexes by a single polyvalent allergen molecule induces the immediate energy-dependent degranulation of the pre-formed mediators stored in MCs granules (histamine, tryptase…). MCs and basophil activation also leads to the de novo synthesis of eicosanoids (leukotrienes and prostaglandins) within minutes [257]. MC degranulation promotes vasodilatation, blood vessel leakage, edema and mucus secretion from glandular and goblet cells which results in sneezing, itching, rhinorrhea and nasal blockade in the nose and obstruction in the bronchi. Furthermore, cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) are potent broncho-constrictors able to act on smooth muscle inducing bronchospasm and trigger asthma attacks [258]. Moreover, MC and basophil activation induces the synthesis within hours of different Th2 cytokines with the ability to tailor the late immune reaction [257].

b. The Late Phase Reaction

The activation of epithelial cells by the allergen triggers the release of chemo-attractants such as CCL13 and CX3CL1 [259, 260] which act as recruiters of CCR2+ CX3CR1+ CD14+ monocytes from the circulation [27]. In experimental animal models of allergic inflammation [76] and in human disease [42] these cells have been reported to differentiate into inflammatory DCs under the influence of Th2 cytokines and to act as potent inducers of inflammation. In mouse models of allergic asthma, monocyte-derived DCs (moDCs) were shown to accumulate at high numbers in the challenged tissue [261] and to release vast amounts of chemokines such as CCL17, CCL22 and CCL24 [45]. These mediators are able to recruit CCR3+ CCR4+ CCR8+ circulating memory Th2 cells that accumulate at the sites of allergen exposure together with moDCs. Both in mice and humans inflammatory DCs express a trimeric form of FcεRI (lacking the β chain) [256] and thus display IgE on the surface [43]. Memory Th2 cells need to be reactivated to be functional and this re-stimulation is performed locally by moDCs which capture the allergen utilizing their surface IgE [45]. Attachment of the allergen to IgE/FcεRI induces a rapid internalization of the complex with subsequent processing and presentation of allergen peptides to recruited memory Th2 cells. This modality is usually called “IgE-facilitated antigen presentation” and has been reported to be 100-1000 times more efficient than regular antigen presentation [262]. Re-activated Th2 cells release IL-4, IL-13 and IL-5 that will promote the inflammatory response. In this regard, IL-5 and other inflammatory DC-derived chemokines will induce the recruitment of CCR3+ circulating eosinophils [138]. Inflammatory DCs are regarded as the main local orchestrators of allergic inflammation in mouse models of allergic asthma [157], but their contribution to AR and asthma in humans has not been confirmed and this was the aim of the first and second papers included in this thesis. Recruited eosinophils accumulate at high

numbers in the mucosa and lumen of the airways and their protease mediators together with IL-13, promote airway remodeling (thickening of the basement membrane, goblet cell metaplasia and smooth muscle hyperplasia) [177]. Airway hyper-responsiveness, the key clinical feature of asthma, can be partially explained by these structural changes that in patients with long-lasting inflammation become increasingly irreversible.

A summary of the effector phase of allergic inflammation can be seen in **Figure 8**.

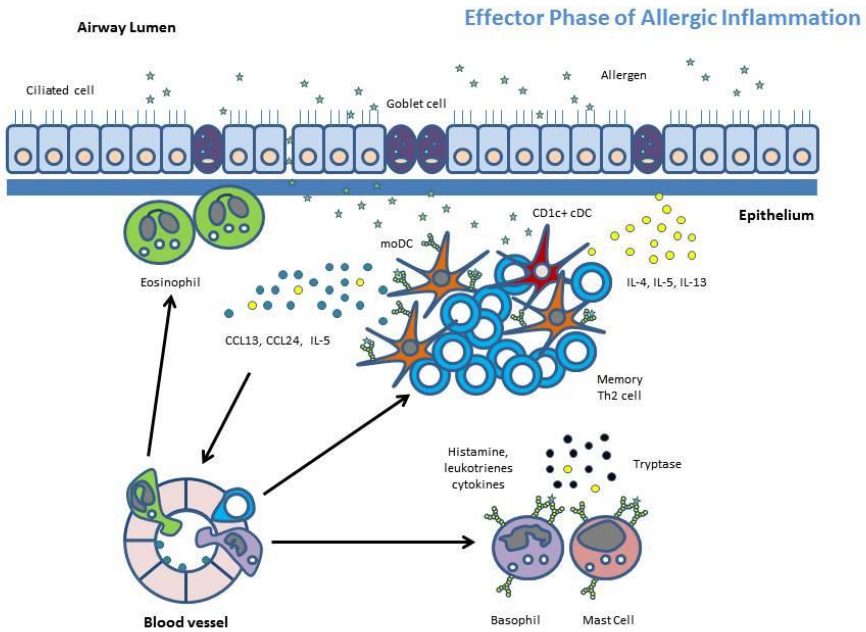

Figure 8: Effector phase of allergic inflammation

During ongoing allergen exposure, inflammatory moDCs secrete different chemokines that recruit in turn Th2 cells, eosinophils and basophils. Th2 cells form lymphoid clusters with DCs in the allergic mucosa and are locally reactivated by IgE+ inflammatory moDCs. Re-activated Th2 cells release type 2 cytokines that further the inflammatory response. IgE/FcεRI complexes on basophils and mast cells can be cross-linked by a single polyvalent allergen molecule, thus inducing the release of high amounts of pre-stored mediators (histamine, tryptase…) and start to synthetize eicosanoid and cytokines. Recruited eosinophils after stimulation by type 2 cytokines release toxic mediators and reactive oxygen species (ROS). Eosinophil mediators and IL-13 induce airway remodeling (thickening of the basement membrane, goblet cell metaplasia…) in chronic allergic inflammation.

