Mucosal dendritic cells in immune homeostasis and upper airway allergy

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Oslo 19.12.2014

Guro Reinholt Melum

APC antigen presenting cells

BDCA blood dendritic cell antigen

CCL chemokine ligand

CCR chemokine receptor

CDP common dendritic cell precursor

DAMP danger-associated molecular pattern

DCs dendritic cells

FcεRI high affinity receptor for IgE

Flt3L FMS-like tyrosine kinase 3 ligand

GMP granulocyte macrophage precursor

HEV high endothelial venules

HSC hematopoietic stem cell

IDO indoelamine 2,3 dioxygenase

IFN interferon

Ig immunoglobulin

IL interleukin

LPS lipopolysaccharide

mDC myeloid dendritic cell

MHC major histocompability complex

MLP multilymphoid progenitor

MPS mononuclear phagocytic system

nT naïve T cells

PAMP pathogen-associated molecular pattern

pDC plasmacytoid dendritic cell

PGE2 prostaglandin E 2

PRR pattern recognition receptor

RA retinoic acid

SOCS suppressors of cytokine signaling

STAT signal transducers and activators of transcription

Tfh follicular helper T cell

TGF-β transforming growth factor beta

Th T helper cell

TLR toll like receptor

Tm T memory cell

Treg regulatory T cell

TSLP thymic stromal lymphopoietin

TSLPR thymic stromal lymphopoietin receptor

PAPERS INCLUDED

This thesis is based on the following papers:

I. A thymic stromal lymphopoietin–responsive dendritic cell subset mediates allergic responses in the upper airway mucosa
Journal of Allergy and Clinical Immunology 2014; 134, 3, 613–621

II. Identification of gene networks activated during experimental allergic rhinitis in humans
Manuscript December 2014

III. Human mucosal CD1a+ dendritic cells display immunoregulatory properties
Manuscript December 2014

1. Introduction

1.1 The immune system - basic immunological concepts

The immune system is a highly effective defense system occupied with the important task of protecting the body against threats from the outside world, such as harmful microorganisms as well as threats from within the body like cancer cells or damaged tissue. To accomplish this task, the immune system must be able to separate harmful from innocent substances and initiate effective immune responses when necessary[1]. If this process fails, the immune system faces the risk of inadequate responses to infections, in addition to mounting attack against healthy tissue (autoimmune diseases) or harmless substances (allergic diseases)[2].

The immune system can be divided into two arms; the innate and the adaptive component. The innate immune system is the first line of defense, and involves the epithelial barrier, a humoral component (the complement system and cytokines), and a variety of different hematopoietic immune cells like mast cells, granulocytes, the mononuclear phagocytic system (MPS), (dendritic cells, monocytes and macrophages), among others[2].

The main task of the innate immune system is to initiate a swift and effective response upon recognition of harmful substances[3]. The adaptive immune system is slower to respond, but characterized by higher degree of specificity and immunological memory. The adaptive component consists of antibody responses carried out by B cells, and cell-mediated responses mediated by T cells. In an optimal response to a potential threat, the innate and adaptive parts of the immune system cooperate to mount a rapid and effective immune reaction, while at the same time develop memory for later encounters with the same antigen[3, 4].

Dendritic cells (DCs), central to this thesis, act as the bridge between the innate and adaptive part of the immune system, consequently these cells face the important task of deciding when to trigger an immune response in the presence of danger – and when to "keep calm and carry on", thus maintaining tolerance to harmless substances and self molecules[3].

1.2 THE MUCOSAL IMMUNE SYSTEM IN THE UPPER AIRWAYS

The upper airway mucosa is in close contact with the external environment, and is exposed to a large variety of antigens constantly challenging the immune system to mount effective protection.

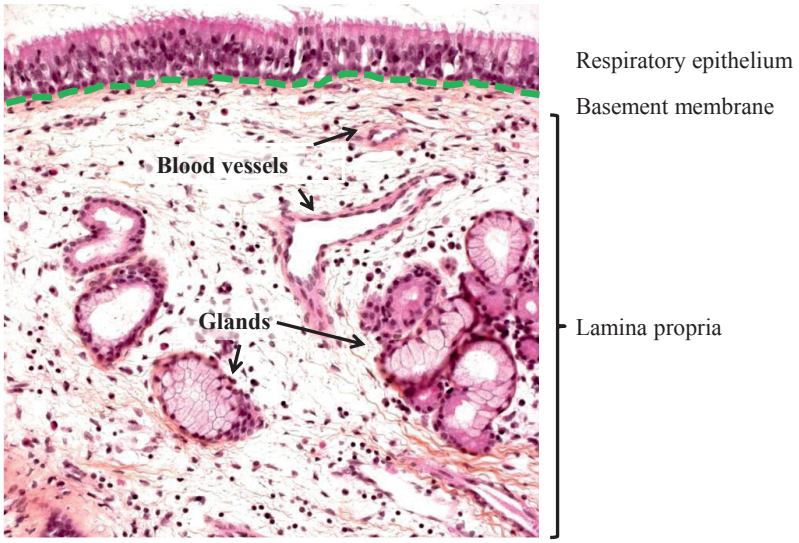

Figure 1. Histology of normal nasal mucosa. Epithelial layer with ciliated epithelium. Basement membrane separating the epithelial layer from the lamina propria. Lamina propria with vessels, glands and immune cells.
Adapted from www.humpath.com – Human pathology

The nasal mucosa is covered by a layer of pseudostratified columnar epithelium, including scattered goblet cells (Figure 1). The lamina propria contains a large number of glands, where the glandular epithelium produce mucus and is important for translocating immunoglobulin A (IgA) and IgM, produced by lamina propria-resident plasma cells. The goblet cells and the glands are responsible for a protective layer of mucus, which together with secretory Igs and ciliated epithelial cells are important for removal of foreign substances.

The epithelial cells comprise a first line of defense of the mucosal immune system. In addition to being a physical barrier, epithelial cells express a broad range of pattern recognition receptors (PRRs), that can detect pathogen associated molecular patterns (PAMPs) expressed by microbes, and damage-associated molecular patterns (DAMPs)

released upon tissue damage, cell death or cellular stress. Activation of these PRRs leads to the secretion of antimicrobial peptides as well as cytokines and chemokines that attract and activate immune cells that reside underneath and within the epithelial surface[5]. Crosstalk between epithelial cells and immune cells is crucial for the immunological barrier function, and aberrant crosstalk may result in inflammatory disease at mucosal surfaces[6]. In addition, commensal microorganisms contribute to host defense through metabolic competition and by enforcing the host's immune barrier[7].

1.2.1 Immune cells in the nasal mucosa

The lamina propria contains a dense network of immune cells with various functions.

Mast cells

Mast cells are found in all mucosal tissues, and play a central role in inflammatory and immediate allergic reactions. They harbor vast amounts of potent inflammatory mediators that can be released swiftly and mediate inflammatory responses by blood vessel dilation, increased vascular permeability and recruitment of immune cells to the tissue.

Mast cells respond to antigenic stimulation through cross-linking of IgE bound to the high affinity receptors for IgE (FcεRI). Upon activation, mast cells release either prestored mediators such as histamine and proteases, or newly generated mediators such as eicosanoids, cytokines and chemokines[8]. In addition to their central role in allergic responses, mast cells are recognized as effector cells in various pathological conditions including chronic inflammation, autoimmune diseases, cardiovascular disorders, bacterial clearance and resistance to infections with parasites[9, 10]. Their diverse nature is reflected in the vast amounts of mediators that can be released from these cells[11].

Eosinophils

Eosinophils are involved in the initiation and maintenance of diverse inflammatory responses. In particular, they have long been associated with parasitic and allergen driven inflammation, and the infiltration of eosinophils to the airways is one of the hallmark characteristics of allergic asthma.

Eosinophils are quickly recruited to sites of inflammation where they release proinflammatoy cytokines, chemokines, growth factors, lipid mediators and cytotoxic granules. Some of these substances are pre-stored and can be released rapidly within minutes to initiate inflammatory responses. Eosinophils were shown to be a major source of the cytokine interleukin 4 (IL-4), and this may serve as a major initial source of IL-4 required for the differentiation of naïve T cells into T helper 2 (Th2)-cells, as will be described later[12, 13].

The mononuclear phagocyte system

The MPS is defined as a family of cells comprising monocytes, DCs and macrophages[14, 15]. Macrophages and DCs are tissue-residing cells that express PRRs and are strategically situated underneath and within the epithelium of the mucosa and continuously scan the environment to sense potentially harmful antigens (Figure 2). Monocytes are mainly present in the circulation and may serve as precursor for tissue-residing macrophages and inflammatory DCs. They are all heterogeneous populations, and can differentiate into different functional subsets[14].

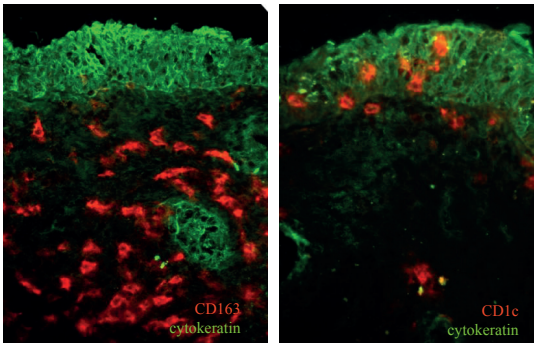

Figure 2. Immunohistochemistry of the main antigen presenting cell subsets of the upper airway mucosa.
CD163+/CD14+ macrophages and CD1c+ DCs reside in the epithelium and in the lamina propria underneath the epithelial surfaces. Green: cytokeratin staining in epithelial cells, red: CD163 and CD1c respectively

Monocytes and DCs are derived from a common bone marrow-derived hematopoietic stem cell (HSC) as will be described later. They differentiate into various subsets in response to different mediators that act as growth and differentiation factors[16].

Tissue-residing macrophages were initially thought to be derived from blood monocytes, however this notion has been challenged by the fact that in mice, a proportion of resident tissue macrophages arise from yolk sac and foetal liver precursors. These cells seed the tissue during embryonic development, and are able to proliferate and self-maintain locally[17, 18, 19 , 20]. However, the relative proportion of embryonic or monocytic origin to the tissue-resident macrophage population depends to a large extent on the tissue they reside in. For example, tissue-resident macrophages (microglia) in the brain seems to be exclusively derived from embryonic stem cells[21, 22], while studies from the intestinal mucosa in mice have shown that the macrophage population requires constant replenishment from blood monocytes[23, 24, 25, 26, 27].

In addition, the balance between monocyte- and stem cell derived macrophages may vary with inflammatory conditions and with age. Monocytes may be recruited in inflammatory conditions, furthermore in mice cardiac macrophages derived from embryonic precursors gradually lose their capacity to self-renew and are continually replaced by monocyte-derived macrophages even in the absence of inflammation[28]. How this correlates to the macrophage population in human tissues is unknown. Interestingly, recent work in our lab shows that in transplanted gut tissue between humans where immune cells can be traced as donor or recipient derived, all donor macrophages are replaced by recipient monocytes one year after transplantation. (Bujko et al, unpublished data).