AIMS

The main objective of this thesis was to study the role of the mononuclear phagocyte system (MPS), and in particular monocytes, in the pathogenesis of allergic airway diseases. Because novel information from studies in mice suggests that macrophages are embryonically-derived, long-lived cells, independent of monocyte recruitment, we also wanted to investigate the longevity of human alveolar macrophages.

Specific aims:

a. To examine the involvement of the MPS, including the recruitment of circulating monocytes, in an in vivo experimental model of allergic rhinitis.

b. To examine the recruitment of monocytes into the lower airways of patients with fatal allergic asthma attacks.

c. To determine the longevity of human alveolar macrophages and assess whether circulating monocytes can give rise to alveolar macrophages in vivo.

SUMMARY OF RESULTS

Paper I

In this study we found that CD14+ monocytes rapidly (within hours) accumulate in the allergen-challenged nasal mucosa from allergic rhinitis (AR) patients whereas DCs accumulate after days of continuous challenge. Global transcriptomics of sorted CD45+HLA-DR+ cells from the nasal mucosa of six AR patients showed that several chemokine genes (CCL13, CCL17, CCL18 and CCL24) were up-regulated after one week of continuous pollen challenge. The cognate receptors for these chemokines are known to be expressed in cells involved in Th2 immune responses, and we found increased numbers of both mucosal Th2 cells and eosinophils paralleling the accumulation of CD14+ monocytes.

Paper II

Analysing tissue specimens from challenged AR patients we found that co-expression of CD68 and S100A8/A9 was a reliable method to identify recently recruited monocytes. Using this information we demonstrated that monocytes were significantly increased in the bronchial mucosa and in the alveoli of fatal asthma patients compared with control individuals. Interestingly, aggregates of CD68+S100A8/A9+ monocytes obstructing the lumen of bronchioles were found in asthmatics but not in controls. We also found that the vast majority of accumulating monocytes both in the lungs and nasal mucosa, expressed matrix metalloproteinase 10, suggesting that this protein may be involved in their migration within the tissue.

Paper III

In this study we assessed the longevity of alveolar macrophages (AMs) by examining their replacement kinetics in lung transplanted patients without rejection. We found a rapid decrease in the density of donor-derived AMs paralleled by a rapid increase of incoming monocytes (2 weeks post-transplantation). However, between 2 weeks and a follow up period of two years post transplantation the density of donor AMs remained stable, demonstrating that AMs in humans are long-lived cells. Furthermore, two years after transplantation, incoming monocytes appeared to have developed into mature AMs intermingled with donor-derived cells. To further validate this finding in vivo we analyzed lung biopsies from immune-deficient mice reconstituted with human cord blood mononuclear cells (humanized mice) and we demonstrated that the lungs of those animals contained human AMs, which were likely derived from human monocytes. To study the mechanisms of AM self-maintenance we examined their proliferative ability in lung transplanted patients without rejection and we found a significant proportion of proliferating AMs over time. Interestingly we detected both donor- and recipient-derived proliferating AMs, indicating a potential for long-term self-maintenance of AMs.

METHODOLOGICAL CONSIDERATIONS

This section discusses advantages and limitations of the main methods included in this thesis. A detailed description of material and methods can be found in the methods sections of the individual manuscripts.

In vivo Allergen Challenge

In paper I and II, several cohorts of allergic rhinitis (AR) patients and healthy non-atopic control subjects were recruited. Patients were selected on the basis of confirmed allergic sensitization to pollen and a clinical history compatible with AR for a minimum of three pollen seasons. Sensitization can be assessed by skin prick test (SPT) or the measurement of specific IgE (sIgE) in serum. SPT attempts to provoke a small, controlled allergic response (a wheal) which will reveal the presence of IgE attached to skin mast cells. Both SPT and sIgE are equally accepted to assess allergic sensitization, yet SPT is easier and cheaper. On the other hand serum sIgE allows the interrogation for specific allergens (proteins) in addition to allergenic sources and is superior to guide allergen-specific immunotherapy. In paper I and II we assessed allergic sensitization by SPT.

All AR patients and non-atopic controls were challenged intra-nasally with the relevant allergen for different periods of time ranging from 1 to 7 days. The allergen was self-administered by the participants with a hand-driven pump that delivered a fixed amount of 50 µl (100.000 SQ/ml in paper I and 30 HEP/ml in paper II). All the provocation protocols induced typical allergic symptoms and increases in mucosal eosinophils in the patients but not in the control subjects, thus validating our experimental model of AR. In this thesis we used extracts of two different pollens: timothy grass (Phleum pratense) and birch (Betula alba). In previous studies [84, 102] and in papers I and II of this thesis we demonstrate that

both pollen allergens produce a very similar inflammatory response in allergic individuals. This model induces a controlled and synchronized inflammation and enables the study of factors affecting the immune system during respiratory allergy in vivo. One of its limitations is that the administered dose is stable, in contrast with the pollen season where the allergen dose is highly variable.