Macrophages are resident phagocytic cells that engulf and eliminate apoptotic cells, cellular debris and incoming pathogens[15]. In response to bacterial infections, macrophages capture and clear microorganisms and secrete pro-inflammatory molecules, thus playing a crucial role in host defense[14]. They are present in every tissue of the body, display great functional diversity, and are divided into subpopulations depending on their anatomical location. Macrophages perform important homeostatic functions by clearance of pathogens and toxins, as well as suppression of inflammation. Thus, macrophages contribute to the restoration of homeostasis following infection or injury[29, 30].

Tissue-resident macrophages may differentiate into different subsets depending on the signals they sense in their environment. Based on their function, macrophages are divided broadly into two categories; classical M1 and alternatively activated M2 macrophages. M1 macrophages differentiate in response to interferon (IFN)-γ and lipopolysaccharide (LPS),

and are involved in inflammatory responses, pathogen clearance, and antitumor immunity. M2 macrophages take part in anti-inflammatory responses, wound healing, tumor progression and immunosupression[31]. This subset of macrophages can further be divided into four different subsets, one being M2a macrophages which differentiate upon stimulation with IL-4 and IL-13 and are involved in Th2 responses, allergy and parasite clearance[32, 33]. These findings are primarily derived from in vitro experiments, thus the functions of M2 macrophages in vivo remains to be determined.

Monocytes circulate in the blood stream, and may replenish resident tissue macrophages under steady state as previously described. In response to tissue damage and inflammatory signals they move quickly to sites of inflammation and may differentiate into inflammatory macrophages and DCs[22, 34].

Two main human monocyte populations have been described based on the expression of the receptors CD14 and CD16; classical CD14+monocytes that are precursors of peripheral mononuclear phagocytes, and non-classical CD16+ monocytes that reside in the vascular lumen and survey endothelial integrity[22, 35].

Dendritic cells excel in antigen-presenting capacity and ability for migration to lymph nodes compared to monocytes and macrophages[26]. The presence of different mucosal DC subsets with distinct functional specialization will be discussed later. In case of encounter with a pathogen, tissue resident DCs take up pathogens by macropinocytosis or receptor-mediated phagocytosis and migrate via the lymphatics to regional lymph nodes, where they arrive as mature nonphagocytic DCs[36]. Here, the mature DCs activate antigen-specific naïve CD4+ helper T cells[37] (Figure 3).

DC-migration to lymph nodes is dependent on the chemokine receptor 7 (CCR7) and its ligands chemokine ligand 19 (CCL19) and CCL21, both during steady state and during inflammation[38]. In response to mediators of inflammation DCs up-regulate CCR7 and increase their capacity for lymph node migration. The importance of CCR7 for lymph node migration was demonstrated in studies where CCR7 deficient mice had a marked defect in DC migration to lymph nodes[38]. CCR7 and its ligands control DC chemotaxis towards

lymphatic capillaries and docking on the vessels. In the lymph nodes, CCR7 is critical for correct localization to the T cell zone, ensuring interplay between DCs and naïve T cells[39].

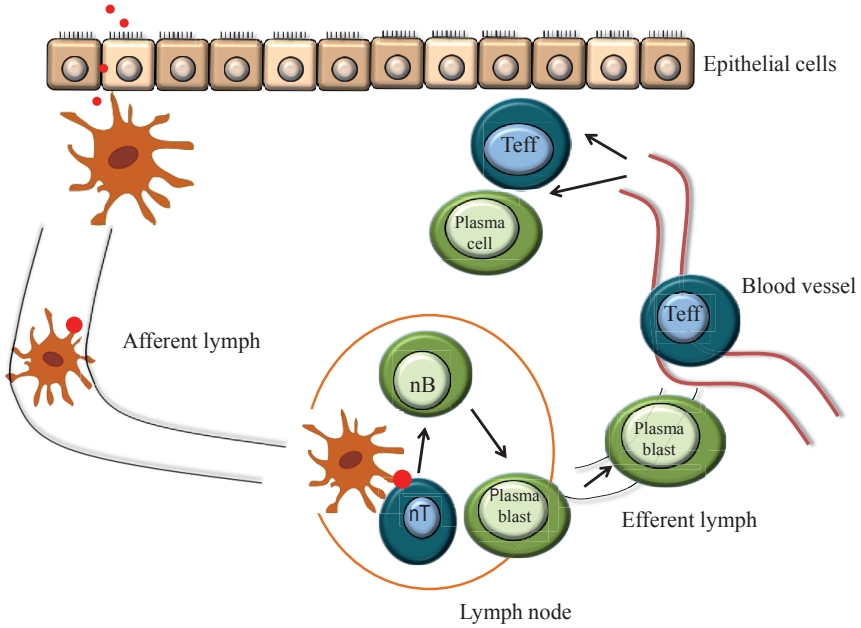

Figure 3. The adaptive immune response in the nasal mucosa is initiated by dendritic cells. Antigen is ingested by DCs in the tissue, transported to the lymph nodes and presented for naïve T cells (nT). Naïve T cells become activated; contribute to the activation of naïve B (nB) cells and both subsets may home back to the nasal mucosa as effector T cells (Teff) and plasma cells, respectively.

Lymphocytes

In draining lymph nodes, activated T cells interact with naïve B cells which subsequently differentiate into antibody-secreting plasmablasts that travel to the mucosal effector sites, and differentiate into plasma cells for antibody production and later encounter with the antigen[40, 41](Figure 3).

Activated T cells undergo clonal proliferation in the lymph node, and may reside in the lymphoid tissues to activate more B cells, or exit the lymph nodes through efferent lymphatics and home to the mucosa as effector T cells in the process of an ongoing

inflammation. After the inflammation has been resolved, most effector T cells die, but a minority differentiate into memory T (T_M) cells weeks after the resolution of inflammation.

Different subsets of T_M cells reside in different locations and perform distinct types of immune surveillance under homeostatic conditions. Resident memory T cells (TR_M) reside in peripheral tissue and do not recirculate, central memory T cells (T_{CM}) recirculate between lymphoid tissue and blood, and effector memory T (T_{EM}) cells primarily recirculate between the blood and peripheral tissue[42, 43].

1.2.2 T-CELL PRIMING

T cells mature in the thymus, where they undergo positive and negative selection to be able to recognize self major histocompatibility complex (MHC), but at the same time autoreactive clones are eliminated (central tolerance)[44].

After development in the thymus, naïve T cells migrate to secondary lymphoid organs where activation by DCs may take place[45]. Naïve CD4+T helper cells differentiate into different functional effector subsets depending on the signals they receive from antigen presenting cells (APCs) and the stimulatory cytokines that are present in the microenvironment during activation (Figure 4)[46, 47, 48].

The pathogen type and the local mediators from neighboring immune and stromal cells at the site of antigen capture are integrated by tissue-resident DCs which is important for the type of T-cell differentiation[49]. Thus, DCs determine, to a large extent, the adaptive immune response that is initiated in response to a specific pathogen via the signals by which they activate and differentiate naive T cells[50]. Through production of subset-specific cytokines these specific CD4+ helper T cells provide support to B lymphocytes and CD8+ cytotoxic T cells, and activate cells of the innate immune system[46, 51].

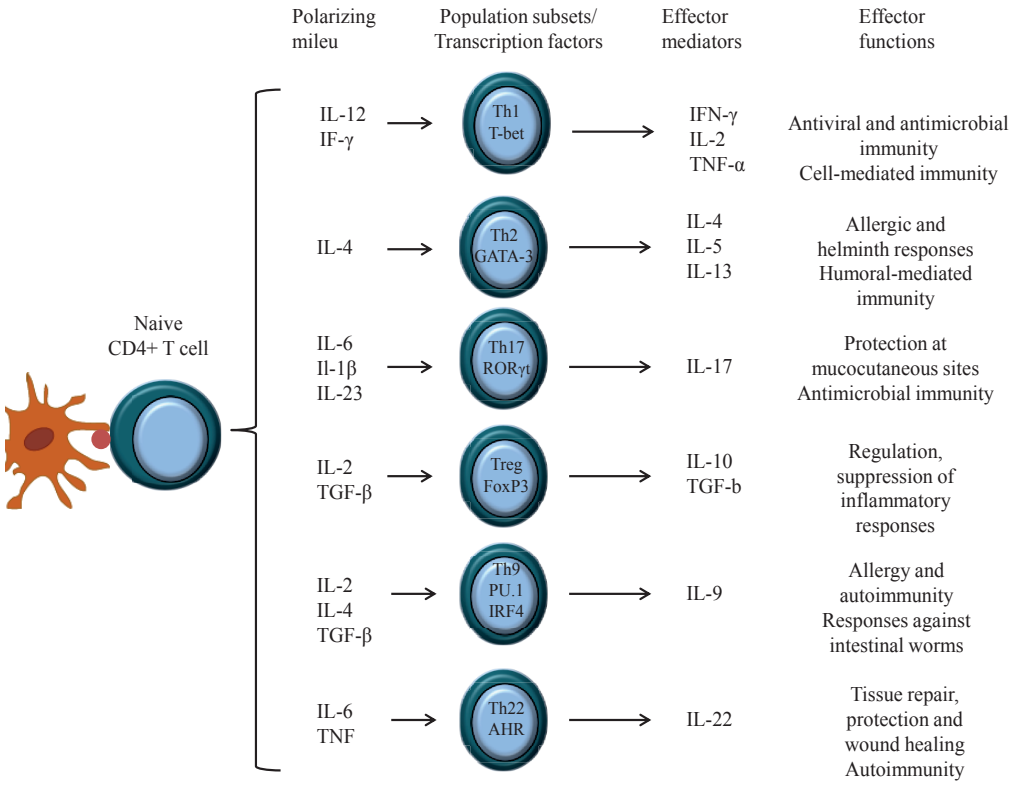

Figure 4. Dendritic cells control the development of distinct T-cell responses. Antigen-specific naïve T cells are stimulated to expand in interaction with APCs expressing MHC class II / peptide. These T cells specialize to become distinct subsets and produce restricted patterns of cytokines, depending on the cytokine milieu and the signals they receive from the antigen presenting cell.
Adapted from; Raphael I et al. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine (2014),published ahead of print; http://www.sciencedirect.com/science/article/pii/S1043466614005390

Following activation and differentiation, CD4+ helper T cells may migrate back to the mucosal effector sites as effector T cells to promote local immune responses (Figure 3)[40], thus they are crucial for host defense. CD4+ helper T cells are also implicated in immune-mediated diseases like allergy and autoimmune disease[46].

Naïve conventional CD4+ T cells may be polarized into several different effector subtypes; Th1, Th2, Th17, Th9, Th22, Tfh and inducible T-regulatory cells (Tregs), with specialized functions to control immune responses[52]. T-cell differentiation is a two- phase process , consisting of a T-cell receptor (TCR)-driven induction phase, where key transcription factors

are induced or activated, and a cytokine-driven polarization phase, in which expression of key factors is amplified and their differentiation is completed[53]. The lineage acquired depends upon the network of cytokines present and transcription factors expressed in the activated cells[54]. For each Th lineage differentiation, more than one cytokine is involved, and cytokines involved in differentiation of one lineage might suppress the differentiation of other subsets, thus driving the immune response in specific directions[54].