Several methods have been described to perform allergen nasal challenges for both diagnostic and research purposes [263]. The different devices used to administer the allergen intra-nasally include syringes, nose droppers, micropipettes, nasal sprays and impregnated cottons or disks. Syringes and nose droppers have an unpredictable area distribution with associated risk of laryngeal or bronchial aspiration whereas application by micropipettes or impregnated devices requires some training. Therefore we decided to use a nasal spray which ensures a higher delivery to the nasal cavity and less leakage into bronchi while being easy and reproducible and thus constituting an optimal option for self-administration at home.

Biopsies from Human Respiratory Tract

All the studies included in this thesis used specimens from human respiratory tract. In paper I and II the nasal biopsies were obtained from the lower edge of the inferior turbinate and in paper III the lung parenchyma was biopsied trans-bronchially. In both cases topical anesthesia was pre-administered but the individuals remained conscious during the procedure. In paper III open lung biopsies from cancer patients undergoing thoracic surgery were obtained under general anesthesia from areas of the parenchyma distal from the tumor. In paper II autopsy lung specimens from individuals dying from asthma attack or traffic traumatism were utilized. In both cases the biopsies were considerably larger than those obtained by topical anesthesia. Performing thoracic surgery is not ethically acceptable

unless the patient fulfills well defined diagnostic or therapeutic indications. Trans-bronchial biopsies can have life-threatening complications including bronchial hemorrhage, decrease in arterial oxygen, pneumothorax or incoercible hemoptysis and in general they must be warranted by clinical necessities [264]. On the other hand, nasal biopsies can be taken with research purposes because complications associated to them are limited and largely preventable (e.g. the use of topical vasoconstrictor prevents epistaxis). The size of the nasal biopsies from paper I was not sufficient to perform both flow cytometry-based cell sorting and immunohistochemistry (IHC) in the same tissue specimens and therefore several cohorts were necessary to complete these studies.

Animal Models

In the third paper included in this thesis, NOD/SCID/IL-2Rγ-null (NSG) mice [265, 266] were engrafted with human cord blood mononuclear cells to generate humanized mice. NSG mice are severe combined immunodeficient (SCID) animals that display a complete absence of T cells and B cells and more profound defects in innate immunity than other SCID strains. These abnormalities include the lack of complement system and NK cells and reduced activity of antigen presenting cells [267], making them good candidates for transplantation studies with human cells. However, some authors consider that humanized mice are poor models for myeloid development because mouse growth factors do not cross bind human receptors. Nevertheless it was shown that hematopoietic stem cell (HSC)-engrafted NSG mice developed mature and functional human myeloid subsets [266] and in paper III of this thesis we proved that AMs in cord blood-reconstituted NSG mice arose from human cells 10 weeks after engraftment. In this regard, we hypothesize that HSCs or cord blood cells could act as an autocrine or paracrine source of the human growth factors required for myeloid development in humanized mice.

Immunofluorescence Staining and Fluorescence in situ Hybridization

In situ identification and phenotyping of cells in the papers included in this thesis was performed by immunofluorescence staining (IFS). This technique is a variant of IHC that uses fluorochromes as reporter molecules as opposed to enzyme-based chromogenic detection. The protocols of IFS involve using primary antibodies (up to 4 different in our set-up) of different isotypes or species that can be recognized by secondary fluorescent labeled antibodies. Primary antibodies in IHC can be either monoclonal (produced by a single clone of B cells) or polyclonal. Polyclonal antibodies are heterogeneous mixtures of immunoglobulins that recognize different epitopes within a protein or peptide of interest. The signal provided by either chromogenic or fluorescence detection can be enhanced using amplification methods. For IFS the most common enhancement involves using a secondary antibody combined with biotin which is detected by a tertiary reagent composed by streptavidin labeled with a fluorochrome.

Tissue sections utilized in the studies included in this thesis were either cryosections that were frozen at -20 degrees after acetone fixation, or biopsies fixed in formalin and subsequently embedded in paraffin. Tissue architecture in paraffin sections is better preserved than in cryosections but the protein epitopes can be chemically masked or conformationally altered during formalin fixation. To override this limitation, paraffin sections have to be subjected to antigen retrieval which aims at reversing these chemical modifications of the epitopes. In the papers included in this thesis antigen retrieval was performed by heat treatment using either high or low pH buffers. In this regard, heat acts by cleaving cross-links, exposing buried epitopes and promoting protein unfolding or refolding.

In paper III of this thesis the identification of the sexual chromosomes (X/Y) was performed by fluorescence in situ hybridization (FISH). FISH is a cytogenetic technique that uses

fluorescence probes that hybridize to only those parts of the chromosome with a high degree of sequence complementarity. X/Y-FISH detects segments of nucleotides that are specific of either X or Y chromosome. The probes are tagged directly with fluorophores using different methods such as nick transplantation or polymerase chain reaction with tagged nucelotides.

Fluorescence Microscopy

In both IFS- and FISH-subjected sections fluorescence microscopy was used for analysis. This variant of light microscopy uses fluorescence or phosphorescence instead of (or in addition to) reflection and absorption to study the properties of organic or inorganic substances. For counting the cells in the nasal mucosa, a conventional wide-field fluorescence microscope was utilized. With this microscope the entire specimen is excited evenly by a light source and the resulting fluorescence detected by the camera includes a large part of unfocused background. For counting the cells in the lung biopsies, for analysis of the chromosomal configuration in paper III and for image acquisition for illustration in all the papers a confocal microscope was used. This modality of fluorescence microscopy utilizes an optical pinhole that allows point illumination to eliminate out-of-focus signal. As only light produced by fluorescence very close to the focal plane is detected, the optical resolution is much better than the one provided by wide-field microscopes. However, much of the light generated from the section fluorescence is blocked by the pinhole and obtaining increased signal resolution with confocal technology often requires longer exposures than those of wide-field microscopes.

Flow Cytometry-based Cell Sorting

Flow cytometry (FC) is a well stablished technology with multiple uses in clinical diagnostics, medical therapy and scientific research. Flow cytometers are essentially fluorescence microscopes that collect light signals from cells (or other particles) flowing across a laser interception point by photomultiplier tubes (PMTs) fitted with barrier filters with specific wave-length cutoffs. This method allows interrogating the cells for the presence of fluorescence and light scattering signals that correlate to protein expression and cell morphology respectively. The cells must be put in suspension and pressurized into a fluid stream for analysis. This stream contains aligned cells that sequentially intersect with one or more laser beams (up to 4) placed orthogonal to the flow of fluid. By using multiple lasers of different wavelengths, the potential number of fluorochrome-labeled markers that can be detected is increased up to 15, as fluorescence signals are emitted sequentially from the same cell intersecting each laser. Fluorochromes are defined by a range of light wavelengths that add energy to the fluorochromes (the excitation spectrum), causing them to emit light in another range of wavelengths (the emission spectrum). When emission spectra from different fluorochromes overlap, the fluorescence from more than one fluorochrome may be detected. In this scenario, a process of compensation is needed to ensure that the fluorescence detected in a particular detector derives from the fluorochrome that is being measured.

In the paper I of in this thesis FC-based cell sorting was used to separate the cells undergoing microarray analysis. In this technique the fluid containing the cells passes across a nozzle with a defined size. During cell sorting the nozzle is applied a slight vibration to generate small waves on the surface of the jet as it emerges from the nozzle, causing it to break into regular droplets downstream of the point of laser intersection. An electric charge is applied

to the drops containing the cells with the desired combination of fluorescent and light scattering parameters. These cell-containing drops can be thus deflected in an electric field and collected in a tube while the remaining uncharged drops are discarded. The amount of charge applied will affect the degree of deflection of individual drops, implying that multiple populations of cells can be separated simultaneously by using charges of different polarity and intensity.

Immunofluorescence Staining versus Flow Cytometry

IFS and FC are among the commonest techniques utilized for cell phenotyping studies. For detailed characterization of cell populations FC is usually superior because permits the interrogation for >10 surface or intracellular markers whereas IFS is usually restricted to a maximum of 4 molecules. Additionally, the information on cell morphology provided by FC is usually more accurate and objective whereas in IFS depends usually on researcher's interpretation. On the other hand, IFS excels in the study of the relationships of a cell population with the surrounding tissue, as it gives precise information of tissue architecture, whereas the investigation of cell distribution by FC is limited to broad tissue compartments (e.g. epithelium versus lamina propia). In the three papers included in this thesis cells with a specific phenotype were quantified by directly counting them superposing a grid on tissue specimens subjected to IFS. The election of the technique was imposed by the reduced size of the nasal and trans-bronchial biopsies, but IFS is probably superior to FC for cell quantification in most circumstances. In a recent mouse study investigating the presence of memory T cell subsets in different organs, cell suspension followed by FC was reported to significantly underestimate the total number of memory CD8 T cells, whereas the total number of nucleated cells as determined by IFS was similar to that obtained by whole organ DNA content [268]. Furthermore, this discrepancy did not affect evenly the different tissues

or tissue compartments. Many mucosal sites, including the lung, contained 50- to 70-fold more memory CD8 T cells when evaluated by IFS as compared to FC, whereas in lymph nodes this difference decreased to <2 folds. Moreover, the analyzed cells in mucosal surfaces were more efficiently recovered from the epithelium than from the lamina propia. These phenomena could lead to the conclusion that some organs or tissue compartments preferentially host the population of interest when in fact the differences with other anatomical locations are much smaller.

Analysis of Microarray Data

Microarray analysis is a transcriptional profiling method enabling the study of the expression level of thousands of genes simultaneously, usually by comparing a basal state with an altered (often pathological) condition. A DNA microarray is a collection of microscopic DNA spots attached to a solid surface. Each spot contains multiple identical unique strands of DNA, known as a probe. Each spot represents one gene, and is a short sequence of a gene transcript that can pair with a complementary DNA strand in the process of hybridization.

In paper I of this thesis we used transcriptional profiling to discover genes central to the allergic response. A subset of cells from nasal mucosa defined by their surface expression of CD45 and human leukocyte antigen (HLA)-DR was sorted out before and after allergen challenge. The amount of cells required for microarray analysis represents one of the limitations of this technology. The number of CD45+HLA-DR+ cells separated by FC-based cell sorting from a cell suspension of digested nasal mucosa ranged from 1000-6000 cells/biopsy. This yield did not permit the analysis of the gene expression in the separate subsets of mucosal macrophages and DCs, meaning that all the distinct APC subsets had to be studied as a whole population. This constituted one of the limitations of paper I of this thesis.