The main cytokines involved in T-cell differentiation, as well as transcription factors and cytokines specific for each subset are shown in figure 4. Interestingly, emerging data has suggested a certain degree of flexibility and plasticity of helper T cell polarization, indicating that induction of specific cytokine-producing and transcription factor T cell subsets is not a sign of terminally differentiation, but rather that the T cells remain responsive to stimuli from APCs, and can change their polarization[47, 55]. Whether these cells alter their cytokine-producing potential and change their phenotype under physiological conditions *in vivo* is still uncertain[47, 56, 57].

1.2.3 CD4+ T CELL SUBSETS

Th1 cells are competent effectors against intracellular bacterial and viral infections and promote cell mediated immune responses. Such cells secrete IFN-γ, IL-2, IL-10, TNF-α and TNF-β. Although Th1 cells are critical for the clearance of intracellular pathogens, exaggerated Th1 responses are associated with autoimmune diseases, including rheumatoid arthritis, multiple sclerosis and type 1 diabetes

Th1 cells depend on the T-box transcription factor expressed in T cells (T-bet) together with signal transducer and activator of transcription (STAT) 4 during differentiation from naïve T cells. Furthermore, IL-12 and IFN-γ are important cytokines for the differentiation of Th1 cells[52].

Th2 cells are critical for expelling extracellular parasites, and differentiate from naïve T cells in response to the cytokines thymic stromal lymphopoietin (TSLP), IL-4 and IL-2.

The Th2 master regulatory transcription factor is GATA-3, which in combination with STAT6 induces differentiation of Th2 cells. In addition, aberrant Th2 cells are involved in

allergic responses and produce the classical Th2 cytokines IL-4, IL-13 and IL-5. These cytokines are pivotal for the induction and maintenance of allergic responses, i.e. IL-4 and IL-13 induce class switching and IgE-production in B cells and contribute to further differentiation of naïve T cells towards a Th2 phenotype. Furthermore, IL-5 is crucial for the activation of eosinophils, and IL-13 is involved in activation of mast cells and regulation of mucus production[53,54,58].

Th17 cells are effective in the defense against extracellular pathogens including bacteria and fungi. They are implicated in a broad spectrum of chronic inflammatory conditions and autoimmune diseases[59]

Th17 cells differentiate from naïve T cells in the presence of IL-1β, IL-23, IL-6 and transforming growth factor β (TGF-β), and control extracellular bacteria and fungi through production of IL-17 and IL-22. Th17 cell differentiation depends on induction of the transcription factor RORγt together with STAT3.

Th22-cells

Th22 cells produce IL-22 and were initially described to be involved in the immunopathology of skin diseases. IL-22 was shown to be prominently expressed by T cells in skin inflammation[60].

Th22 cells are closely associated with Th17 cells, but represent a separate Th subset with distinct gene expression and functions. These cells produce IL-22 and IL-13, but not IL-17 or IFN-γ and express CCR4, CCR6 and CCR10. The transcription factor aryl hydrocarbon receptor (AhR) is required for IL-22 production in both Th17 and Th22-cells[61, 62]. Th22- cells are involved in tissue protection and wound healing at epithelial surfaces through induction of epithelial cell proliferation and antimicrobial peptides. In addition they may be engaged in the pathogenesis of autoimmune and allergic diseases[63].

Regulatory T cells (Tregs)

Tregs can be generated in the thymus in the process of positive and negative selection, so-called natural Tregs (nTregs), or in secondary lymphoid organs and tissues, so-called inducible Tregs (iTregs).

DCs play a major role in the induction of peripheral tolerance through generation of iTregs, in addition to induction of T-cell anergy and deletion[64]. iTregs are essential for maintaining peripheral tolerance, and execute suppressive functions by several different mechanisms, including modulation of the cytokine microenvironment, suppression of development of effector T cells, and suppression by targeting DCs[65, 66].

Tregs play a vital role in fine-tuning the balance between effector and tolerogenic responses. FoxP3 is a master regulator in Treg development, and is expressed in both nTregs and iTregs. iTregs express FoxP3 in response to defined tolerogenic stimuli such as TGF-β and retinoic acid (RA) in lymphoid tissues[65].

Other subsets of T cells with suppressive capacity have been described. One such subset is the inducible Type 1 regulatory (Tr1) cells, which produce the immunosuppressive cytokine IL-10. Distinct intracellular and surface markers and cytokine expression profile distinguish FoxP3+ Tregs and Tr1 cells from each other[67]. Interestingly studies in humans have demonstrated that the T cell response to allergens depends on a fine-tuned balance between allergen-specific effector cells and IL-10 producing Tr1 cells[68, 69].

Th9 cells

IL-9 was initially viewed as a Th2 cell cytokine, however it is now recognized that a subset of CD4+ T cells preferentially produce IL-9, and is distinct from Th2 cells. Such cells depend on IL-2, TGF-β and IL-4 for their differentiation and survival, and their transcriptional regulation is controlled by PU.1 and IRF4, which synergistically regulate IL-9 production in Th9 cells[52, 70]. These cells facilitate immune responses against intestinal worms, but are also implicated in the immunopathology of allergy and autoimmunity[71, 72].

Tfh cells

Follicular helper T (Tfh) cells provide help to B cells in lymphatic tissues, and are crucial for germinal center formation, affinity maturation and the development memory B cells. Their differentiation is regulated by IL-6, inducible costimulator (ICOS) and IL-12, and depend on the transcription factor Bcl6[73].

1.3 The immunobiology of Dendritic cells

DCs are present throughout the body, and studies on their ontogeny and functions have revealed important roles during tissue homeostasis and disease, with the potential to induce both immunity and tolerance[74].

1.3.1 Dendritic cell subsets

DC subsets and their functional specialization have been extensively studied in mouse models, whereas knowledge of human DC subsets is mainly derived from studies of skin and blood DCs.

DCs comprise a heterogeneous group of cells, and can be defined according to expression of surface markers, their functional specialization, developmental origin, transcriptional regulation, patterns of migration or residence, and anatomical and micro-environmental localization[75].

In human blood, three main DC subsets have been identified based on their expression of surface markers; the CD1c+ (BDCA-1) DCs, CD141+ (BDCA-3) DCs and CD303+ (BDCA-2+/ CD123+) plasmacytoid DCs (pDCs)[76, 77]. Traditionally these subsets can be broadly divided into two main groups; (i) myeloid (m)DCs that include CD1c+ DCs and CD141+ DCs, and (ii) plasmacytoid (p)DCs (CD123/303 + DCs)

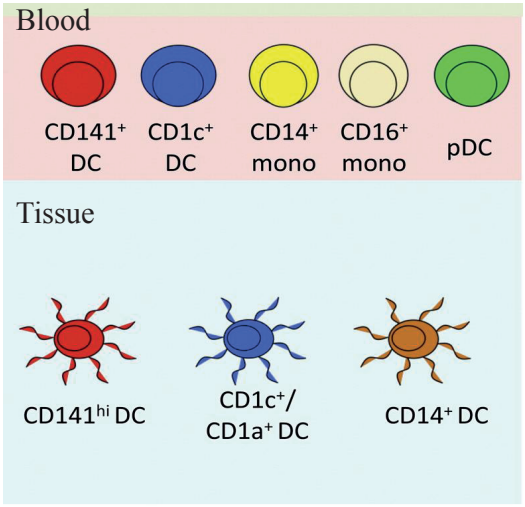

Figure 5. Human dendritic cell subsets. Blood DCs are immature precursors of tissue-residing DCs. pDCs are mainly found in secondary lymphoid tissue, and blood-derived pDCs enter the lymphatic tissue through high endothelial venules. CD14 + monocytes may give rise to CD14+ tissue-residing DCs especially in case of inflammatory conditions in the tissue. *Reprinted under the terms of the Creative Commons Attribution License (CC-BY); Haniffa et al. Human Tissues Contain CD141hi Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103+ Nonlymphoid Dendritic Cells. Immunity 2012, 37(1): 60-73. © 2012 Immunity published by Elsevier*

Circulating human blood DCs display an immature phenotype[78], and serve as precursors for tissue-residing DCs (Figure 5).

As previously described, there are two main subsets of monocytes in blood; CD14+ monocytes that may differentiate into inflammatory DCs during inflammatory responses in the tissue, and CD16+ monocytes with potential functions in the circulation (Figure 5)[79]. CD16+ monocytes were shown to crawl on the luminal side of the endothelium, sensing viruses and immune complexes via toll like receptor 7 (TLR7)- and 8, thus being involved in local surveillance of tissues[80].

In an inflammatory setting, cytokines and chemokines are produced at the site of inflammation, which attract CD14+ monocytes that migrate into the tissue and differentiate into inflammatory DCs. In several inflammatory conditions, such as atopic dermatitis, psoriasis, rheumatoid arthritis and tumor ascites, the presence of this subset of inflammatory

DCs has been described. Also, trancriptomic analysis revealed that they likely derive from monocytes that differentiate at the site of inflammation[34, 81].

1.3.2 Functional specialization of human dendritic cell subsets

The different DC subsets have unique surface receptor expression patterns and different functional capacities, thus arguing for a division of labor between the three main DC subsets in human mucosa[79].

CD141+ DCs uniquely express the lectin CLEC9A (DNGR-1)[76, 35], and have been detected in several organs such as skin, lung and intestine, where they show a more mature phenotype compared to CLEC9A+/BDCA-3+ DCs in blood, indicating a more mature stage of differentiation[76]. Such cells were reported to produce high levels of IFN-α after recognition of synthetic dsRNA[82] and high levels of IL-12[83], thereby enabling Th1 polarization, which could be significant for a protective immune response against viral infections[81]. In the skin, resident CD141+ DCs were shown to produce IL-10 and induce regulatory T cells that suppress skin inflammation[84,85].

The initiation of CD8+ cytotoxic T cell responses is dependent on presentation of exogenous antigens on MHC class I molecules. This process is known as cross-presentation and is mainly utilized by DCs. In mice a subset of tissue-resident CD8+ DCs excel in cross-presentation compared to other DC subsets[86]. CD141+ DCs have been proposed to be homologues to mouse CD8+ DCs and it was therefore suggested that they could be specialized in cross-presentation[87]. In line with this CD141+ DCs can cross-present antigens from dead cells better than other DC subtypes[83], however they seem equally able to cross-present soluble antigens when compared to other DCs[88,87] Furthermore: the lectin CLEC9A, uniquely expressed on CD141+ DCs was shown to be required for cross-presentation of dead-cell associated antigens, and uptake of necrotic cells in mice[89]. This suggests a specific role for CLEC9A expressed on CD141+ DCs in uptake and presentation of necrotic cells.