Statistical Analysis

Due to small number of samples in many experiments, the normality of data distribution could not be assumed in the studies included in this thesis and it was assessed by Kolmogorov-Smirnov test. Normally distributed samples were compared by either t-student for paired/unpaired samples (2 time points) or one-way analysis of variance (ANOVA) for matched/unmatched observations followed by Newman-Keuls test (>2 time points). Paired measurements not normally distributed were analyzed by either Wilcoxon test (2 time points) or Friedman test followed by Dunn's test (>2 time points). Unpaired not normally distributed samples were compared by Mann-Whitney test (2 time points) or Kruskal-Wallis test followed by Dunn's test (<2 time points). GraphPad Prism 5.0 was utilized to perform the analysis and a p value <0.05 was considered significant.

DISCUSSION

Discussions on the isolated results of the individual papers can be found in the respective manuscripts. This chapter provides an integrated discussion on the results in relation to the aims of the thesis.

CD14+ classical monocytes are regarded as potent pro-inflammatory cells that produce many different cytokines upon activation. However, after entering the tissue, monocytes have been shown to differentiate into dendritic cells (DCs) and anti-inflammatory macrophages. The classical immunological dogma postulates that blood monocytes constantly migrate to the peripheral tissues where they maintain the pool of tissue-resident macrophages [30]. This idea was later amended by studies in mice demonstrating that microglia cells, Langerhans cells and alveolar macrophages (AMs) are derived from precursors in yolk sac and fetal liver that populate peripheral tissues during embryogenesis. These progenitors differentiate to macrophages locally and are self-maintained throughout life with minimal contribution of bone marrow-derived precursors [32, 56, 57]. Nevertheless in organs like the intestine, tissue macrophages are clearly replenished by blood monocytes [36]. In parallel, different animal studies showed that during inflammatory responses large numbers of CD14+ monocytes were recruited to the airways and did not differentiate into regulatory macrophages but into inflammatory DCs [45, 269, 270]. These monocyte-derived DCs (moDCs) shared many markers with CD11b+/CD1c+ conventional (c)DCs but retained expression of CD14 and became positive for FcεRI and other inflammatory markers [29, 73]. Inflammatory moDCs have been described to play distinct roles in respiratory infections [271], asthma [45] and dermatitis [272]. During viral infections they contribute to host defense by releasing type I interferons which limits the replication of the virus within

infected cells [273], but over-activation of inflammatory moDCs was also related to acute lung injury and death from respiratory insufficiency [77]. Animal models of both allergic asthma and respiratory infections have demonstrated that these moDCs are the master local orchestrators of airway inflammation by recruiting and reactivating memory T cells [76, 274]. In addition, inflammatory moDCs have been reported to migrate to the lymph nodes and drive sensitization in mouse models of asthma, but they were much less efficient in this task than CD11b+ cDCs [45]. To further complicate this issue in humans during homeostasis a small proportion of dermal and intestinal DCs are suggested to be derived from CD14+monocytes [39, 80, 82].

In papers I and II we show that the recruitment of monocytes is part of the inflammatory response observed in patients with allergic rhinitis (AR) and allergic asthma. Work from our group has shown that the APC network during homeostasis in the nasal mucosa consists of spatially closely related macrophages and DCs [275]. DCs in the human intestine can be further divided into CD1c+SIRPα+CD103+ and CD141+CD103+SIRPα- cDCs, CD103-SIRPα+ DCs and CD123+ pDCs [39]. Tissue cDCs and pDCs originate from blood DC precursors in humans [276], whereas CD103-SIRPα+ DCs are thought to be monocyte-derived [39]. Unlike AMs, mucosal macrophages seem to be constantly replenished by circulating monocytes. This suggests that CD14+ monocytes recruited to the upper airways during homeostasis can differentiate into two distinct populations: resident macrophages that retain expression of CD14 and CD103-SIRPα+ DCs that become CD14 negative [39]. Applying the same in vivo model of AR that we used in the papers I and II we have shown that both CD1c+cDCs and pDCs significantly increase after several days of continuous nasal challenge in patients with AR, whereas the number of CD141+ cDCs remained unaltered [84, 102]. In paper I of this thesis we found that AR patients but not non-atopic controls experienced a rapid

recruitment of CD14+ monocytes to the challenged mucosa, within hours after allergen exposure. In addition, results in paper II also suggest that the massive recruitment of monocytes is also an essential part of the inflammatory response in patients with asthma. Of note, in fatal asthma patients but not in non-atopic controls aggregates of monocytes were found in the airway lumen, implying the migration of these cells through the respiratory epithelium. This observation is in line with animal models of respiratory viral infections where over-activation of moDCs contributed to severe lung injury and death [76, 271]. On the other hand, the number of macrophages remained unchanged for the first two days of nasal challenge in AR individuals (paper II), strongly suggesting that the accumulating HLA-DR+CD68+ cells represent the human counterparts of mouse inflammatory moDCs (**Figure 9**). These findings might indicate that the function of monocytes recruited to mucosal tissues is context-dependent; during homeostasis they differentiate into CD14+ immunoregulatory macrophages and CD14-CD103-SIRPα+ DCs, whereas in an inflammatory microenvironment they preferentially become inflammatory moDCs.