CD1c+ DCs comprise the largest population of DCs in blood, tissues and lymphoid organs. They express a wide range of TLRs, migrate to draining lymph nodes and stimulate naïve T cells efficiently[76,79]. This population of cells was shown to efficiently respond to Mycobacterium tubercolosis (M. tuberculosis) infection by inducing CD4+ T cell

proliferation[90]. Moreover CD1c+ DCs stimulated with Escheria coli (E-coli) suppressed T cell activation and produced high levels of the anti-inflammatory cytokine IL-10 and the regulatory molecule indoelamine 2,3 deoxygenase (IDO) suggesting the induction of a immunoregulatory CD1c+ DC phenotype upon E-coli infection[91]. CD1c+ DCs in tissue variably express the protein CD1a involved in presentation of lipids to T cells, making them candidates for processing and mounting immune responses against mycobacteria[92]. In the intestine CD1c+ DCs were shown to display an activated phenotype under homeostatic conditions, and produce IL-23[93].

In a study of DCs in human inflammatory fluids, a subset of CD1c+ DCs secreted Th17 cell-polarizing cytokines and induced Th17 cells. Inflammatory CD1c+ DCs were shown to represent a distinct DC subset, enriched for gene signatures of monocyte-derived DCs. Thus under inflammatory conditions distinct subsets of monocyte derived CD1c+ DCs may mediate inflammation through activation of Th17-cells[94].

Plasmacytoid dendritic cells (pDCs) express CD123, CD45RA, CD303 (BDCA-2) and CD304 (BDCA-4), but lack expression of CD11c and CD14 which separate them from other DC subsets. They are crucial mediators of antiviral immunity and secrete large amounts of type 1 IFN in response to viruses[79,95], which they sense with TLR9 and TLR7. They represent a rare subset of cells and are primarily found in the circulation (0.3-0.5 % of peripheral blood mononuclear cells (PBMCs)) and peripheral lymphoid organs during steady state[96].

Recent studies indicate that chronic pDC activation and secretion of type1 IFN in a non-inflammatory setting may result in autoimmune diseases, with the strongest evidence found in diseases like systemic lupus erytheromatosus (SLE) and psoriasis[97, 98]. Furthermore; pDCs have been implicated in allergy and asthma[99], antitumor immunity[100] and responses to nonviral pathogens[101, 102]. Notably, depletion of pDCs in mice has been shown to cause sensitization and lung inflammation to a harmless antigen [103]. Furthermore, human tonsillar pDCs induce allergen-specific FOXP3+ Tregs that can suppress effector T cells *in vitro*[104] suggesting that pDCs have tolerogenic functions in airway allergy[105].

1.3.3 ONTOGENY OF DENDRITIC CELLS

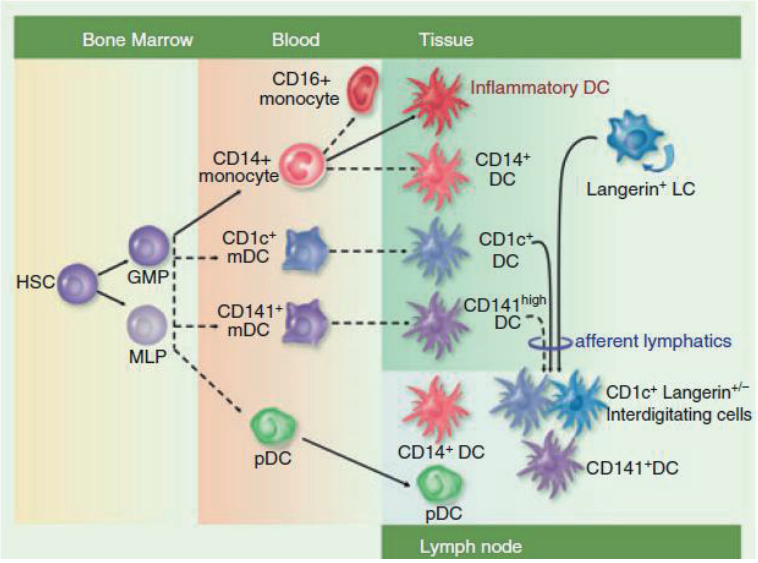

Figure 6. The distribution of major human dendritic cell subsets in blood, epithelial tissues and lymph nodes. Broken arrows indicate relationships that require further confirmation in humans. Human DCs can be generated either from granulocyte-macrophage progenitors (GMP) or multi-lymphoid progenitors (MLP) both of which ultimately arise from hematopoietic stem cells (HSC). Classical monocytes, blood mDCs and pDCs are putative precursors of tissue and lymphoid DCs.

Blood DC subsets are derived from hematopoietic stem cells, but the exact precursor-progeny relationship in human DCs is not clear.

Classical monocytes, blood mDCs and pDCs are precursors of tissue and lymphoid DCs (Figure 6)[75]. In mice, DC subsets derive from a common DC precursor (CDP) that can differentiate into immature DCs in response to lineage restricted differentiation factors[15, 16]. Even though it is unknown whether committed DC precursors exist in humans it has been demonstrated that both granulocyte macrophage precursors (GMPs) and multilymphoid progenitors (MLPs) can give rise to DCs *in vitro*[106] (Figure 6).

Important for the development of all DC subsets is the growth factor FMS-like tyrosine kinase 3 ligand (Flt3L) and its receptor Flt3, underscored by the fact that mice lacking Flt3L have deficient hematopoiesis affecting hematopoietic stem cells and DCs[107]. Furthermore; the injection of Flt3L into humans dramatically increased the number of all blood DC subsets[108] and several studies have shown that the number of DCs and level of Flt3L are inversely correlated[107,109].

Recently, three genetically defined syndromes of DC deficiency were described in humans. One of them, caused by a mutation in GATA2, which encodes a transcription factor involved in the homeostasis of hematopoietic stem cells, resulted in complete loss of MLPs, in addition to DC, monocyte, B and NK cell (DCML) deficiency. This mutation also resulted in massively elevated serum levels of Flt3L in line with the notion that Flt3L is a key protein in the development and maintenance of DCs[110]. In another set of patients, a specific autosomal dominant sporadic mutation (T80A) of the transcriptional regulator IRF 8 resulted in selective reduction in the number of CD1c+ DCs, but not CD141+ DCs or pDCs, implicating an important role for IRF8 during CD1c+ DC development. This mutation was linked to increased susceptibility to mycobacterial infection[111], in line with the reports that CD1c+ DCs have important functions in the immune response towards mycobacterial infections[90]. In one patient an autosomal recessive mutation (K108E) of IRF8 resulted in complete loss of peripheral blood mDCs, pDCs and monocytes.

Human DC subsets have been proposed to depend on unique and specific master transcription factors. The transcription factor E2-2 is specifically required for pDC development, and its expression is crucial to maintain the pDC phenotype[102, 112, 113]. CD141+ DCs depend on the transcription factor Batf3 for their ontogeny, as development of CD141+ DCs was selectively prevented by knockdown of Batf3 in vitro[114]. Furthermore, IRF-4 has been demonstrated to stabilize the lineage commitment of CD1c+ DCs[35, 115].
Recently a zinc finger transcription factor, zDC (zbtb46), was found to be specifically expressed by mDCs and committed mDC precursors in mice, but not by monocytes, pDCs or other immune cell populations, supporting the notion that mDCs constitute a unique immune cell lineage[116, 117]. In our hands this transcription factor was unable to differentiate human macrophages and DCs (Melum, unpublished results) which might represent differences between species.

Langerhans cells (LCs) expressing CD1a is a notable exception to the rule that DCs are derived from blood and bone marrow precursors. Such cells are maintained in the epidermis of the skin independently of circulating precursors[118] and animal studies have shown that LCs are derived from yolk sac cells seeded during the embryonic period. Notably, after a human limb transplantation LCs of donor origin have been observed in the skin of the host for up to 10 years[119 120].

1.4 Dendritic cells during Immune homeostasis

The maintenance of balance between tolerance and immunity is a complex process that can easily be disturbed. In addition to recognition of danger and thus initiation of immune responses, DCs have a central role in keeping the immune system "at ease" when no danger is present in the tissue. To accomplish this task DCs continuously process and present self- and non-pathogenic antigens to T cells. In this context, effector T cells are not induced to proliferate, but rather, the differentiation of immunosuppressive Tregs producing immunosuppressive cytokines is favored[50].

DCs continuously interact with T cells to induce antigen-specific refractoriness or tolerance in organized lymphoid tissue and in the periphery[74,121]. The maintenance of self- tolerance and unresponsiveness by DCs is influenced by several factors, including the activation status and type of DCs, and the local cytokine milieu[75]. Thus, the tissue environment is crucial for maintenance of immune homeostasis, and depends to a large extent on cytokines produced continuously by epithelial and stromal cells in the tissue. During non-inflammatory conditions, epithelial cells produce the immune-modulating cytokines TGF-β and RA, shown to be crucial for the DC-mediated induction of Tregs in mice[122, 123]. A subset of intestinal DCs in mice was shown to activate the latent form of TGF-β and thereby inducing Foxp3+ Tregs[122].

In addition, DC characteristics like their maturation status or expression of certain receptors are involved in maintenance of immune homeostasis. Antigen presentation by immature DCs typically results in immune tolerance because of deficiency of co-stimulatory molecules[64,74],

whereas activated mature antigen-bearing DCs may initiate the differentiation of antigen-specific effector T-cells as previously described.

Exposure to various anti-inflammatory cytokines and immunosuppressive agents can shape DCs to a tolerogenic state. For example DCs generated in vitro, in the presence of anti-inflammatory factors such as vitamin A, prostaglandin E2 (PGE2), IDO, IL-10 and TGF-β exhibit tolerogenic functions[64]. The enzyme IDO is expressed by DCs, and is a negative immune regulator that depletes tryptophan by catalyzation of the first step in tryptophan catabolism. Depletion of tryptophan modulates the activity of the immune system and mediates Treg proliferation and activation, inhibition of effector T cell responses and decreased cytotoxic T-cell activity[124].

Furthermore, receptors expressed on DCs may mediate tolerogenic responses. One such receptor is AXL, a member of the TAM (TYRO3, AXL and MER) receptor tyrosine kinase family, which inhibits inflammation in DCs and macrophages and promotes phagocytosis of apoptotic cells[125]. AXL expressed on DCs mediates increased uptake of apoptotic cells and blocks proinflammatory cytokine production upon TLR stimulation[126]. This is mediated through induction of suppressors of cytokine signaling (SOCS) molecules, which inhibit both TLR and cytokine receptor cascades, thereby attenuating the inflammatory response[125].

In summary, DCs are important immune modulators and mediate immune homeostasis through various mechanisms.

1.5 Airway allergy and Allergic rhinitis

The prevalence of allergic diseases worldwide is rising dramatically in both developed and developing countries. Allergic rhinitis is becoming increasingly common, affecting more than 30 % of the population in the western society. A high proportion of this increase is occurring in young individuals, thus the burden of allergic disease is expected to increase even more[127]. Allergic rhinitis is most often a chronic disease which can impose major impact in quality of life for the patients, and also inflict a significant burden on health care resources. Furthermore, allergic rhinitis is a known risk factor for asthma and effective treatment of allergic rhinitis has a preventive effect in the development of asthma[128].

1.5.1 Immunopathology of allergic rhinitis

An allergic reaction occurs when normally harmless substances trigger an immune response. Key players in this process are immune cells (e.g. mast cells, DCs, T cells, and B cells) and stromal cells. The process has two phases; the sensitization phase where genetically susceptible individuals react towards a harmless substance (allergen) by producing antigen-specific IgE. Secondly, in the allergic effector phase the allergen triggers an immune reaction which leads to an allergic inflammation in the tissue[129]. Mast cells armed with allergen-specific IgE may induce a rapid allergic response upon binding to the allergen and subsequent cross-linking of IgE-FcεRI complexes.

However, it is important to emphasize that not all sensitized individuals develop an allergic inflammation[130] and the question why only some sensitized people develop allergy is still unresolved. Several factors might be important, such as differences in the local microenvironment including the stromal cell compartment. For instance in the upper airway mucosa of allergic rhinitis individuals an increased local production of IgE from resident B cells has been shown[131, 132]. In addition, local class switching to IgE may take place in the nasal mucosa of allergic rhinitis patients but not in healthy individuals[133]. Thus, higher IgE present at effector sites in allergic individuals may be a factor that contributes to development from sensitization to allergic disease. In addition, clinical trials of allergen specific immunotherapy (SIT) have demonstrated that the induction of a tolerant state in peripheral T cells represents a key factor in the development of healthy immune responses towards allergens. Thus the balance between effector Th2 and regulatory T cells might represent

important differences in the immune response towards allergens in healthy and atopic individuals[134].

Furthermore, genetic regions have been linked to atopy and allergy[135]. Genome-wide association studies (GWAS) allow for the discovery of novel genes and pathways involved in disease pathogenesis, and nearly 100 asthma and genes/loci in addition to multiple genes/ loci for allergic rhinitis have been identified by GWAS studies[136] A large meta analysis of GWAS in ethnically diverse asthmatic patients showed that only 5 genes reached statistic significance among which 3 of them are related to cytokines secreted from stromal cells and epithelium; TSLP, IL-33, and its receptor ST2[137].

However a rapid increase in allergy over the last decades implies that genetic factors can be only partially responsible, favoring a significant contribution from environmental factors[136]. It has become clear that environmental conditions, for instance microbes or air pollution present during allergen exposure, may cause tissue damage and interfere with the normal sensitization process[138]. The hygiene hypothesis suggests that decreased exposure to pathogens during infancy leads to defective maturation of the immune system and results in increased risk of developing allergies[139]. Furthermore, asymptomatic subjects may be sensitized to only one allergen (monosensitization), as opposed to patients who more often may be sensitized to several different substances (polysensitization)[140].

To further understand the processes that lead to development of allergic inflammation, it is important to study the key players involved in allergic immune responses. Understanding the functions of DCs in these immunological processes may be a key for novel therapeutic approaches in allergic disease.

Allergen sensitization phase of allergic rhinitis

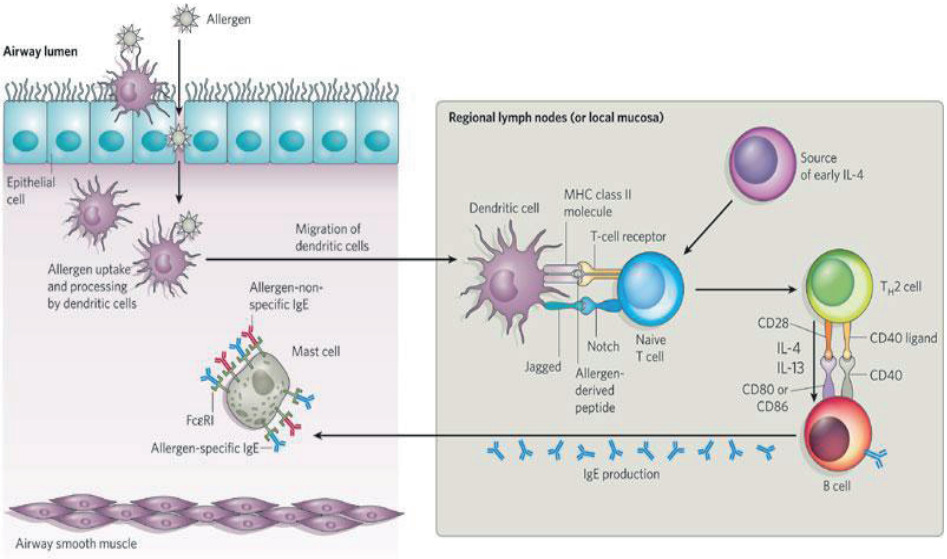

Figure 7: The allergic sensitization phase. Allergens in the airway mucosa can be sampled by DCs, transported to regional lymph nodes and presented to naïve T cells. Under certain conditions naïve T cells acquire the characteristics of Th2 cells, and activate B cells to undergo immunoglobulin class-switch recombination, such that the antibody of the IgE class is produced. IgE is then distributed systemically and binds to the FcεRI on tissue-resident mast cells, thereby sensitizing them to respond when the host is later re-exposed to the allergen.
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Allergens are sampled by DCs in the airway lumen, processed and transported to a regional lymph node (Figure 7). In the presence of the cytokine IL-4, DCs differentiate naïve T cells into Th2 cells, producing IL4 and IL13. These cytokines contribute to activation and Ig class switch in B cells, a process were the gene segments that encode the Ig heavy chain are rearranged such that antibody of the IgE class is produced. Allergen specific IgE is distributed systemically, and after gaining access to the mucosa they bind to the high-affinity receptor for IgE (FcέRI) on tissue-resident mast cells, thereby sensitizing these to respond when the host is re-exposed to the allergen[129].

The effector phase of allergic rhinitis

The effector phase of an allergic reaction is divided into an immediate response and a late-phase response.

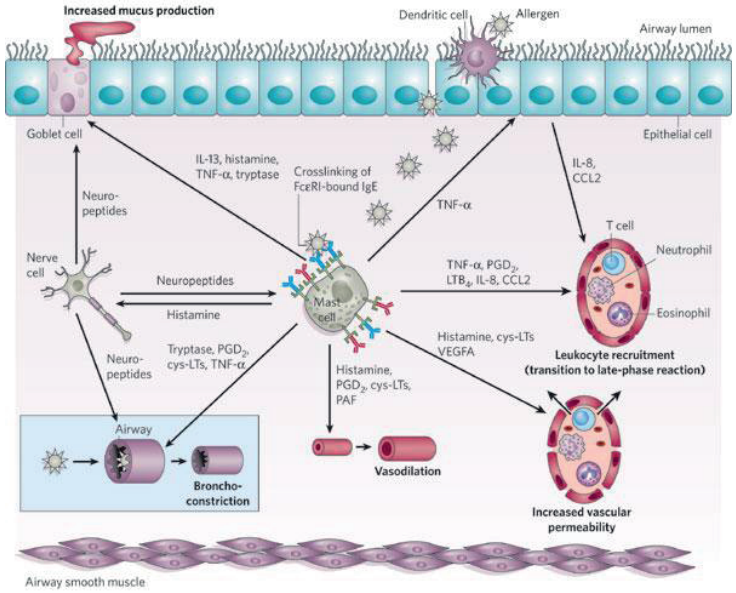

Figure 8. The immediate effector phase of allergen induced airway inflammation
Activation of IgE armed mast cells through FcéRI aggregation results in rapidly secreted preformed mediators and increased synthesis of cytokines, chemokines and growth factors. The rapidly secreted mediators result in bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production. Mast cells also contribute to the transition to late-phase reaction by promoting influx of inflammatory leukocytes.
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The immediate reaction in allergic rhinitis

In sensitized individuals, mast cells armed with allergen specific IgE reside in the mucosa. Encounter with allergen may lead to cross-linking of FcεRI and release of a number of inflammatory mediators like vasoactive amines, lipid mediators and chemokines that create the immediate phase of the allergic reaction (Figure 8). This occurs within an hour after the initial exposure, and brings on the classical symptoms seen during the acute phase reaction in allergic rhinitis; sneeze, itchy and red eyes and coughing, due to vasodilation of blood vessels, leucocyte recruitment, increased mucus production by goblet cells and airway

bronchoconstriction. Mast cell activation also leads to de novo generation of mediators, contributing to the ongoing inflammatory response.

The late phase response

4-12 hours after the initial encounter with allergen, memory Th2 cells, DCs, as well as other members of the immune cell family will contribute to an ongoing inflammatory response[129, 141]. This may lead to chronic allergic inflammation, tissue damage and consequently airway remodeling, leaving permanent damage to the tissue, especially if the lower airways are involved[129].

1.5.3 Dendritic cells in upper airway allergy

DCs have been implicated as essential initiators of allergic responses, both in the sensitization phase and during an ongoing allergic inflammation[142]. Studies in mice demonstrated that DCs are required for the initiation of allergic responses[143] Furthermore, early studies of grass pollen allergic individuals showed accumulation of DCs in the upper airways during pollen season[144]. In line with this it was demonstrated that DCs have an essential role in experimental allergic rhinitis[145].

There is compelling evidence to show that the tissue environment the DCs reside in upon allergen encounter is essential for the outcome of these processes[146, 147, 148]. Interactions of allergens with epithelial cells and various pro-inflammatory substances promote disruption of epithelial integrity and production of Th2-skewing cytokines like TSLP, IL-33 and IL-25 by epithelial cells[149]. By integrating signals from the environment, DCs activate naïve T cells to become Th2 cells, producing the hallmark Th2-cytokines responsible for induction of the allergic inflammatory response (Figure 9). Thus, DCs are initiators of the allergic cascade, directing the process of sensitization and the effector phase of an allergic inflammation.

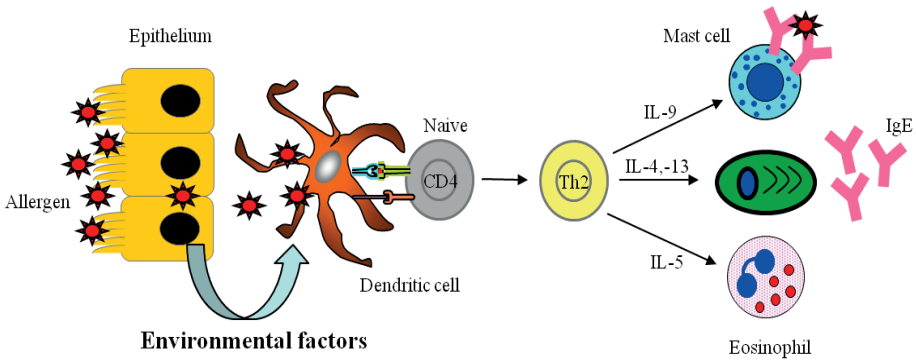

Figure 9. DCs integrate signals from the airway epithelium to mount Th2-responses

Monocytes and macrophages in allergic rhinitis

Antigen presenting cells (APCs) other than DCs might contribute to the immunopathology of allergic rhinitis.