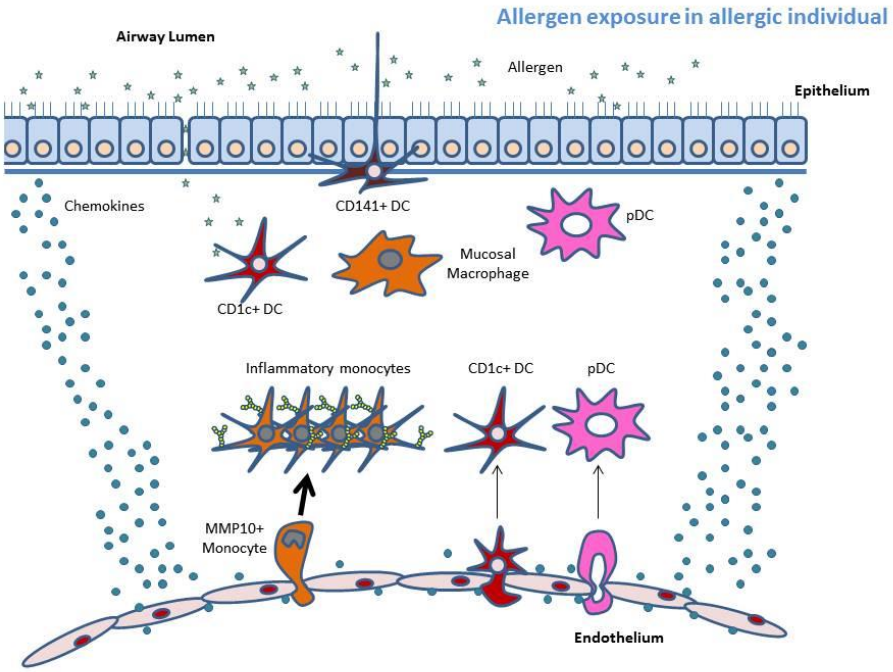

Figure 9: Inflammatory monocytes are involved in allergic rhinitis and asthma in humans

Allergen challenge in allergic individuals induces a rapid and massive recruitment of blood monocytes that is followed after several days by a smaller accumulation of CD1c+ conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs). The number of CD141+ cDCs and mucosal macrophages does not significantly vary during the first days of allergen challenge.

This idea was underscored by the results of our microarray analysis. During the allergen challenge CD45+HLA-DR+ cells increased expression of several pro-inflammatory genes and almost half of them were regulated by IL-4 or IL-13 signaling. On the other hand, HLA-DR+CD14+ cells were found to start accumulating before CD1c+ cDCs and pDCs and after seven days of continuous provocation CD14+ cells constituted the most abundant subset of HLA-DR+ cells within the nasal mucosa (paper I). These findings suggest that during allergic responses the differentiation of monocytes into inflammatory moDCs is governed by IL-4

and IL-13. Potential sources of these Th2 cytokines in allergic individuals include allergen-specific Trm cells [217], ILC2 cells, and IgE-armed mast cells and basophils [143]. Interestingly, four of the up-regulated genes corresponded to chemokines usually involved in Th2 responses, namely CCL13, CCL17, CCL18 and CCL24 [206]. Among them, CCL13 and CCL24 are ligands of CCR3, a chemokine receptor expressed in eosinophils [138], basophils and Th2 cells while CCL17 and CCL18 bind to CCR4 and CCR8 respectively, receptors expressed by memory Th2 cells [206]. In paper I of this thesis we demonstrate that the recruitment of HLA-DR+CD14+ cells was paralleled by a significant accumulation of mucosal eosinophils in AR patients but not in non-atopic controls. Additionally the nasal provocation induced increased recruitment of CD4+ T cells and increased Th2 phenotype within the accumulating CD4+ T cells in AR patients. It is thus tempting to speculate that mucosal CD14+ monocytes recruited during allergic responses in humans exert similar roles than inflammatory moDCs in mouse models of allergic asthma [45] and that they are also master orchestrators of the local allergic reaction by both recruiting and reactivating other immune cells (**Figure 10**).

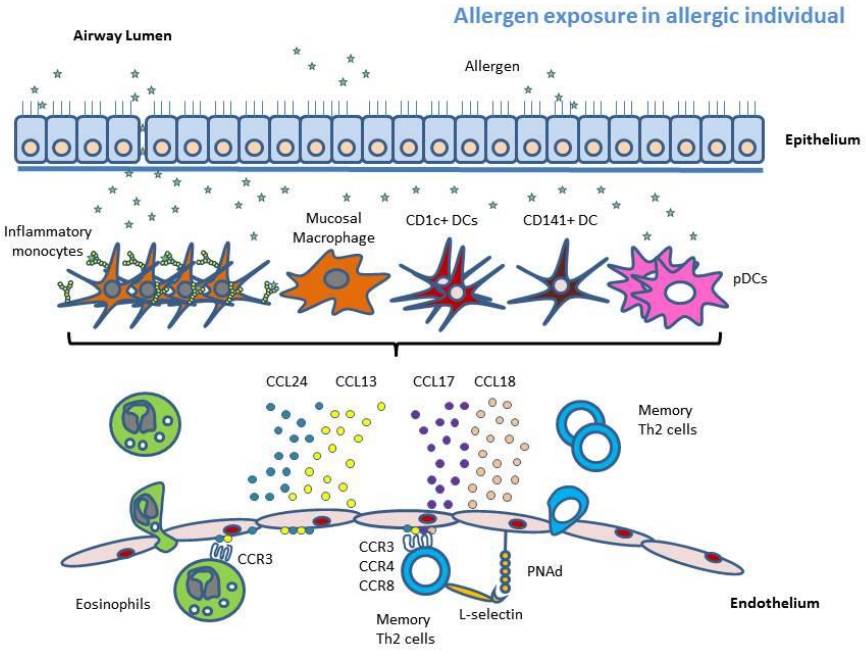

Figure 10: The mononuclear phagocyte system (MPS) produces chemokines that recruit memory Th2 cells and eosinophils in allergic rhinitis (AR) patients.

Inflammatory monocytes are the most abundant mucosal MPS subset after one week of continuous allergen challenge in AR patients. The MPS upregulates the expression of CCL24, CCL13, CCL17 and CCL18 during allergic responses and contributes to the recruitment of CCR3+ eosinophils and CCR3+ CCR4+ CCR8+ memory Th2 cells. Peripheral lymph node addressin (PNAd)/L-selectin interactions also contribute to Th2 cell recruitment in human AR.

The interaction with the CCL13+CX3CL1+ endothelium probably mediates the recruitment of CCR2+CX3CL1+ circulating monocytes to the respiratory mucosa [27, 259, 260]. Nevertheless, other mechanisms may also play a role in monocyte extravasation. The results of paper II of this thesis suggest that the collagenolytic activity of matrix metalloproteinase 10 (MMP10) [277, 278] could be involved in the high intra-tissue motility of monocytes.

MMP10 is a proteolytic enzyme highly expressed by the invasive front of several tumors [279] and by epithelial cells during wound healing [280] or allergic inflammation [281, 282]. More recently MMP10 was reported to be expressed by mouse bone marrow-derived macrophages during inflammatory conditions [277]. MMP10 has the ability to cleave the large biomolecules of the extracellular matrix, thus facilitating the movement of the migrating cell [278]. Because aggregates of MMP10+ monocytes were found in the lumen of bronchioles of asthmatic patients, it is possible that recruited monocytes use this molecule to invade the respiratory mucosa.

In the third paper of this thesis we studied the longevity of human AMs after lung transplantation. We demonstrated that in cross-gender lung-transplanted patients without rejection, AMs can survive for longer than two years and that the density of donor-derived AMs did not significantly change during the first two years after the surgery. AMs in transplanted patients displayed a sustained ability to in situ proliferate as assessed by Ki67 expression and this observation possibly explains their capacity to self-maintain or expand. On the other hand, ~50% of AMs were recipient-derived two weeks after transplantation and interestingly their density did not significantly change during the studied period. Additionally we were able to show that the lung parenchyma of transplanted patients was populated by both mature AMs and monocytes differentiating into AMs two weeks after the surgery, whereas only mature AMs were found after two years. The ability of human monocytes to differentiate in vivo into AMs was further assessed by our experiments in humanized mice. To complete the picture, the finding of Ki67+ recipient-derived AMs showed that the capacity to self-perpetuate or expand was not restricted to donor-derived macrophages. Studies investigating the biological changes experienced by grafts during transplantation have reported that a significant amount of both stromal and immune cells in

the lung parenchyma undergo apoptosis during the procedure [283, 284]. In line with this, our observations might indicate that donor-derived AMs dying from apoptosis during graft transportation or reperfusion are replaced in the first weeks after the surgery by incoming recipient-derived monocytes that differentiate into long-lived AMs (**Figure 11**).

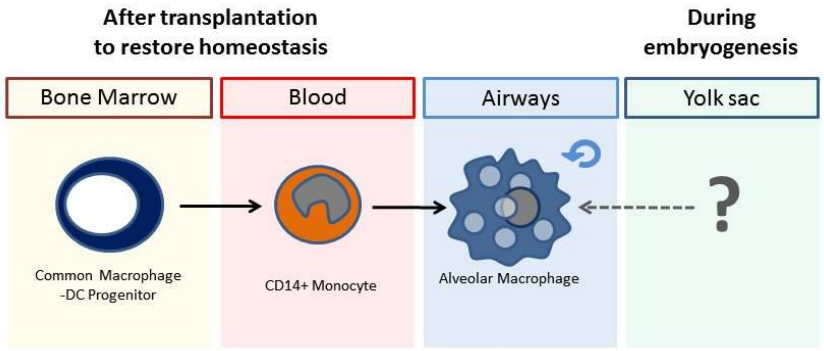

Figure 11: Blood monocytes give rise to long-lived alveolar macrophages (AMs) to restore lung homeostasis after transplantation.

Blood monocytes are recruited to the lung after transplantation and differentiate into long-lived anti-inflammatory AMs that replace the donor-derived AMs not surviving engraftment. Human AMs may also have embryonic progenitors as was shown for their mouse counterparts.

Results in paper III suggest that a stable chimerism is achieved between donor-derived AMs that survive the transplantation and AMs derived from monocytes recruited after the surgery. Of note, in lung cancer patients the density of AMs in areas distal from the tumor was similar to that resulting from the addition of donor- and recipient-derived AMs in lung transplanted patients one year after the surgery. Mouse models of lung inflammation have shown that during respiratory infections most resident macrophages die and high numbers of monocytes are recruited to the lung [269]. When inflammation resolves, the majority of recruited monocytes undergo apoptosis and the remaining population of resident macrophages proliferates and repopulates the organ [270]. Only when mouse lungs are

exposed to genotoxic insults recruited monocytes become long-lived resident macrophages [57]. Our results suggest that in humans resident macrophages surviving transplantation are able to self-maintain but not to repopulate the entire organ and that recruited monocyte can differentiate into non-inflammatory long-lived macrophages. This apparent disagreement can be related to the distinct life spam between species or to the transplantation model itself. In paper III we were not able to determine whether donor-derived AMs arose from fetal precursors as has been shown in mice [38]. The intake of immunosuppressive drugs constitutes another limitation of the study in paper III. In this regard, corticosteroids were part of both the induction and maintenance anti-rejection therapy and could have influenced our results [285]. The rest of the medications administered (see Supplementary Table I in paper III) principally targeted T cells (and indirectly B cells) and had little effects over monocytes or macrophages [286]. All the patients included in paper III had a good clinical course with no evidence of clinical or histological rejection during the studied period, and they did not require any rescue medications. Nevertheless, our model reflects homeostasis only several months after the surgery because the engraftment likely induced a massive recruitment of blood monocytes to replace AMs not surviving the transplantation

In conclusion, this thesis has focused on the capacity of blood CD14+ monocytes to migrate to the respiratory tract and participate in various pathological conditions. As deduced from paper I and II monocytes exposed to a Th2 microenvironment become potent inflammatory cells with the ability to invade the tissue and to attract other immune cells. On the other hand, monocytes recruited to the lung after transplantation aim at restoring lung homeostasis and differentiate into anti-inflammatory long-lived macrophages. These divergent maturation programs are governed by different tissue microenvironments, and

this fact reinforces the idea of stromal cells tailoring immune responses to organ requirements.

FUTURE PERSPECTIVES

The finding that pro-inflammatory monocytes accumulate in allergic airway diseases makes them good therapeutic targets for the treatment of AR and asthma. Glucocorticosteroids have been shown to antagonize monocytes by inducing transient monocytopenia followed by transient monocytosis [287]. The monocytopenia is thought to be the consequence of reduced release of monocytes from the bone marrow, whereas the monocytosis can be explained by a diminished egress of these cells from peripheral blood [287]. When inhaled corticosteroids are not sufficient for disease control in asthma patients, systemic drugs are often required [2]. Systemic glucocorticosteroids display an unacceptable safety profile [288], and the search of medications with more restricted immunomodulatory effects is an interesting field of investigation. Several rodent models have shown that blocking monocyte recruitment by targeting either CCR2 or monocyte chemoattractant protein-1 (MCP-1) has beneficial effects over the formation of arteriosclerosis plaques or allograft rejection [289, 290]. Nevertheless monocytes play multiple roles in host defense [26] and translating this therapeutic approach into humans would probably face many safety issues.

Our microarray data showed that IL-4 and/or IL-13 induced the maturation of mononuclear phagocytes during the allergic challenge. Several drugs have been developed to target IL-4 including VK694, a fully humanized anti-IL-4 monoclonal antibody (mAb). It was proposed that blocking IL-4 would favor the induction of regulatory T cells during allergen-specific immunotherapy (AIT), but when VK694 was combined with AIT no additional benefit was observed over AIT alone in the suppression of allergen-induced skin responses [291]. Dupilumab, a fully-human mAb directed against IL-4Rα, was reported effective for the treatment of adults with moderate-to-severe atopic dermatitis [292]. Strategies targeting IL-

13 include the mAbs lebrikizumab and tralokinumab and both of them have been proposed to treat uncontrolled asthma patients displaying high levels of serum periostin [293, 294]. Periostin is a signaling protein produced by respiratory epithelial cells that promotes bronchial hyperresponsiveness and airway remodeling [295]. The allergen challenge in paper I induced the upregulation of four chemokine genes within mononuclear phagocytes. Among them, CCL17 binds to CCR4 on Th2 cells and CCL17 and CCL24 attach to CCR3 on Th2 cells, eosinophils and basophils [138, 206]. Mogamulizumab is a humanized anti-CCR4 mAb that is approved in Japan for the treatment of some patients with T-cell lymphoma [296]. Both mogamulizumab and the anti-CCR3 drug ASM8, are being currently tested for the treatment of adult patients with asthma [297].

Even though none of the above mentioned studies investigated the effects of blocking IL-4 or IL-13 on mononuclear phagocytes, some of the described clinical effects might be related to the inhibition of monocyte differentiation into inflammatory DCs. The search of new biological drugs for respiratory allergy including agents targeting monocyte-derived DCs will probably continue in future years.

The management of patients with hereditary pulmonary alveolar proteinosis (hPAP) usually constitutes a clinical challenge because only symptomatic treatments such as repeated whole lung lavages are currently available [69]. Hematopoietic stem cell transplantation (HSCT) was shown to permanently ameliorate experimental models of hPAP [72], but the complications related to myeloablative conditioning in humans have limited the usefulness of this approach. Recently it was suggested that hPAP patients could be treated by intra-tracheal transplantation of gene-corrected in vitro-generated alveolar macrophages (AMs) or AM precursors [298]. In this regard, mouse models of experimental hPAP have

demonstrated that a single instillation of either AMs or AM precursors is able to restore lung homeostasis for ~1 year [299, 300], consistent with the fact of AMs being long-lived cells. To translate this approach into humans, information on the longevity and precursors of human AMs is crucial. Our findings in paper III suggest that human monocytes can differentiate into long-lived AMs and this observation might pave the way to new cost-efficient strategies of intra-tracheal transplantation in patients with hPAP.

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