Monocytes may serve as precursors for inflammatory DCs, and animal studies have shown that monocyte-derived DCs (moDCs) are necessary and sufficient for induction of Th2 immunity and features of asthma[150, 151]. MoDCs generated Th2 responses in the tissue by chemokine production and antigen presentation, but were dependent on high doses of antigen to induce Th2-inflammatory responses. Also, DCs were needed to induce Th2 cell-mediated immunity in the lymph node, suggesting a division of labor between moDCs and DCs in an allergic inflammatory response[150].

As previously described, alternatively activated M2 macrophages may be effector cells in allergic inflammatory responses. Interestingly, a study in mice reported that imbalance in the gut microbiota altered the macrophage phenotype towards M2 via PGE2, resulting in enhancement of allergic airway inflammation[152]. The role of monocyte-derived cell subsets in human allergic airway responses is poorly characterized.

TSLP and immunoregulation

TSLP is a cytokine that plays essential roles in allergic inflammatory disorders in the skin and airways. TSLP belongs to the IL-7 cytokine family, and was originally shown to promote B-cell growth and development[153], but was later demonstrated to be crucial for the induction

of Th2 responses by driving DC maturation[154, 155]. Co-culture of TSLP-stimulated DCs with naïve allogeneic T-cells results in the generation of inflammatory Th2 cells that produce IL-4, IL-5, IL-13 and TNF-α[156]. This Th2-driving phenotype was shown to depend on upregulation of the costimulatory molecule OX40L on TSLP-treated DCs in addition to lack of IL-12[155].

TSLP signaling is mediated by a heterodimer composed of the IL-7 receptor α-chain, and TSLP receptor (TSLPR). Activation of TSLPR by TSLP leads to activation of nuclear factor κB (NfκB) and phosphorylation and activation of STAT5 and STAT 6[157], with the subsequent upregulation of OX40L and production of chemokines necessary for the recruitment of Th2 cells[158].

TSLP is produced by epithelial cells, keratinocytes and stromal cells, and induction of TSLP has been demonstrated in several allergic diseases including atopic dermatitis, allergic rhinitis and asthma[156, 159]. In vitro studies of human keratinocytes and bronchial epithelial cells demonstrated that Th2 cytokines, rhinovirus infection, TLR3 ligands and allergens with protease activity are potent inducers of TSLP[160, 161].

In contrast to its role in the development of allergic disorders, TSLP was reported to be constitutively expressed by human intestinal epithelial cells (IECs) implying a role in maintenance of immune homeostasis at this site. In fact, TSLP produced by IECs conditioned DCs to become non-inflammatory, secreting less IL-12p40, and driving the generation of FoxP3+ Tregs[162]. In the setting of TSLP-related homeostatic maintenance in the gut, TSLP was produced by DCs, thus controlling Th17 and Treg cell development[163].

Recent work highlighted the previous unrecognized fact that TSLP consists of at least two splice variants that have different functions; the short form TSLP, being constitutively expressed and implicated in immune homeostasis, and the long form TSLP which is the only variant that encodes a protein that has been shown to activate TSLPR, and thus associated with allergic inflammation[164, 165, 166]. These findings might explain the reported presence of TSLP under both inflammatory and homeostatic conditions, and highlight the necessity of analyzing the two isoforms separately.

In conclusion, DCs have been shown to play important roles in maintenance of immune homeostasis and initiation of allergic responses, however relatively little is known about the heterogeneity and functional characteristics of human DC subsets.

2. AIMS OF THE STUDY

The main focus of this thesis is human DCs and their functional properties during steady state and allergic airway inflammation.

We aimed to identify mechanisms involved in immune homeostasis and allergic responses in the human airway mucosa.

To this end our objectives were to

- Define the DC compartment in the upper airway mucosa at steady-state. (paper I and III)
- Assess mechanisms by which airway mucosal DCs promote tolerance at steady state. (paperIII)
- Determine phenotypes and functions of DCs in human upper airway mucosa during airway allergy (paper I)
- Explore the feasibility of performing system biology studies on cell populations isolated from human upper airway biopsies during experimentally induced allergic rhinitis (paper II)

3. MATERIALS AND METHODS

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.

3.1 IN VIVO ALLERGEN CHALLENGE (Paper I and II)

For study I and II allergic volunteers with a typical history of pollen-induced upper airway allergy and positive skin-prick test or allergen-specific IgE in serum to the relevant pollen were included. In study I, healthy volunteers were included as controls. All subjects were non-smoking, had no nasal polyps, nasal deformities or recent nasal surgery.

Participants were challenged with relevant allergen for seven days, and biopsies were obtained from the lower edge of the inferior turbinate at day 0 and 7 (study I and II a), and at day 0-3 and 7 (study II b) (Figure 10). The challenge was performed outside of pollen season when the patients had no symptoms.

In this model the dose of allergen is standardized, in contrast to the allergy season where the allergen dose is highly variable.

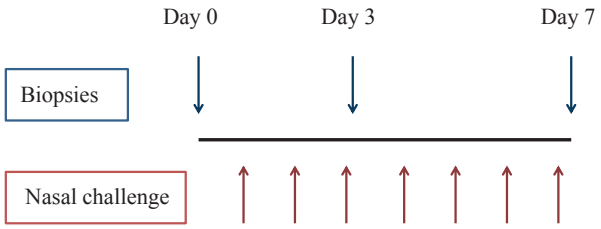

Figure 10. Outline of the experimental setup in article I and II

The main advantage of this experimental model for allergic rhinitis is that it enables the study of factors affecting the immune system during allergic inflammation *in vivo*, thus allowing for studies of the immune cells in their natural habitat. Moreover, it is unique because it is possible to follow the initiation phase of an inflammatory reaction in humans and the dynamics over time.

For phenotyping of mucosal DCs (study I and III), and in vitro studies on blood DCs in (study I and III) we used blood-derived primary DCs, nasal mucosal biopsies obtained during septum deviation surgery in otherwise healthy donors, as well as macroscopically healthy small intestinal tissue obtained during pancreas cancer surgery.

3.2 Quantitative Real-Time PCR and culture of human biopsies

For the study of TSLP induction in human nasal mucosa we measured mRNA expression, since we wanted to differentiate between the long and short form of TLSP. Currently no antibodies can differentiate between the two different TSLP-isoforms making it difficult to investigate the induction of long-form TSLP protein in our in-vivo challenged material.

Nasal mucosal biopsies were cultured 4 hours with or without the cytokines IL-4, IL-13 and TNF-α, and the biopsies were minced in TRI-reagent prior to RNA purification and cDNA synthesis. The quantity and purity of the RNA was assessed with a NanoDrop spectrophotometer, and the RNA integrity was analyzed on an Agilent 2100 Bioanalyzer.

3.3 Analysis of microarray data

Microarray analysis is a transcriptional profiling method that enables the study of the expression levels of thousands of genes simultaneously, usually by pairwise comparison (i.e. healthy vs. pathological conditions). 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 base pair with a complementary DNA strand in the process of hybridization.

In paper II and III, we made use of transcriptional profiling to discover genes or gene interactions central to the allergic disease process (paper II), and genes differentially expressed in circulating and upper airway DCs (paper III).

In paper II, defined cell subsets from nasal mucosal biopsies were sorted based on expression of surface markers (CD4+ T cells and CD45+ HLA-DR+ APCs). Purity check after sorting revealed high purity within each population (>95%), however analyses of data from the T cell population uncovered probable contamination with non- T cells, most likely stromal cells

since they comprise a relatively large proportion of cells within the biopsy, and even small amounts of stromal cells will have an impact on the end result. To overcome this challenge we used a filter created based on allergen challenged T cells, ensuring the genes analysed was relevant for the T cell population. One could argue that this procedure would miss out on novel findings since the filter will narrow down the number of genes available, however novel genes within the T cell population would still be possible to uncover.

In the same study, we used immunohistochemistry to validate a selection of the upregulated genes on a protein level. This was done on a separate study population to further strengthen the validity in our findings. Due to practical limitations it was not feasible to perform immunohistochemistry on nasal biopsies in parallel with gene expression analysis as the amount of tissue one can sample during allergen challenge from each donor is limited.

3.4 Immunofluorescence staining

The principle of immunofluorescence staining is that primary antibodies of different Ig subclasses or species can be detected with secondary fluorescent labeled antibody, specific for the relevant Ig.

In paper I and II two- or three-color immunofluorescence staining was performed on frozen sections from biopsies taken pre- and post allergen challenge. As a control for unspecific binding, parallel staining experiments were performed with concentration-matched irrelevant isotype control. To determine cell densities, the cells of interest were counted in a fluorescence microscope by superimposing a grid parallel to the basement membrane of the surface epithelium.

3.5 Flow cytometry

Flow cytometry was used for phenotypic characterization of cells (paper I and III), detection of intracellular signaling by means of phosphoflow assays (paper I), measurement of cytokine concentrations in supernatants after cell culture experiments by means of cytometric bead arrays (CBAS) (paper I),sorting of cells from tissue biopsies (paper II) and for cytokine production (paper I and III).

Flow cytometry allows for the study of large amounts of cells and several different markers in the same sample. It is a sensitive technique that makes it possible to detect relatively rare cell subsets, such as APCs and T cells from small mucosal biopsies.

To avoid misleading results, caution must be made to avoid improper gating, and "bleed through" signals from overlapping fluorochromes. This can be avoided by running compensation controls to reveal overlapping signals.

Phosphorylation of proteins is a crucial post-translational modification that regulates a broad range of cellular activities such as cell differentiation and metabolism. As previously described, phosphorylation of STAT5, an intracellular signaling molecule, has been shown to be an event downstream of TSLPR activation. To detect TSLPR activation in DCs, we made use of a phospho-STAT5-specific antibody to detect intracellular signaling upon TSLP activation of DCs. This makes it possible to perform single cell analysis on a defined population of cells. Because of the transient nature of intracellular signaling events, fixation techniques used for phospho-protein analysis must be rapid and efficient to prevent dephosphorylation. To find the optimal time for stimulation and fixation/permeabilization procedure, experiments with STAT5 phosphorylation upon IL-4 stimulation in peripheral blood mononuclear cells (PBMCs) were performed.

3.6 Statistical analysis

During analysis of data it's important to make use of the proper statistical methods to be sure to end up with reliable results. Because of small number of samples in some of the experiments, normal distribution could not be expected and non-parametric methods were applied. Wilcoxon signed rank test was used for paired data, and differences between groups were determined by Mann Whitney U test. For correlation analysis Spearman correlation test was used. 2-way ANOVA was used for differences between groups where the response was affected by two factors. To perform the statistical tests, Graph pad Prism 5.0 was used. A p-value of < 0.05 was considered significant.

4. Summary of results

4.1 Paper I: A thymic stromal lymphopoietin–responsive dendritic cell subset mediates allergic responses in the upper airway mucosa

In this paper the role of DCs in human allergic rhinitis was studied. A distinct subset of CD1c+ DCs was shown to express the receptor for TSLP, a cytokine involved in aggravation of Th2 responses and in particular allergic airway disease. The number of these cells increased in the upper airways of allergic individuals during an allergic inflammation. Upon stimulation with TSLP these cells obtained enhanced capacity for activation of allergen-specific memory Th2 cells and up regulated CCR7, indicating an increased capacity for lymph node migration. The CCR7- effect was abrogated by the Th2 cytokines IL-4 and IL-13, suggesting that during an allergic inflammation, DCs are retained in the tissue to further aggravate the allergic response by activating memory Th2 cells residing in the tissue.

Conclusion of the study: A subset of human airway DCs is involved in the induction of allergic airway inflammation through their responsiveness to TSLP. TSLP activation triggers CCR-7 dependent migration to draining lymph nodes and enhances their capacity to initiate Th2 responses.

4.2 Paper II: Identification of gene networks activated during experimental allergic rhinitis in humans

In this study we describe gene networks activated during experimental rhinitis in humans. By taking a system biology approach we could perform comprehensive analysis of gene networks relevant to the allergic immune response in the tissue. Allergic rhinitis is a complex immunological disease involving various cell subsets including immune cells and stromal cells, making it beneficial and necessary to study whole system biology as opposed to limited groups of selected biomarkers.

We aimed to construct a cell-to-cell co-expression network of genes to identify cellular relationships during the inflammatory reaction. In a challenge model of allergic rhinitis, APCs and T cells were sorted from the tissue after allergen challenge in allergic subjects. Bioinformatic analyses revealed T cells expressing a mixed Th2/ regulatory phenotype, with

evidence of recent migration into the tissue. APCs displayed increased capacity for antigen presentation in addition to production of Th2 related cytokines. Central in gene expression in DCs were IL-4/ IL-13 inducible genes.

Conclusion of the study: It is possible to perform gene network analysis on various specific cell populations from small biopsies. Furthermore, our findings suggest that CD45+HLA-DR+ cells (which include monocyte-derived cells, macrophages and DCs) are producers of Th2-associated chemokines, important for the recruitment of Th2 cells and eosinophils.

4.3 Paper III: Steady-state mucosal CD1a+ Dendritic cells display immunoregulatory properties

In this paper we identify a subset of CD1c+ DCs expressing CD1a in human upper airways and small intestinal mucosa that display signs of immune regulatory functions, potentially involved in maintenance of immune homeostasis. Blood-derived CD1c+ DCs displayed similar characteristics when incubated with TGF-β. TGF-β-stimulated CD1c+DCs upregulate CD1a, express AXL, a receptor involved in maintenance of immune regulation, and produce low amount of TNF-α in response to activation with LPS.

Conclusion of study: A subset of upper airway and small intestinal DCs display properties suggesting involvement in immune regulation. Thus, CD1c+ DCs coexpressing CD1a may represent human tolerogenic DCs.

5. Results and general discussion

DCs are important for the generation of effective immune responses and maintenance of immune homeostasis. Allergic asthma and rhinitis are T cell-mediated diseases, where susceptible individuals develop a Th2-dominated inflammatory immune response against allergens[167]. To initiate these responses DCs are both sufficient and necessary, as demonstrated in mice where DC depletion abolished the initiation of Th2 responses against house dust mites[151].

Early studies showed that in allergic rhinitis there is an increase of HLA-DR+ cells, thus indicating a role for APCs in the effector phase of allergic rhinitis[168]. Also, during homeostatic conditions, a subset of intestinal mouse CD103+ DCs has been shown to be important by promoting differentiation of FoxP3+ regulatory T cells from naïve T cells through mechanisms involving TGF-β and the metabolite RA[123], suggesting that this subset represent a tolerogenic subset in mice. The possibility that a similar tolerogenic subset exists in humans remains to be explored.

In this thesis, we provide evidence that CD1c+ DCs in human upper airways play a role in allergic inflammatory reactions and display tolerogenic properties during immune homeostasis.

CD1c+ DCs are central in human allergic rhinitis

The basis for study I was emerging data revealing that the pathogenesis of allergic inflammation is a combined result of barrier dysfunction and loss of immunological tolerance to harmless antigens. Thus damaged epithelium, caused by genetic and/ or environmental factors, leads to the production of various proteins (eg IL-33, CD25 and TSLP) that can activate immune cells to initiate allergic responses[169]. In particular; the epithelial cytokine TSLP has been shown to promote Th2 responses and play a crucial role in the development of allergic inflammations through activation of DCs[170 171]. However, whether DCs in the human upper airways respond to TSLP had not been investigated.

Mucosal DCs may be involved both in the sensitization and the allergic effector phase of an allergic inflammatory response. As previously described, the sensitization phase is initiated

by DCs, sampling antigens in the periphery and subsequently migrating to the draining lymph nodes for presentation and activation of naïve T cells[172]. In addition, allergen-specific memory T cells reside in the airway mucosa and may be activated locally when inhaled allergens are presented by DCs in situ. Furthermore, during the effector phase of an allergic reaction there might be a continued sensitization of naïve T cells to further enhance the inflammatory process, thus DCs may be involved in allergen-triggering of recall responses, making them attractive targets for allergy treatment[173].

We found that expression of the receptor for TSLP was almost confined to a subset of CD1c+ DCs, being expressed by the majority of these cells (paper I).

Furthermore, TSLP-stimulated CD1c+ DCs upregulated CD1a, a molecule involved in lipid presentation to T cells, and had an increased capacity to activate memory Th2-cells supporting the notion that DCs are involved in the recall phase of the allergic reaction. Plant pollen act as vehicles for foreign protein antigens and are important initiators of allergic responses through activation of DCs. In addition, plant pollen contain lipids that are recognized by human CD1 restricted T cells[174]. Thus the observed increased expression of CD1a might contribute to increased T cell recognition of lipids from pollen, thus exacerbating the allergic response.

In addition, TSLP-stimulated DCs upregulate CCR7, thus making them capable of lymph node migration. However, the Th2 cytokines IL-4 and IL-13 abrogate the TSLP-mediated upregulation of CCR7, indicating that during an allergic inflammatory reaction in the mucosa, DCs are retained in the tissue to further exacerbate the allergic inflammatory response locally. This was reflected in the in vivo allergen challenge model where CD1c+CD1a+ DCs accumulated in the tissue, potentially caused by both increased recruitment of DCs from blood and decreased migration from the tissue due to IL-4/IL-13 dependent down regulation of CCR7. This is in line with previous reports showing that CD1a+ DCs are increased in the nasal mucosa of allergics during the grass pollen season[173]. A recent study reported that in mice, aeroallergen challenge promotes DC proliferation in the airways, thus leading to accumulation of DCs in the tissue[175], however in our allergen challenge model in human upper airways, staining for Ki-67 revealed no proliferating cells within the HLA-DR+ cell population (Melum, unpublished data).

Upregulation of CCR7 by TSLP might be important in the sensitization phase, as well as in the early effector phase when DCs migrate to the lymph nodes to recruit memory T cells. On the other hand, downregulation of CCR7 by IL-4 and IL-13 might also serve to dampen the inflammatory reaction since reduced migration of DCs will reduce the activation and homing of new allergen-reactive T cells to the site of inflammation, and furthermore reduce the possibility for a systemic inflammatory reaction. In the upper airway mucosa; mast cells armed with allergen-specific IgE might be an early source of IL-4 and IL-13, thus initiating TSLP production by epithelial cells after encounter with the allergen[176, 177]. Allergen proteases might also induce TSLP secretion from epithelial cells directly[161].

Different factors like allergens with protease activity and the Th2 cytokines IL4 and IL13 have been shown to trigger TSLP production in epithelial cells[161, 160]; however few studies have taken into account that TSLP consists of at least two splice variants, one being involved in immune homeostasis, whereas the other activates the TSLPr and induces Th2 responses[164, 165]. To investigate if the long form of TSLP, associated with allergic inflammation is produced by cells in the nasal mucosa, we cultured nasal mucosal biopsies with IL-4 and IL-13 and found induction of long from TSLP after 4 hours culture. This indicated that TSLP is involved in the early phase of the recall response of an allergic reaction, priming DCs to activate resident memory Th2 cells.

TSLP has been shown to play a role in the immunopathology of several different disorders[178, 179, 180]; however without any discrimination between isoform specific subtypes one cannot be sure which of the two functionally different forms was detected during examination. This raises the important question of whether the short form TSLP, which was shown to have antimicrobial and homeostatic properties[165] is upregulated in various inflammatory conditions. Future investigations of TSLP should include isotype-specific primers or antibodies to seek answers to these questions.

Recently, the first clinical trial of TSLP blockade in allergic asthma showed promising results with evidence for attenuation of measures of allergen-induced early and late allergic asthmatic responses. Following allergen bronchoprovocation, patients with allergic asthma pretreated with anti-TSLP displayed lower blood and airway eosinophil counts and reduced

fraction of exhaled nitric oxide as compared to the control group[181]. The biological mechanisms on how blocking of TSLP serves to ameliorate both the early and late phase of asthmatic responses is not clear[182], but based on our findings one can speculate that it involves reduced activation of DCs, thus dampening the allergic response.

CD4+ T cells and APCs actively participate and interact in the chronic phase of allergic airway inflammation

A systems biology approach to investigate the immunopathology during the allergic inflammatory response may prove beneficial. To this end our goal was to explore the possibility of using an in vivo challenge method to study networks of gene expression induced in upper airway APCs and T cells during pollen induced allergic rhinitis in allergic individuals.

The allergic inflammatory upper airway mucosa showed signs of recently recruited activated Th2 cells and Foxp3 regulatory T cells (study II), in line with the notion that activation of effector T cells is accompanied by a set of regulatory T cells to dampen the response and avoid collateral damage[65].

Analysis of HLA-DR+ APCs showed signs of activation and upregulation of receptors important in antigen presentation, suggesting a role for APCs in the tissue in the chronic phase of allergic inflammation. As observed in study I, there was an increased expression of CD1a on HLA-DR+ cells, in addition to several other genes downstream of IL-4 and IL-13 showing that "Th2-activated"APCs accumulated in the nasal mucosa during the allergic inflammation. However; the contribution of the monocyte/ macrophage population in this setting has not been resolved, and which subsets of APCs that contribute to the increase in HLA-DR+ APCs was not investigated in this study. Based on our findings in study I it is reasonable to assume that CD1c+CD1a+ DCs contribute to this accumulation. Furthermore, we have previously shown that pDCs are recruited to the nasal mucosa during an allergic inflammatory response[183], however no increase was seen in CD141+ DCs (paper I).

CD14+CD68+ macrophages comprise the largest subset of APCs in the upper airway mucosa, during steady state[184] however their role in an allergic inflammatory response has not been investigated. Interestingly, we recently found that in a similar challenge model as in

study I, CD14+HLA-DR+ monocytes were recruited to upper airways at an earlier time point compared to CD1c+ DCs (Figure 11.), (Eguiluz Garcia et al, unpublished results).

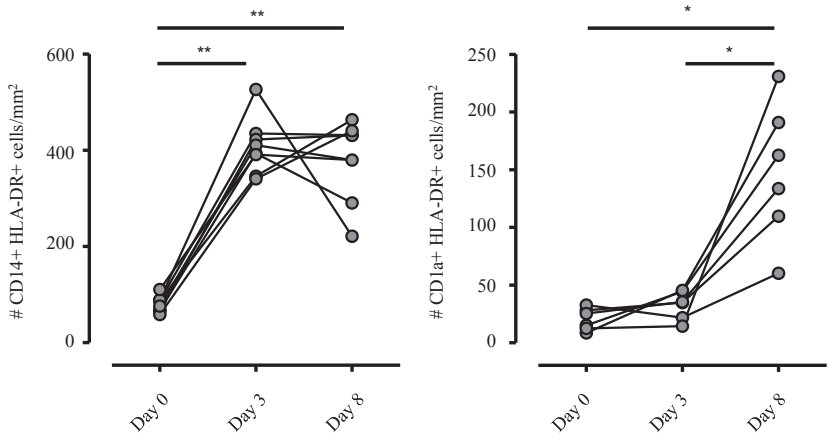

Figure 11. Early recruitment of CD14+ HLA-DR+ monocytes in human upper airways during experimentally induced allergic rhinitis. CD14+ monocytes accumulate in the tissue earlier than CD1a+ DCs.

This indicates a division of labor between macrophages and DCs in the allergic inflammatory response, where monocytes are recruited to the mucosa in the early phase of the response. DCs might be involved in lymph node migration during early responses, and therefore accumulate in the mucosa at a later stage when CCR7 is downregulated due to the increased presence of the cytokines IL4 and IL13 (study I). This is in line with a study in mice where monocyte-derived DCs (moDCs) were sufficient to drive Th2 responses in the mucosa, whereas CD11b+ classical DCs induced Th2 responses in the lymph node[150].

In addition APCs showed signs of secretion of chemokines such as CCL18, CCL24 and CCL17 that attract T cells to the site of inflammation, thus in our model of allergic rhinitis we demonstrate the presence of parallel core elements of a Th2 inflammatory response in the upper airway mucosa, thus the method can be widened to apply to other cell subsets like stromal cells, to reveal functional interactions between novel genes and previously recognized members of the allergic cascade.

Particularly interesting was the fact that gene expression of four chemokines was upregulated. The receptors for these chemokines are CCR3, CCR4, and CCR8; all shown to be involved in the recruitment of Th2 cells and eosinophils. These receptor-ligand pairs should be tested as targets for therapy in allergic rhinitis, either by blocking the receptor-ligand interaction or by blocking the local production of these chemokines. Identifying hub genes that are central to these processes should be addressed in future studies.

CD1c+ dendritic cells have a potential role in immune regulation and homeostasis in human small intestine and upper airway mucosa

How DCs contribute to the induction and maintenance of tolerance and immune homeostasis is a matter of vast investigation. The concept of a tolerogenic DC subset has been proposed for a subset of CD103+ DCs in mice, which induces tolerance by induction and activation of FoxP3+ Tregs[122 123]. Whether or not a specific subset of tolerogenic DCs exists in humans remains elusive. As previously described DC subsets have a high degree of plasticity and are able to change their functional properties in response to environmental signals. Thus different subsets might be responsible for induction of tolerogenic responses depending on the signals they are exposed to in the environment.

In paper III we identify a subset of CD1c+ DCs present in the upper airway and small intestinal mucosa during steady state. These cell express CD1a, initially described on Langerhans cells in the skin, but later shown to be expressed on DCs at other sites[185, 186, 88].

CD1a is not present on blood DCs, suggesting that CD1a is induced on CD1c+ DCs due to factors in the tissue environment. TGF-β is a cytokine that is highly expressed in the mucosa at epithelial barrier interfaces during steady state, and a central regulator of immune cell development and function. TGF-β is important for induction of regulatory T cells thus maintaining immune homeostasis[187, 188]. We observed that TGF-β induced CD1a on blood derived CD1c+ DCs suggesting that TGF-β produced in airway and small intestinal mucosa induces CD1a expression on tissue-residing CD1c+ DCs.

HLA-DR molecules are constitutively expressed by DCs and induced together with costimulatory molecules on tissue-residing DCs in response to inflammation, thus making the cells more efficient in antigen presentation to T cells[189]. Blood-derived CD1a+CD1c+ DCs induced after culture with TGF-β displayed a lower DR expression than their CD1a-

counterparts, suggesting a lower capacity for antigen presentation and thus activation of T cells. Furthermore; CD1a+CD1c+ DCs from the small intestinal mucosa produced less TNF-α after activation with LPS implying a role for TGF-β in the maintenance of a immune-regulatory DC subset in the small intestinal and upper airway mucosa.

TGF-β also induced the tyrosine kinase AXL on blood CD1c+ DCs. AXL belongs to the family of TAM receptors (Tyrosine, Axl and Mer). These receptors have pivotal roles in innate immunity demonstrated by the observation that TAM mutant mice develop severe autoimmune diseases[190], probably as a combined result of loss of regulation of the innate inflammatory responses to pathogens and loss of phagocytosis by DCs and macrophages. The innate immune system relies on mechanisms to turn off the response after activation to avoid unrestrained signaling and chronic inflammation. TAM receptors prevent this dysregulation through induction of SOCS[190].

It has been shown that AXL is present on a population of langerhans cell in the skin enhancing their capacity for apoptotic cell uptake, and blocking of proinflammatory cytokine production. Our findings support the notion that AXL expression on DCs may be an important factor for homeostatic maintenance at other sites than the skin.

In conclusion we provide data showing that CD1a is expressed on a subset of mucosal DCs with potential functions in immune homeostasis in the small intestinal and upper airway mucosa. Future efforts will be aimed at studies of their modulatory effect on T cells.

In summary, studies in this thesis gave new insights into the understanding of the role of a subset of DCs in human mucosa during allergic inflammation and immune homeostasis.

6. Conclusions

The work in this thesis has shown that

- A defined subset of CD1c+DCs in human upper airways responds to TSLP and activates allergen specific T cells to aggravate an allergic response.

- A defined subset of CD1c+ DCs is present in human upper airways and small intestinal mucosa during steady state and may play a role in maintenance of immune homeostasis.

- Defined cellular gene networks can be identified in human upper airway mucosa to reveal novel pathways involved in the pathogenesis of allergic rhinitis.

7. Future perspectives

The central role of DCs in maintenance of immune homeostasis and in the initiation of allergic responses makes them attractive targets for intervention to suppress allergic inflammatory responses.

The heterogeneity of DCs argues for a division of labor between DCs subsets during steady state and possibly also with monocytes recruited to the tissue during allergic inflammation. Further investigations on how the different human DC subsets cooperate to mount immune protection will be important.

Our understanding of the mechanisms involved in the initiation and development of Th2 responses in the airway mucosa is advancing, however, much of our knowledge is derived from animal studies. Whether these models derived from murine studies are identical in humans need further investigation.

In vitro studies have revealed that many allergens can activate epithelial cells, or promote the formation of cytokines that activate epithelial cells and DCs. This emerging knowledge of stromal cells being important effector cells in the initiation of allergic responses makes the study of their function *in vivo* important. Further investigations on their role in the sensitization and effector phase of allergic inflammatory responses should take into account that their function relies on the interaction with other immune cells.

The knowledge of DC responses in allergic rhinitis might also have implications for our understanding of the pathogenesis of other diseases like allergic asthma, which share many features with allergic rhinitis.

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ERRATA

Please note the following corrections to the thesis:

Spaces have been added between paragraphs.

1.1

Page1: *B-cells* changed to *B cells*

1.2.1

Page 4: "*Eosinophils was shown to be"* changed to "*were shown to be"* and

"Tissue residing macrophages was initially" changed to *"were initially"*

Page 6: *"These findings primarily derive from in vitro experiments..."* changed to *"These findings are primarily derived from…"*

1.2.2

Page 8: *"differentiation[49] Thus DCs determine…"* changed to *"differentiation[49]**.** Thus DCs…"*

Page 9:"*Therefore these cells are crucial for host defense but are also implicated in immune-mediated disease like allergy and autoimmune disease."* sentence rephrased to *"…to promote local immune responses (Figure 3)[40], thus they are crucial for host defense. CD4+ helper T cells are also implicated in immune-mediated diseases like allergy and autoimmune disease[46]."*

1.3.2

Page 15 "*Resident CD141+DCs **was** shown to*" changed to "***were** shown to"* Page 16: *"CD1c+ DCs **was** shown to display"* changed to "***were** shown to display"* and *"Furthermore; pDCs **has** been implicated"* changed to *"**have** been"*

Page 18: *not able* changed to *unable*

Page 18*: "animal studies have shown that **they** are"* changed to *"animal studies have shown that **LCs** are"*

1.4

Page 20: *"Furthermore**;** receptors"* changed to *"Furthermore**,** receptors"*, "***block** proinflammatory cytokine production"* changed to "***blocks** proinflammatory"* and *inhibits* changed to *inhibit*

1.5.1

Page 21: *"genetically susceptible individuals **reacts**"* changed to "*individuals **react**"*, *"However**;** it is important to emphasize" changed to "However**,** it is important to emphasize"*and *"In addition, local class switching to IgE **may takes** place"* changed to *"**may take**"*.

Page 22:*"Genome-wide association studies (GWAS) **allows** for"* changed to *"**allow** for"*

1.5.3

Page 25: *"during **pollens** seasons"* changed to *"during **pollen** season"* and *"initiation of allergic responses[144]Furthermore"* point added in between sentences and changed to *"initiation of allergic responses[144]. Furthermore"*

3.1

Page 30: *"allergic volunteers **was** included*" changed to "***were** included"*

3.3

Page 31: "*In paper II **a defined cell** subsets"* changed to: "*In paper II, **defined cell subsets"***

Page 32: *avaable* changed to *available*

3.4

Page 32: *staning* changed to *staining* and*;"To control for"* changed to *"As a control for"*

3.5 Page 33: "*Using flow cytometric analysis it is possible to study large amounts of cells and several different markers in the same sample, and it is a sensitive technique that allows for the detection of relatively rare cell subsets*" changed to "*Flow cytometry*

allows for the study of large amounts of cells and several different markers in the same sample. It is a sensitive technique that makes it possible to detect relatively rare cell subsets".

4.2

Page 35: *Human beings* changed to *humans*

4.3

Page 36: "*DCs display properties suggesting involvement **role** in immune regulation"*, role deleted.

5

Page 40: activation of effector T cells *are* changed to *is*

8 Two duplicated references were **removed:**

124. Worthington JJ, Czajkowska BI, Melton AC, Travis MA. Intestinal Dendritic Cells Specialize to Activate Transforming Growth Factor-β and Induce Foxp3(+) Regulatory T Cells via Integrin αvβ8. *Gastroenterology* 2011, **141**(5)**:** 1802-1812.

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Page 50 Reference # 74; *RM S, DH, MC N* changed to *Steinman RM, Hawiger D, Nussenzweig MC*

Page 51 Reference # 85; *A K, K C, K N, W S* changed to *Kitani A, Chua K, Nakamura K, Strober W.*

Page 60 Reference # 189 *JC M, JJ L, M G, AY R* changed to *Marie JC, Letterio JJ, Gavin M, Rudensky AY*

Page 61 Reference #190 *MC F, C B, G M, F P, PR G, MF N* changed to *Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF*