Center for Infectious Medicine, Department of Medicine
Karolinska Institutet, Stockholm, Sweden

TISSUE REGULATION OF DENDRITIC CELLS:

with focus on chemokines, function and migration

Anh Thu Nguyen Hoang

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet
Printed by Larserics Digital Print AB

ISBN 978-91-7549-176-9

“Wherever you go,
go with all your heart”

Confucius

The image on the cover shows a dendritic cell, expressing HLA-DR (green) and DC-SIGN (red), situated close to the epithelial layer (blue-nuclei staining).

ABSTRACT

Tissue-specific cells, such as fibroblasts and epithelial cells in local microenvironments have been recognized to influence the function and phenotype of hematopoietic cells, such as dendritic cells (DC). The interaction and cooperation between DC and the cells of the tissue is important for the maintenance of immune homeostasis as well as orchestrating immune responses against pathogens. However, a majority of studies on human DC are performed under conditions absent of a relevant physiological milieu allowing interactions between different cell types. Thus, there is a need to develop *in vitro* human tissue models with immune cells that can capture cellular responses under conditions similar to those found in real tissue. In my thesis work, I have developed a human three-dimensional (3D) lung tissue model that has morphological and functional features mimicking those of human lung epithelial tissue. The model has a stratified epithelial layer with human DC that are situated closely to the epithelium and an underlying collagen matrix rich in fibroblasts. We have found that the lung tissue model supports DC survival for at least eleven days in the absence of exogenous growth factors. The tissue model also regulates chemokine production by DC leading to enhanced production of CCL18 and downregulation of CCL17 and CCL22, which resemble chemokine production under physiological conditions in lung tissue. In addition, using live cell imaging, we could observe that stimulation with toll-like receptor-ligands and CCL2 attracted DC to the epithelial layer as well as increased their speed and their ability to survey a larger area in the tissue model. We also found, using our newly established 3D tumour spheroid tissue model of non-small cell lung cancer, that DC are recruited to the tumour area and engulf tumour cells more readily than normal epithelial cells.

Another major focus of this thesis work is the study of stromal cell-derived chemokines supporting regulatory DC development during *L. donovani* infection. Stromal cells are known to regulate hematopoiesis in the bone marrow and spleen by secretion of chemokines, cytokines and growth factors. Studies have shown that murine splenic stromal cells have the ability to support differentiation of hematopoietic stem and progenitor cells (HSPC) into regulatory DC and this ability is enhanced during *L. donovani* infection. We further showed that stromal cell-derived chemokines CXCL12 and CCL8 cooperate to recruit HSPC with the ability to differentiate into regulatory DC. In addition, we observed that direct infection of MBA-1 cells by *L. donovani* enhanced their ability to support regulatory DC as well as their ability to produce CCL8. Interestingly, CCL8 expression was strongly induced in splenic stromal cells of mice infected with *L. donovani*, which enhanced their ability to attract HSPC. Our findings suggest that *L. donovani* infection modulates the ability of stromal cells to recruit and support HSPC differentiation into regulatory DC, and this may be a mechanism used by the parasite to establish persistent infection.

Together, the studies in this thesis show the impact of tissue specific cells on DC differentiation and function, and highlights the importance of taking into account tissue-specific components when studying DC biology.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vävnadsreglering av dendritceller: med fokus på kemokiner, funktion och migration

Dendritceller (DC) finns i alla kroppsvävnader och är viktiga för att generera ett starkt immunsvar mot bakterie- och virusinfektioner. Dessa celler är immunsvarets bevakare och har som uppgift att söka efter farliga inkräktare, till exempel bakterier och virus, som kan orsaka sjukdomar. När DC upptäcker ett hot mot kroppen, som vid en bakterieinfektion, tar de med sig delar av bakterien och presenterar det för immunförsvarets soldater som kallas för T-celler. Presentationen av bakterien leder till att T-cellerna blir aktiverade och ökar i antal, för att sedan förflytta sig till den infekterade vävnaden där de kan hjälpa till att eliminera dessa farliga bakterier. Denna immunologiska process är mycket viktig för att skydda oss mot alla typer av infektioner. Dendritceller har också en annan viktig uppgift; de kan presentera det som är kroppseget för T-celler, vilket gör att T-cellerna lär sig att känna igen det som är kroppseget och inte blir reaktiva mot kroppens egna celler och vävnader. Därför är DC både viktiga för att hjälpa till vid infektioner och för att upprätthålla den immunologiska balansen i kroppen. Forskning har visat att DC inte är ensamma i sina uppdrag utan får instruktioner från vävnadspecifika celler såsom fibroblaster och epitelceller. Dessa celler är viktiga då de bidrar till att upprätthålla strukturen och funktionen hos vävnaden. Samtidigt har forskningen också visat att vävnadspecifika celler kan påverka och interagera med DC för att hjälpa till att upprätthålla den immunologiska balansen och reglera immunsvar mot patogener. Därför är det viktigt att kunna studera DC i en mikromiljö som efterliknar den som finns i riktig vävnad. De flesta studier på humana DC har dock utförts i odlingsflaskor som saknar viktiga vävnadsspecifika celler och en mikromiljö som omger DC i riktig vävnad. Forskning på DC som är baserad på *in vivo* data har utförts på möss, vilka har visats vara användbara modellsystem för att ge en bättre förståelse för mänskligt immunförsvar. Men på grund av att många patogener endast infekterar människor kommer djurmodeller inte att kunna ge fullständig förståelse för hur ett mänskligt immunsvar fungerar.

Därför har jag, i detta avhandlingsarbete utvecklat en *in vitro* human tre-dimensionell lungvävnadsmodell som består av DC, fibroblaster och epitelceller. Denna modell möjliggör studier av DC i en mikromiljö som efterliknar den som finns i riktig lungvävnad. Jag har visat att lungvävnadsmodellen kan stödja DCs överlevnad i minst elva dagar, till skillnad från DC odlade i ett cellodlingsmedium; dessa överlever endast i några dagar. Vi har observerat att vävnadsmodellen även kan påverka DC:s förmåga att producera molekyler, så kallade kemokiner som kan attrahera andra celler. Dendritceller i modellen har även kunnat följas i realtid med hjälp av ett fluorescens mikroskop där vi har observerat att DC i modellen reagerar på inflammatoriska stimuli, vilket leder till att de migrerar fortare och har en ökad förmåga att övervaka ett större område i vävnaden. Vår vävnadsmodell har också visat sig vara användbar för att studera DCs interaktion med tumörceller. I detta avseende har vi utvecklat en tumörvävnadsmodell och observerat att DC i denna modell attraheras till tumörområdet och tar upp tumörcellerna mer frekvent än vad de attraheras av och tar upp normala epitelceller.

I detta avhandlingsarbete har jag också studerat hur vävnadsspecifika celler som fibroblaster påverkar regulatoriska DCs utveckling vid infektion av parasiten *Leishmania donovani*. Regulatoriska DC är celler som har förmåga att dämpa immunförsvaret och är viktiga för att upprätthålla den immunologiska balansen. Men forskning har visat att vid en kronisk infektion, som orsakas av den ovan nämnda parasiten, ser man en ökad produktion av regulatoriska DC. Denna ökade produktion visade sig bero på att infektionen ledde till att fibroblaster fick en ökad förmåga att kunna stödja utvecklingen av stamceller till regulatoriska DC. Vilka faktorer som produceras av fibroblaster för att möjliggöra denna process var dock okända. Därför har vi, i detta arbete, studerat huruvida kemokiner, som utsöndras av fibroblaster har förmåga att bidra till utvecklingen av regulatoriska DC. Vår studie visade att två kemokiner, CXCL12 och CCL8, samverkar för att stödja rekrytering av stamceller som har förmåga att utvecklas till regulatoriska DC. Vi kunde också observera att CCL8 uttrycket i fibroblaster ökade vid parasitinfektion och detta ledde till en ökad förmåga hos fibroblasterna att rekrytera stamceller. Resultat av våra studier indikerar att infektion med *L. donovani* ökar fibroblasternas förmåga att rekrytera och stödja utvecklingen av stamceller till regulatoriska DC, vilket skulle kunna vara en mekanism med vilken parasiten dämpar immunförsvaret och etablerar en kronisk infektion.

Sammantaget visar arbetena i avhandlingen att vävnadsspecifika celler spelar en viktig roll för DCs utveckling och funktion samt lyfter fram vikten av att studera DC tillsammans med vävnadsspecifika cellerna och dess komponenter. En ökad förståelse av DCs reglering i vävnad möjliggör att vi kan utveckla nya målinriktade behandlingsstrategier som syftar till att styra DC cellers funktion vid sjukdomar som, exempelvis, kronisk infektion och inflammation, samt cancer.

LIST OF PUBLICATIONS

I. **Anh Thu Nguyen Hoang**, Puran Chen, Julius Juarez, Patty Sachamitr, Bo Billing, Lidija Bosnjak, Barbro Dahlén, Mark Coles and Mattias Svensson. Dendritic cell functional properties in a three-dimensional tissue model of human lung mucosa.
Am J Physiol Lung Cell Mol Physiol 2012, 302: 226–237

II. **Anh Thu Nguyen Hoang**, Puran Chen, Kari Högstrand, John Lock, Alf Grandien, Mark Coles and Mattias Svensson. Live imaging analysis of dendritic cell migrating behaviour under the influence of immune stimulating reagents in an organotypic model of human lung.
Manuscript

III. Puran Chen, **Anh Thu Nguyen Hoang** and Mattias Svensson. Advances in evaluation of dendritic cell behaviour and function in human lung cancer using an organotypic-based epithelial spheroid model of non-small cell lung cancer.
Manuscript

IV. **Anh Thu Nguyen Hoang**, Hao Liu, Julius Juarez, Naveed Aziz, Paul M. Kaye and Mattias Svensson. Stromal cell-derived CXCL12 and CCL8 cooperate to support increased development of regulatory dendritic cells following *Leishmania* infection.
The Journal of Immunology, 2010, 185: 2360–2371

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

DC	Dendritic cells
cDC	Conventional Dendritic cells
3D	Three-dimensional
L. donovani	*Leishmania donovani*
HSPC	Hematopoietic stem and progenitor cells
TGF-β	Transforming growth factor beta
MHC	Major histocompatibility complex
TLR	Toll-like receptor
ECM	Extracellular matrix
TSLP	Thymic stromal lymphopoietin
HLA	Human leukocyte antigen
CSF-2	Colony stimulating factor 2
IL-4	Interleukin 4
PCR	Polymerase chain reaction
QRT-PCR	Quantitative reverse transcription-PCR
NSCLC	Non-small cell lung cancer
Flt3	Fms-like tyrosine kinase 3
GFP	Green fluorescent protein

1 INTRODUCTION TO THE THESIS

The immune system is a delicate network of cells that interact and cooperate with each other to maintain tissue homeostasis in the body as well as regulate immune responses to pathogens. Within the tissue, immune cells are in close contact with tissue-specific cells such as stromal cells, i.e. fibroblasts, and epithelial cells. The tissue-specific cells play important roles in the immune system by creating tissue specific niches and secrete chemokines and cytokines that regulate hematopoietic cell differentiation and function [1]. For example tissue specialized niches in the bone marrow and spleen, are recognized for their important function supporting homing, migration, proliferation and differentiation of hematopoietic stem and progenitor cells (HSPC) into terminally differentiated blood cells [2]. In this context, chemokines secreted by stromal cells are crucial for the regulation of HSPC homing and migration between the circulation and peripheral tissues and organs [1]. At peripheral sites, tissue also has the ability to shape the phenotype and regulate functional properties of hematopoietic cells, such as DC, and thereby influence the outcome of immune responses [3, 4]. An increased understanding of the mechanisms involved in tissue-specific regulation of DC differentiation and function may enable the development of potential strategies to restore tissue homeostasis in chronic inflammatory and infectious diseases as well as cancer.

The work of this thesis is based on four studies. In the first three studies, I investigated how the lung tissue microenvironment regulates and influences DC function and migratory behaviour, as well as DC interaction with tumour cells. This was approached by establishing and using a 3D tissue model, so-called organotypic model, of human lung. In the fourth study I explored the role of stromal cell-derived chemokines in supporting the migration of HSPC that have the ability to differentiate into regulatory DC during *L. donovani* infection. Common for all studies is that there is a specific focus on chemokines. Overall the thesis includes studies on the role of chemokines in directing DC migration in lung tissue, and how the lung tissue influences DC production of chemokines, as well as the role of chemokines in supporting the development of regulatory DC differentiation in steady state and in response to infection. The following two sections will give a brief introduction to the studies of this thesis.

1.1 INTRODUCTION TO THE STUDY OF THREE-DIMENSIONAL LUNG TISSUE MODELS WITH DENDRITIC CELLS

Dendritic cells belong to a heterogeneous population of innate immune cells and are widely distributed in all tissues [5]. The main functions of DC are to initiate and orchestrate immune responses to pathogens, as well as maintain immune homeostasis and tolerance to self [6, 7]. The orchestration of immune responses in the local microenvironment by DC includes production of cytokines and chemokines that are important in the activation and recruitment of other inflammatory cells with specific effector functions [8]. However, most studies on human DC functions have been performed culturing cells on plastic surfaces in tissue culture flasks that lack the multicellular interactions and extracellular matrix components, present in real tissues.

Alternatively studies on DC have been performed in animal models and, there is extensive data from *in vivo* studies of DC function in animal models, which can provide important knowledge also about human immune responses, but have limitations because many pathogens are species specific. In addition, there are molecules with no homologues counterparts in mice, and some molecules may even have different target cell populations in different species [9]. Thus, there is an increasing need to study human hematopoietic and non-hematopoietic interactions in a multicellular environment that resemble *in vivo* tissue to increase our understanding on the cellular processes that occur in a human setting. However, to investigate DC function in human tissues, such as lung tissue, is difficult. Therefore, the over all aim of the first part of this thesis was to establish a 3D organotypic lung tissue model with DC and investigate the regulation of the tissue microenvironment on DC function. The first aim was to establish a 3D lung tissue model with DC suitable to investigate the regulation of DC chemokine production by the lung microenvironment. As a second aim, the 3D tissue model was further developed for live cell imaging analysis to study DC migration in response to inflammatory stimuli and chemokines in the tissue model. The third aim was to develop the 3D lung tissue model further and make an organotypic-based epithelial spheroid tissue model of human non-small lung cancer, which could be used to study DC interactions with cancer cells in a multicellular system.

1.2 INTRODUCTION TO THE STUDY OF STROMAL CELL-MEDIATED DEVELOPMENT OF REGULATORY DENDRITIC CELLS

Under homeostatic conditions, HSPC are most abundant in the bone marrow and are in close contact with stromal cells that can regulate the homing, migration and differentiation of HSPC [10, 11]. In response to infection by *L. donovani*, increased hematopoietic activity has been observed, followed by altered function of splenic stromal cells leading to increased ability to support the differentiation of HSPC into DC with regulatory properties [12, 13]. Similar changes have also been reported in other experimental infections, such as malaria [14]. However, stromal cell-derived factors that are responsible for the development of regulatory DC at steady state and in response to chronic infections is still unknown. By increasing our understanding on the mechanisms by which stromal cells support the differentiation and function of regulatory DC, we may discover potential targets for manipulating the DC repertoire to reverse the course of chronic infections and restore tissue homeostasis. Therefore, the fourth aim of this thesis was to investigate which stromal cell-derived chemokines may play a role in the recruitment of HSPC with the potential to develop into regulatory DC during homeostasis and in response to *L. donovani* infection.

1.3 STRUCTURE OF THE THESIS

The Introduction (Chapter 1) of this thesis is followed by the Background (Chapter 2), which will start with a brief background of DC function in the immune system. The following section will examine the importance of tissue specialized niches in the regulation of DC differentiation and function. This section will also point out the importance of studying immune cells in a more complex multicellular

microenvironment and argue the needs for an approach of using *in vitro* 3D tissue models with human immune cells. The next section will give an overview of 3D tissue culture systems and how they are generated. The last section will give a description of chemokine function in the immune system and emphasize the relationship between chemokines and DC in tissue. In the Research design (Chapter 3), the approach to develop a 3D *in vitro* lung tissue model for studying tissue-specific regulation of DC function and behaviour, will be described. This chapter will also describe the methodological approach used for dissecting stromal-derived chemokines that may play important role in supporting the development of regulatory DC. Following on chapter 3, the Aims of this thesis (Chapter 4), will outline the hypotheses and aims of the thesis. Thereafter, the Results generated in this thesis work (Chapter 5) are presented, and this chapter follows by the Discussion of the results generated in this thesis (Chapter 6), and the Conclusion (Chapter 7).

2 BACKGROUND

In this chapter I give a brief background to my thesis work, and the first section of this chapter is a general overview of DC biology. This is followed by a section emphasizing the importance of tissue-specific non-immune cells as regulators of DC differentiation and function in health and disease. Thereafter, I discuss the significance of performing studies on human DC function using three-dimensional tissue models, so-called organotypic models, and at the end of the chapter I will provide an overview of chemokines focusing on their role in the immune system.

2.1 DENDRITIC CELLS

2.1.1 Function

Dendritic cells belong to a heterogenous family of hematopoietic cells with professional antigen presenting skills and are often described as "gatekeepers of the immune system", as well as the key link between innate and adaptive immunity [15-17]. In 2011, Ralph Steinman was awarded the Nobel Prize in Medicine and Physiology for his pioneering work on the importance of DC linking innate and adaptive immunity [16]. By, patrolling peripheral tissues, DC are among the first cells to recognize pathogens via a specialized set of pattern recognition receptors. Upon recognition of pathogens, DC undergo a process of cellular activation, so-called maturation, including phenotypic and functional alterations. This includes, for example upregulation of the chemokine receptor CCR7, costimulatory molecules CD80, CD86 and MHC class I and II peptide complexes. Upon maturation, altered chemokine receptor expression directs DC to migrate from the site of infection to the secondary lymphoid tissues, where they present pathogen-derived peptides on their MHC molecules to T cells [6]. Activated DC expressing high levels of costimulatory molecules and MHC/peptide complexes, in combination with increased secretion of cytokines have the ability to initiate T cell priming and differentiation of antigen-specific T cells [6]. The proper activation of specific T cell effector functions, e.g., Th1, Th2 and Th17, mediate protective immunity against infections, and possibly tumours [18-20]. Dendritic cells are also important in controlling inflammation-induced immunopathology through the generation of antigen-specific regulatory T cells [21, 22]. Among these are the IL-10 [23] and TGF-β producing regulatory T cells [24, 25] that possess inhibitory function on immune effector mechanisms. In addition, DC orchestrate immune responses at local sites of infection [5] and furthermore, the tissue where DC originate from as well as the mode and context of DC activation, appear to be highly relevant for the outcome of T cells responses, including T cell-specific homing properties [26, 27].

Although, clearly demonstrated, the ability to induce and regulate immune responses is a pivotal function of DC, another fascinating role of DC is to maintain immune homeostasis and tolerance to self. In this context, DC capture non-harmful antigens from the tissue microenvironment, for example in the respiratory and digestive tracts [28], or self antigens from tissues [29]. The capture of environmental proteins in steady state in the absence of microbial stimuli and DC maturation allow DC to maintain

immune tolerance to self by inducing peripheral T cell tolerance. Tolerance to self and environmental non-harmful proteins is crucial for maintaining immune homeostasis [7].

2.1.2 Classification

In order to fulfil their various tasks in the immune system DC are plastic in nature, and can be instructed by the surrounding tissue microenvironment, but there are also several different subsets of DC that develop independently, have overlapping functions but also unique features. Studies deciphering DC heterogeneity has mostly been performed in mice, and mouse DC are today usually divided into four major subsets based on their surface marker expression and functional properties: conventional DC (cDC), Langerhans cells, plasmacytoid DC and inflammatory DC [30].

Conventional DC dominate at steady state and are specialized in antigen processing and presentation. There are two major groups of cDC: lymphoid tissue-resident DC and migratory DC. The lymphoid tissue-resident DC are the most studied DC in mice and are located in spleen, lymph nodes and thymus. These cells can be further divided into two groups: $CD8^+$ and $CD11b^+$ DC [31] (Figure 1). Their location in lymphoid tissues allows them to sense antigens or pathogens that are transported in the blood. The migratory DC populate the peripheral tissues such as skin, intestines and lungs. They comprise two main groups in mice: $CD11b^+$ DC and $CD103^+$ $CD11b^-$ DC [32]. Tissue $CD103^+$ DC originate from the same precursor as the lymphoid tissue $CD8^+$ DC and they share similar functions. The $CD103^+$ DC are specialized in sensing pathogens and tissue damage and have a critical role in the induction of cytotoxic $CD8^+$ T cells [33]. On the other hand the $CD11b^+$ DC express high levels of MHC class II molecules [34] and are important for mounting $CD4^+$ T cell immune responses [35].

Langerhans cells are DC that populate the epidermal layer of the skin [36]. They are resident in the skin but can also migrate to the lymph nodes to present antigen. Contrary to most of the DC that are derived from HSPC in the bone marrow, Langerhans cells originate from a local precursor cell population in the skin. Their precise function in the immune system is not fully understood. They can induce regulatory T cells [37] and Th17 T cells, but do not mount Th1 responses [38].

Plasmacytoid DC are widely distributed in the body and are recognized by their ability to produce large amounts of type I interferons in response to viral infections. Although, their precise role in shaping immune responses still needs to be elucidate, they are important for, antiviral immunity, but have also been implicated in autoimmune diseases [39].

Inflammatory DC are derived from monocytes [40] or early hematopoietic precursors [41, 42] in response to microbial or inflammatory stimuli. These cells have similar characteristics to conventional DC and express CD11c, MHC class II molecules and DC-specific ICAM3-grabbing non-integrin (DC-SIGN, also known as CD209a) [43]. They are potent antigen-presenting cells and can cross-present antigens, therefore they are thought to serve as a crucial reservoir of professional antigen presenting cells during acute infections [43] [40, 44].

In humans, DC populations show basic similarities to the four subsets described in mice. For technical and ethical reasons the most abundantly studied population of DC are those found in blood or those derived from monocytes, followed by residing in skin DC. [30].

Conventional DC in human blood are characterized as $Lin^{-}MHC\text{-}II^{+}CD11c^{+}$ [45], although CD11c is also expressed on monocytes and macrophages in humans. Conventional DC in humans share similar functions to mouse cDC, however, their surface markers are different [46]. The blood cDC in humans can be subdivided into two major population: $CD1c^{+}$ and $CD141^{+}$ DC [47, 48]. Studies of cDC in the non-lymphoid tissues has been performed using tissue explants from patients with underlying diseases, which could influence the composition of tissue DC. By earlier studies on human dermis, two cDC populations have been identified: $CD1a^{+}CD14^{-}$ and $CD1a^{-}CD14^{+}$ DC [49, 50]. Recent studies have also identified one population of cDC that expresses $CD1c^{+}$ in the dermis [51] and another population that expresses $CD141^{+}$ in dermis [51], lungs, kidney and intestines [52]. In lymphoid tissues, such as spleen and tonsils, $CD1c^{+}$ and $CD141^{+}$ DC that resemble the blood DC have been found [53, 54] and likely are corresponded to lymphoid tissue resident DC. $CD141^{+}$ DC have equivalent antigen cross-presenting function as mouse $CD8^{+}$ and $CD103^{+}$ DC, whereas human $CD1c^{+}$ DC are more related to mouse $CD11b^{+}$ DC [55].

Langerhans cells are the dominant hematopoietic cells in human epidermis and can be easily identified by their expression of epithelial cell adhesion molecule (EPCAM), langerin [56] and CD1a [57]. In humans Langerhans cells have been shown to induce proliferation of epidermal-resident regulatory T cells at steady state, but limit the activation of regulatory T cells during inflammation. This would suggest a role for these cells both in the maintenance of homeostasis as well as in the regulation of immune responses [37].

Plasmacytoid DC have been characterized as $CD303^{+}CD304^{+}$ in human blood and share similar functions to the mouse pDC [39].

Inflammatory DC in humans are also thought to be generated from monocytes but the sequence of events leading to monocyte differentiation in vivo are difficult to study. Therefore relatively little is known how potential monocyte-derived DC participate in immune responses during bacterial infections and inflammatory reactions. However, some recent experimental evidence from studies on rheumatoid artritis and cancer patients points towards monocytes being the source of human inflammatory DC. These DC share similar features with blood $CD141^{+}$ DC and inflammatory macrophages and have the ability to induce Th17 immune responses [58].

2.1.3 Dendritic cell function in immune homeostasis and tolerance

The immune system has important control mechanisms to limit the magnitude of inflammatory responses and thereby avoid destruction of healthy tissue. DC have an important role in controlling immune responses by exerting regulatory functions to maintain immune homeostasis and tolerance [7]. During steady state, DC have the ability to induce both central and peripheral T cell tolerance. Initial experiments have suggested that immature DC can induce tolerance by presenting antigens in the absence of costimulation, which could lead to T cell deletion or generation of regulatory T cells [59-61]. However, recent studies have shown that fully matured DC also acquire regulatory function and can induce tolerance [62-66]. This suggests that the definition of regulatory DC depends on their functional status rather than a specialized subpopulation defined by phenotypical markers. The functional state of DC depends on their ability to respond to environmental signals from different microenvironments [67]. It has become evident that regulatory DC are induced in chronic infections, which

Figure 1

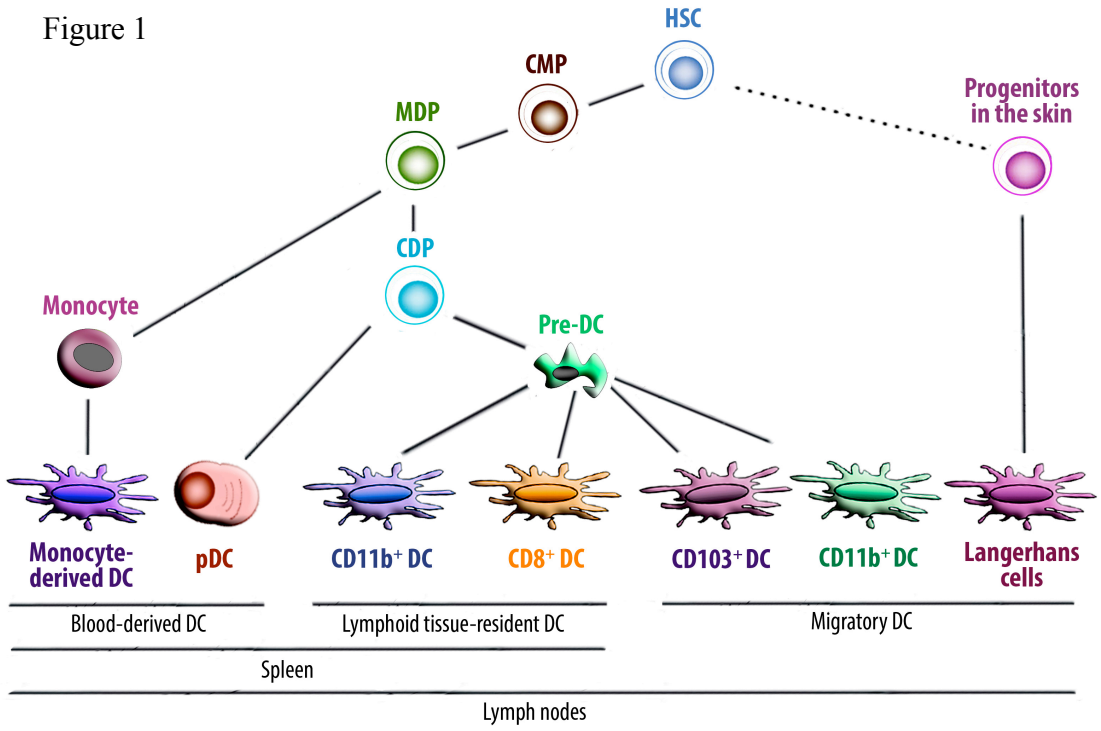

Figure 1. The illustration depicts an overview of DC development, starting from the hematopoietic stem cells (HSC) to the common myeloid progenitors (CMP), macrophage DC progenitors (MDP), common DC progenitors (CDP), circulating DC progenitors (pre-DC) and monocytes. The hematopoietic stem and progenitor cells (HSPC) that have been studied in this thesis is composed of HSC, CMP and MDP. Progenitors for Langerhans cells have been shown to populate the skin prior to birth, however, the origin of those cells remains unknown. Plasmacytoid DC (pDC).

could indicate that pathogens utilize the tolerogenic properties of DC to evade the host immune response [12, 14, 68, 69].

2.1.4 Toll-like receptors – sensors on dendritic cells

DC patrol throughout the body and act as sentinels of the immune system. To perform this task, DCs express a range of microbial recognition receptors that can bind conserved molecular patterns expressed by the pathogens. Amongst the innate immune recognition receptors, toll-like receptors (TLRs) have a key role in microbial detection and initiation of innate immune responses. In 2011, Jules Hoffman and Bruce Beutler shared the Nobel Prize with Ralph Steinman, for their work, which led to the identification of TLRs and their importance in innate immune activation [16, 70, 71]. At least ten TLRs have been characterized in mammalian species and TLRs are highly expressed on DCs, with some differences between distinct DC subsets [72] (Table 1). Each TLR is specialised in the recognition of different microbial molecular patterns. TLR 1,2,4,5, and 6 recognize bacterial products, while TLR 3,7,8 and 9 detect viral components and nucleic acids [73]. Bacterial and viral products recognized by TLRs include LPS that binds TLR4, bacterial lipoproteins that bind TLR1 and 2, flagellin that

binds TLR5, CpG DNA of bacteria and viruses that bind TLR9, double-stranded RNA that bind TLR3 and single-stranded viral RNA that bind TLR7 [74, 75]. Regardless of subset TLR-signalling induce both phenotypic and functional maturation of DC. The production of inflammatory cytokines including IFN-α, IFN-β, IL-12, TNF-α, IL-6 and IL-1 [76] may, however, differ in magnitude depending the DC subset investigated.

Table 1. TLR expression by human DC subsets

	Freshly isolated		*In vitro*-differentiated DC
	DC	pDC	CSF-2 + IL-4
TLR1	+	+	+
TLR2	+	–	+
TLR3	+	–	+
TLR4	–	–	+
TLR5	+	–	+/–
TLR6	+	+	+
TLR7	+	+	–
TLR8	+	–	+
TLR9	–	+	–
TLR10	+	+	

Table 1. Adapted from Iwasaki *et al.* Nature Immunol. 2004;5: 987-995.

2.1.5 Dendritic cell function during acute inflammation

During acute microbial infection, there is an increased recruitment of monocytes from the bone marrow to the blood, and monocytes in circulation migrate to infected and inflamed tissue where they can differentiate into DC with inflammatory properties [44]. However, inflammatory DC can also be differentiated from early hematopoietic progenitors as mentioned before. However, it remains to be explored whether the generation of inflammatory DC differ quantitatively or qualitatively depending on where the DC originate from [30]. The inflammatory DCs play a crucial role in the control of early antimicrobial infection by secreting nitric oxide (NO) and tumor necrosis factor (TNF). Especially, one population of monocyte-derived DC, called TipDC (TNF-iNOS producing), are responsible for TNF and iNOS production during the first three days of systemic infection and have a crucial role in the innate immune defences [77-79]. Inflammatory DC are suggested to have an important back-up role during infection and are important in the clearance of bacterial and parasite infections such as *Brucella melitensis, Leishmania major, Listeria monocytogenes,* and *Trypanosoma brucei* infection [40].

Although, there are many different subsets of DC in the human body, DC generated from blood monocytes have been widely used in many studies because they are readily available. The method of generating a large number of DC from monocytes *in vitro* [80] using CSF-2 and IL-4, has facilitated the studies on human DC. *In vitro* monocyte-

derived DC were believed to resemble the inflammatory DC, first of all, because CSF-2 have been thought to promote the development of inflammatory DC *in vivo*. Second, Monocytes have mostly been studied for their role as precursors of inflammatory DC during infection. However, recent studies suggest a role for CSF-2 also as a steady state cytokine that promotes survival and homeostasis of DC in non-lymphoid tissues, while being less important for the generation of inflammatory DC [81]. Also, monocytes have been recognized to be important for the generation of steady state DC in the lung, spleen, skin and intestines [40]. Although, monocyte-derived DC often gets to represent all human DC, it is important to remember the existence of distinct subsets of DC, and depending on the questions addressed it may be relevant to seek alternative sources of human DC to get an appropriate reflection of the DC capabilities investigated.

Regardless of their origin it is obvious that DC populations are located along side all epithelial linings of body surfaces, e.g., airways, skin, gut, etc, where they can be activated in response to foreign material and tissue damage, followed by the coordinated production of cytokines and chemokines [5] (Figure 2). In tissue, the capacity of DC to orchestrate immune responses is tightly regulated by the tissue microenvironment. In this context, it has become evident that lung tissue homeostasis requires proper tissue regulation of DC [3]; and when this balance is broken immune-mediated tissue pathology can occur as a consequence [82].

Figure 2

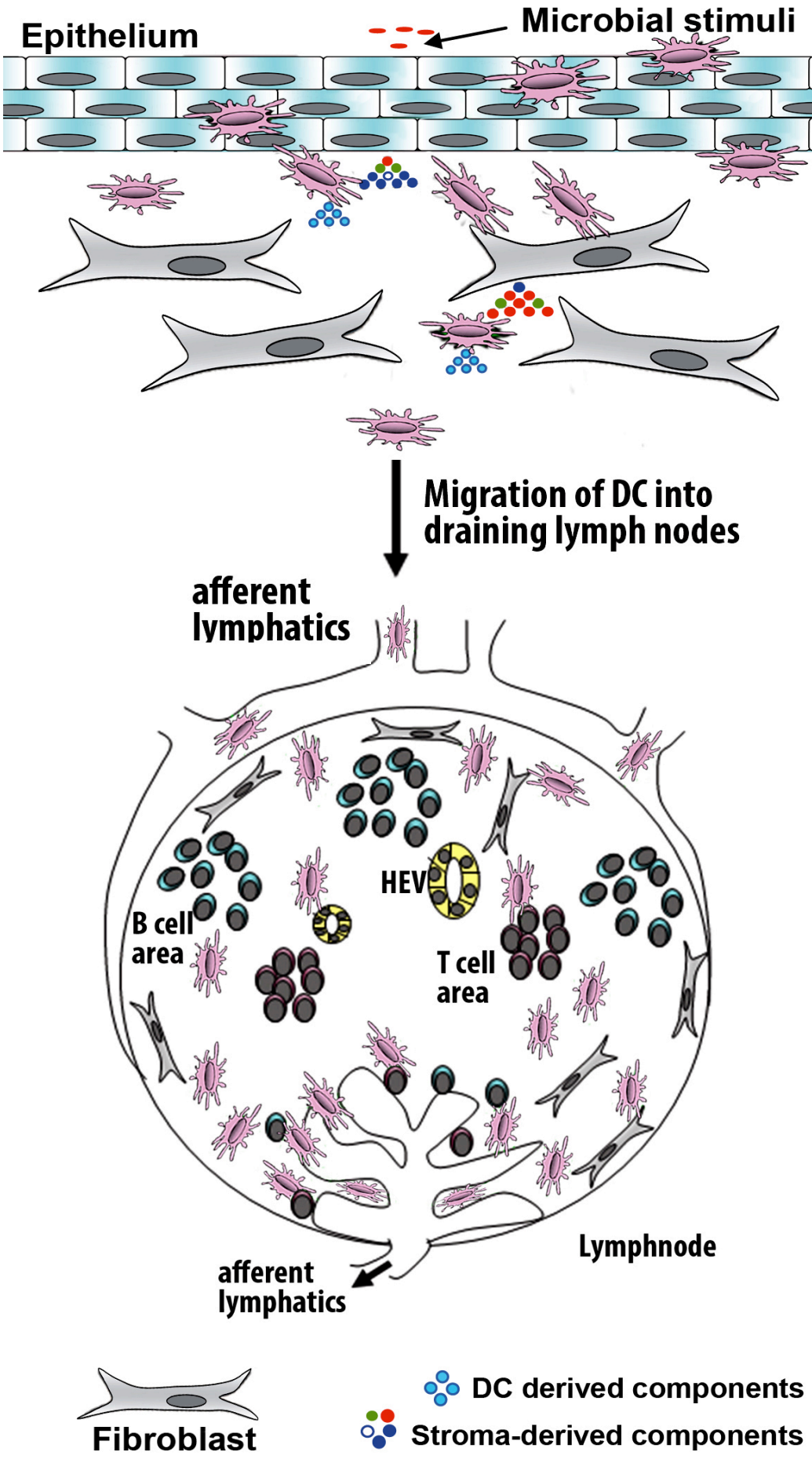
Epithelium
Microbial stimuli
Migration of DC into draining lymph nodes
afferent lymphatics
HEV
B cell area
T cell area
Lymphnode
afferent lymphatics
Fibroblast
DC derived components
Stroma-derived components

Figure 2. This illustration shows DC location in peripheral tissues, such as skin, lung and intestine and highlights the important function of DC in the regulation of adaptive immunity. In peripheral tissues, DC capture antigens and migrate to the draining lymph node where they present antigens to T cells and either induce tolerance or immune responses against pathogens, depending on the nature of the antigen.

2.2 TISSUE REGULATION OF DENDRITIC CELLS

Tissue-specific non-immune cells such as fibroblasts (stromal cells) and epithelial cells, are some of the important cellular components that form the tissue microenvironment in which DC are situated and need to function properly.

2.2.1 Epithelial tissue and their cellular components

2.2.1.1 The structure of epithelial tissue

A typical epithelial tissue of body surfaces, e.g. intestine, lung, and skin consists of a stratified epithelium that grows on a basement membrane and a stromal matrix of fibroblasts, endothelial cells, muscle cells, and lymphatic vessels (Figure 3). The epithelial layer at the apical side is exposed to the outer environment and act as a protective barrier against external intruders. From the basal side, epithelial cells are attached to the basement membrane and receive their nutrition from the underlying stroma. The basement membrane functions as a supportive scaffold for the epithelium and regulates the transport of selective compounds that enter the epithelium from the underlying tissue [83]. The extracellular matrix (ECM) of the stroma constitutes structural proteins including collagens, proteoglycans, elastin and laminin, which provides mechanical support and works as a scaffold for tissue and cells [83]. The ECM also serves as a storage depot for growth factors, chemokines and cytokines [84]. Within this dynamic microenvironment, there is a constant interaction between immune cells and the surrounding tissue cells. They communicate and cooperate through the secretion of chemokines and cytokines and together they orchestrate tissue homeostasis and regulate immune responses.

2.2.1.2 Epithelial cells

Epithelial cells originate from the ectoderm and endoderm of the embryo [83]. To form a proper epithelial cell barrier, epithelial cells are tightly joined together by junctional complexes, including tight, adherence and gap junctions to form a sheet of tissue called epithelia. These epithelial sheets coat many organ surfaces such as skin, intestine and lung, and act as a boundary between tissue and the outer environment. Epithelial cells have many essential functions including protecting the tissue from external danger, regulation of cellular permeability, secretion of hormones and transportation of ions, oxygen and nutrients [83]. Thus, epithelia cell barriers are associated with major functional roles of different tissues, such as hepatocytes and liver metabolism, keratinocytes and the barrier properties of skin and alveolar epithelial cells and gas exchange in the lungs. The development of a well-organized epithelium *in vivo* strictly depends on epithelial interactions with the connective tissue. Thus, transplantation studies have revealed that morphogenesis and differentiation of epithelia is influenced by the underlying mesenchyme [83].

Figure 3

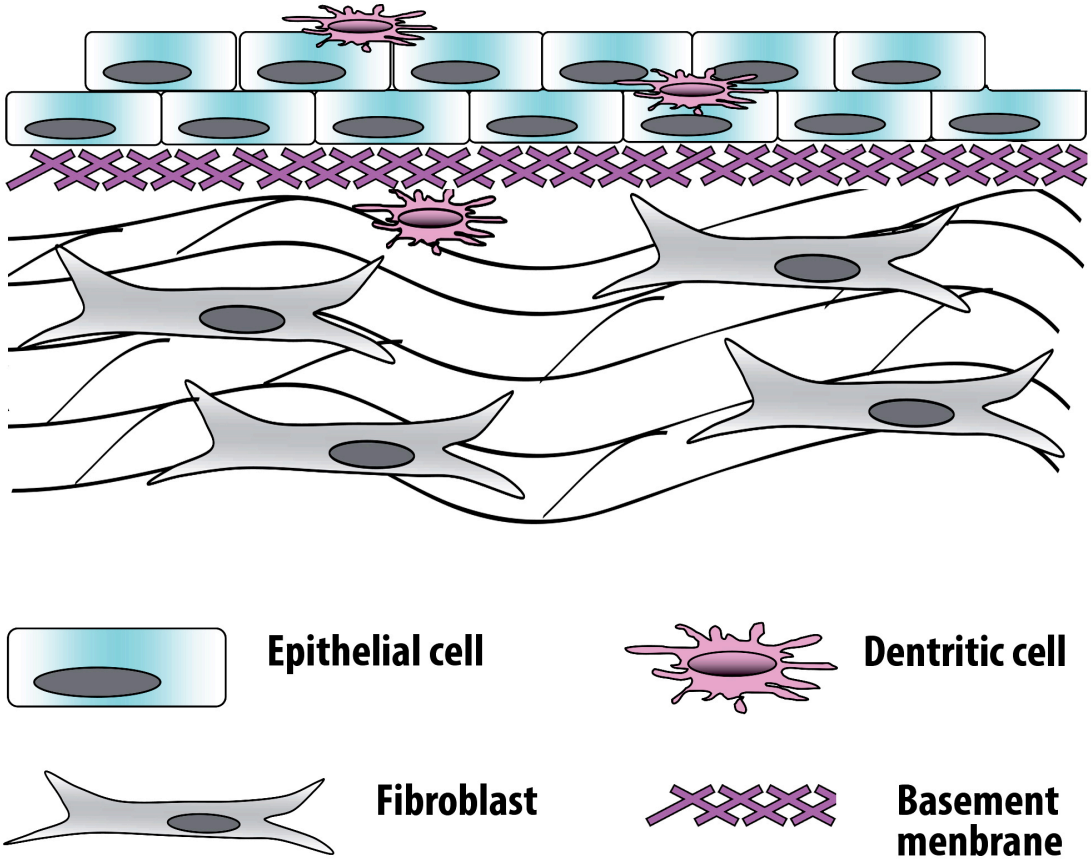

Figure 3. This illustration shows a basic structure of the epithelial tissue with a stratified epithelial layer rested on a basement membrane and an underlying stromal matrix consisted of fibroblasts and immune cells, such as dendritic cells. Epithelia that cover organs such as skin and lungs are exposed to the outer environment and receive nutrition from the underlying stroma, which also provides physical support and interacts with the epithelial layer and immune cells through soluble signals. The extracellular matrix of the stroma comprises of structural proteins such as collagens, fibronectin, laminin and proteoglycans, which together form a supportive scaffold for the cells and also function as a storage depot through their ability to bind growth factors, cytokines, chemokines and other molecules.

The epithelium can be characterized based on their structure, which is also related to their function in different anatomical sites [83]. For example, stratified squamous epithelium in the skin and esophagus functions as a protective barrier, simple columnar epithelium in the intestine usually involves in secretion and absorption, pseudostratified epithelium in the trachea act as a regulatory barrier allowing transport of selective substances and cuboidal epithelium in glandular duct and kidney transports material into or out of the lumen. In common with the hematopoietic system and distinct from the stromal tissues, epithelial cells are constantly regenerated. This process may be rapid, as in the intestines and epidermis, or slow, as in liver and pancreas. The regeneration of epithelial cells is suggested to relay on stem cells that exist in the basal layer of the epidermis or in the crypts of the intestines [83].

2.2.1.3 Fibroblasts

Fibroblasts originate from mesenchymal cells [85] and consist of a heterogeneous family of cells, depending on their location in different tissues. For example, fibroblasts isolated from different anatomical sites of the body are highly diverse in their gene expression patterns of extracellular matrix proteins and growth factors. Chang *et al.* showed that fetal fibroblasts from the skin expressed high level of collagen I and V, whereas fetal lung fibroblasts expressed lung specific transcription factors FOXF1 and FOXP1 [86]. Furthermore, adult fibroblasts maintained HOX gene expression that was established during embryogenesis, which may direct topographic differentiation and positional memory in fibroblasts [86]. In tissue, fibroblasts, are the most abundant cell component of the stroma and fibroblasts are known for their function in promoting tissue survival, remodeling and deposition of matrix components as well as production of extracellular matrix and structural support of the cells in the tissue [87, 88]. In addition to their important functions in remodelling and deposition of the ECM, they also play a key role in the formation and maintenance of epithelial musosa and submucosa including epithelial proliferation and differentiation [89]. Fibroblasts also actively interact with the adjacent epithelial layer and have a key role in tissue inflammation and repair [90].

In immunology, fibroblasts have been recognized as important cells in the regulation of immune responses as well as supporting hematopoiesis [12, 91]. It should also be mentioned that, although, fibroblasts are the major stromal cells [11], also endothelial cells and tissue specific macrophages sometimes are referred to as stromal cells depending on their functional capacities related to hematopoiesis [92-94].

2.2.2 Stromal cells support hematopoietic cell differentiation

Hematopoiesis is dependent on specific niches in the microenvironment [95], which supports hematopoietic stem and progenitor cell (HSPC) survival and proliferation, by providing, specific cytokines, chemokines and adhesion molecules. There is evidence demonstrates that stromal cells in hematopoietic niches such as the bone marrow, fetal liver and spleen, produce extracellular matrix and form supportive structures, for proliferation and differentiation of HSPC [11]. In adults, bone marrow with its stromal cell components is the primary organ that supports HSPC homing, migration, survival and differentiation [2]. The regulation of hematopoietic cell homing and migration in bone marrow involves stromal cell-derived chemokines [96, 97]. For example, the chemokine CXCL12 is highly expressed by bone marrow stromal cells and act as chemoattractant (homing) for HSPC [98]. Under homeostatic conditions, HSPC in the bone marrow have been shown to be highly migratory and recirculate constantly between bone marrow and blood [99]. During inflammation or stimulation with cytokines such as CSF-2 and CSF-3, the blood HSPC numbers are altered [100, 101]. Except their migration between bone marrow and blood, HSPC have also been found in liver, spleen and muscles where they can proliferate and give rise to tissue-resident myeloid cells, preferentially DC [99]. During chronic inflammation or infection such as malaria, leishmaniasis and tuberculosis, there are disturbances in the hematopoietic compartment. For example, during the course of *L. donovani* infection myeloid progenitor cells have been shown to mobilize from the bone marrow into the circulation followed by increased hematopoietic activity in the spleen and bone marrow [102]. In

addition, splenic stromal cells from mice infected with *L. donovani* are altered and support the development of regulatory DC more efficiently compared to splenic stromal cells from non-infected mice [12].

Up to the present time the role of stromal cells in immunology has mostly been studied in lymphoid organ formation and haematopoiesis, while the role of peripheral tissue stromal cells in regulating hematopoietic cell function has largely been ignored.

2.2.3 Tissue-specific cell regulation of dendritic cell function

Within peripheral tissues, DC are embedded in ECM and are surrounded by fibroblasts and epithelial cells that play a key role in the regulation of DC differentiation and function [11]. In lung tissue, DC are in close contact with the respiratory epithelium and together they serve as the first line of defence against inhaled antigen [3, 103]. A rich body of literature suggests that lung tissue homeostasis and immune activation against inhaled antigen depend on the interplay between airway epithelial cells and DC [3, 104, 105]. For example, airway epithelial cells have been shown to express TLRs and can respond to microbial products [106-108]. Ligation of TLRs by epithelial cells leads to upregulation of pro-inflammatory mediators such as IL-6, IL-8, TNF-α, CSF-2, thymic stromal lymphopoietin (TSLP) and chemokines, which can regulate the function of DC [3, 109, 110]. In addition, dysfunction of TLR-expression in airway epithelial cells leads to altered immune activation of DC [104]. The cytokine TSLP produced by keratinocytes, lung epithelial cells and fibroblasts is a potent regulator of DC and can when aberrantly expressed induce activation of DC, which leads to induction of Th2-mediated allergic immune responses [111, 112]. Furthermore, DC trafficking in the lung has been shown to be regulated by the chemokines CCL2 and CCL20 secreted by fibroblasts [4]. It has also been recognized in cases of cancer that DC are strongly influenced by the local tumour microenvironment that can shape the phenotype and function of DC.

2.2.4 Dendritic cell responses in the tumour tissue microenvironment

It has become evident that the tissue microenvironment plays an important role in the promotion of tumour growth [113]. In addition, there is increasing evidence that immunological abnormalities are thriving in the tumour microenvironment. For example it has been shown that cancer cells inhibit immune-stimulating molecules, such as co-stimulatory molecules and cytokines in the local tumour microenvironment [114, 115], which may lead to the generation of regulatory DC and T cells [116, 117]. The tumour microenvironment is likely to produce chemokines that attract DC to the tumour area. The attraction of leukocytes to the tumour microenvironment could resemble the situation during wound healing [118]. These infiltrating leukocytes contain myeloid cells, such as neutrophils, DC, macrophages, eosinophils and mast cells as well as lymphocytes [119]. Leukocytes that are recruited to the wound are attracted by the local production of chemokines, cytokines and growth factors as well as necrotic products from tissue breakdown. Within this pathological site, leucocytes participate in the healing processes that include epithelial cell proliferation and migration, angiogenesis and tissue remodeling [120]. In tumours, similar chemoattractive factors are suggested to mediate the recruitment of leukocytes that may mediate comparable roles to those observed during wound healing. However, the

tumour cells continue to proliferate and attract leucocytes that could support the tumour progression [119]. This concept has led to the dogma of tumours as "wounds that never heal" [118]. As the tumour grow, the cells become less proliferative and more quiescent. In the center of the tumours, cell death and necrosis will occur, due to less accessibility of oxygen and glucose as well as increased accumulation of toxic metabolic products in the center of the tumour [121]. Dendritic cells play a crucial role in the induction of an effective immune response against tumours by engulfment of necrotic tumour cells and cross presentation of tumour antigen to initiate $CD4^+$ and $CD8^+$ T cells responses. However, in numerous types of cancers, tumor progression has been associated with a defective DC function that may be responsible for the failure of the immune system to fight against tumours [116]. Emerging evidence suggests that the tumour microenvironment alter the functional properties of DC and can convert them into potent immunosuppressive cells [122]. Therefore, an increased understanding of the underlying mechanisms that control DC function in the tumour microenvironment will provide important targets for intervention strategies in the clinical management of cancer.

As the interaction and cooperation of DC with stromal cells, epithelial cells and other tissue-specific cells are increasingly appreciated as important components shaping DC heterogeneity and function, as well as maintaining tissue homeostasis and regulating inflammatory processes, it is important to further explore pathways of tissue-specific regulation of DC. Technologies for studies of cell-cell interaction in 3D environments have been established but are often limited to animal models [123, 124]. Therefore, much of our knowledge on DC regulation by tissue-specific non-immune cells is limited to that generated in mice and less is known about tissue regulation of DC in humans. In addition, the disadvantage of using animal models is that many human pathogens induce species-specific responses, for example *Mycobacterium tuberculosis*, group A *streptococcus* and Staphylococcus aureus [125, 126]. The use of human tissue explants from epithelial tissues to study cell-cell interactions exists but is limited by the fact that they are difficult to maintain in culture and also the cellular composition of such organ cultures is difficult, if not impossible, to alter. Therefore, there is an increasing need to develop *in vitro* 3D tissue models that are based on human cells. Those model systems provide unique tools for the exploration of biological processes in human tissue as well as basic mechanisms and early events important for the progression of human diseases associated to specific tissues.

2.3 THREE-DIMENSIONAL TISSUE MODEL SYSTEMS

Most of our knowledge of biological processes and cellular functions are based on results from studies of two-dimensional (2D) cell cultures, where most often one type of cell is grown, at a time on a plastic surface. Monolayer cultures have provided understanding of individual cellular responses but may not capture the physiological behaviour of cells *in vivo* [127-129]. Therefore, moving towards culturing cell in a 3D microenvironment that mimics the morphological and functional features of the *in vivo* human tissue is of utmost interest. Three-dimensional tissue models, also called organotypic cultures, involve cell culture techniques where the cells grow in a 3D environment that comprises the complex network of cells and ECM as well as other important biological molecules found in living tissues [130-132]. The main advantage

of using an *in vitro* tissue model is that the model is created from scratch with different combinations of cells that are cultured together into a multicellular assembly. In contrast to the tissue explants, cellular components of the 3D tissue culture can be manipulated, and genes can be silenced or overexpressed before included in the models. It also allows studies over time and can capture early events important for the regulation of hematopoietic cells by tissue specific cells, investigating the characteristics of immune responses upon stimulation with inflammatory reagents or interventions with pharmaceutical compounds. Mimicking a physiological relevant milieu, in a robust and reproducible manner can quickly provide important information on how immunological processes are regulated in tissue. Thus, human 3D tissue models provide an important and relevant tool to perform studies exploring immune regulation in health and disease.

2.3.1 Scaffolds used in the three-dimensional tissue models

There are some challenges to culture cells *in vitro* to fully mimic their *in vivo* parental tissue with the complex network of the ECM and cellular components present *in vivo*. To generate a 3D-culture, cells need to grow in a structure that mimics the ECM and support production of other biological molecules found in living tissues [84]. The generation of a 3D-culture starts with a suspension of cells in a liquid ECM solution, which then solidifies in a hydrated manner. The ECM scaffold should be porous to enable nutrient and metabolite exchange, and possess sufficient mechanical and biological properties to be self-supporting [84]. There are several commercial natural ECM components available such as Matrigel, type I collagen and fibrin gels. Among these, bovine type I collagen is perhaps the most widely used biologic scaffold due to that it is readily available and have been successfully used in many studies [133]. Also, type I collagen is the main structural protein in mammalian tissue and exhibits high mechanical strength and low biodegradability through cross-linking with other molecules [133, 134]. Even though, type I collagen does not supply all necessary components that exist in the ECM *in vivo*, it could still provide the initial framework that is needed for the generation of a relevant structural network that can be built up by the cells present, forming a more physiological relevant milieu. It has been shown that tissue specific cells secrete their own ECM, which can be incorporated into the local microenvironment. For example, Schwann cells in collagen gels express integrins when treated with TGF-β to promote their spreading and orientation [135].

2.3.2 Existing three-dimensional culture systems

Various cell culture systems for studying host-pathogen interaction are used, including the use of monolayer systems based on one cell type that is cultured in the absence of extracellular matrix and other cellular components [136]. Alternatively, Transwell systems to culture cells on a permeable membrane that allow cells to develop an apical basal polarity are used [137]. The disadvantage of these methods is the lack of multicellular components and the complex 3D cellular interactions, which are crucial for normal tissue function, and will influence infection processes [128]. Yet, another approach is the use of a rotating wall vessel to generate 3D tissue structures using porous beads coated with collagen and basement membrane to culture epithelial cells [132]. These systems are also based on one type of cells and

lack the fibroblast components which is essential regulating several aspects of normal tissue function [89]. Engineering of 3D tissue models of skin [138], oral [139] and lung [140], which include a fibroblast extracellular matrix and a differentiated epithelial cell layer have been established. However, tissue models with relevant features and functions that include immune cells are rare. Combining 3D tissue models with immune cells would allow analysis of immunological responses in live tissue and increase our understanding of tissue regulation on function and behaviour. A skin tissue model containing DC has been developed [141, 142], however when we started this thesis work there was no tissue models available with relevant features and functions of human lung tissue, that included human immune cells.

Overall, 3D tissue models provide powerful tools for the studies of host-pathogen interactions, the onset and progression of human diseases as well as for screening purposes of new drugs before being used in clinical trials.

2.4 CHEMOKINES

2.4.1 Role and classification of chemokines

The proper interaction and communication between immune cells and tissue-specific cells locally are likely to be crucial balancing production of cytokines and chemokines at steady state and during inflammation. Chemokines are small (around 8-14 kDa) cytokines that regulate cell survival, activation and migration [98, 143, 144]. They play a central role in the orchestration of tissue homeostasis and inflammation, and their deregulated production have been implicated in several human infectious, inflammatory and autoimmune diseases such as viral infections [145, 146], atopic asthma [147], rheumatoid arthritis [148] and multiple sclerosis [149]. The work of this thesis has therefore focused on studying chemokine production and cellular migratory behaviour in the tissue microenvironment.

Chemokines regulate cellular migration and can be divided into four subfamilies based on their cysteine residues: CXC, CC, C and CX_3C chemokines [150, 151]. Chemokines act through seven-transmembrane domain G protein-coupled receptors abundantly expressed on leukocytes. More than 40 chemokines and 20 chemokine receptors have been identified in humans [152] (Table 2). In immunology, chemokines have fundamental roles in host defence mechanisms, immune homeostasis, immune regulation and hematopoiesis [153]. In addition to their significant functions in the immune system, chemokines play a major role in the regulation of embryogenesis, wound healing and angiogenesis [154]. Chemokines exert vital roles in all facets of the immune system and biological processes and almost all cells and tissues of the body have the ability to express chemokines.

The capability of cells to migrate from the blood into the tissue, their location within tissue and interaction with other cells is dependent on chemokines. They are important promoting migration of for example neutrophils, monocytes, DC, lymphocytes and eosinophils [144, 155, 156]. It has become evident that chemokines regulate cell movement and localization during both homeostatic and inflammatory conditions. Based on this chemokines sometimes are categorized as constitutive or inflammatory [157, 158]. Under homeostatic conditions, chemokines are expressed constitutively at tissue specific sites such as thymus and secondary lymphoid organs. Their main

function under normal conditions is to regulate movement, homing and survival of cells and promote for example trafficking of lymphocytes and DC into lymphoid tissues [159-161]. In response to pathogenic stimuli, chemokine expression is induced or altered to promote the recruitment of effector immune cells to the site of inflammation and infection [162, 163].

2.4.2 Function of chemokines and their receptors

The trafficking of leukocytes from blood into tissue is mediated by multiple signals generated from chemokines and their receptors [164]. Those highly regulated signals are essential for leukocytes to recognize and bind to the endothelium. The binding of leukocytes allows them to roll on epithelium into arrest and find a suitable locus to extravasate into the tissue [164]. Chemokine receptors have important functions both during inflammation and homeostasis. For example, the chemokine receptors such as CCR2 and CXCR2 are crucial for responses to a wide range of infectious and inflammatory challenges [165], while chemokine receptors such as CCR7 and CXCR5 have been shown to have a central role in lymphoid tissue development as well as lymphocyte homing to lymph nodes during steady state [166, 167]. There is a group of chemokine receptors that do not signal via the G-protein coupled receptor pathway. These receptors share similar structure with the classical receptors but are referred as non-signalling chemokine receptors. The best characterized receptors in this family are DARC and D6 that are believed to have main function as scavenger receptors to bind and internalize chemokines for degradation [168]. DARC, however, has not only scavenger function but is also expressed on endothelial cells to support leukocytes recruitment into tissue during inflammation. This suggests that these atypical receptors play an important role in inflammation by removal of excess chemokines that could mediate tissue pathology and also orchestrate leukocyte entry into tissue in response to pathogens [168].

2.4.3 Chemokines and dendritic cells

The life cycle of DC is believed to include migration of DC precursor from blood into peripheral tissues where DC precursors differentiate into a stage of immature DC. In response to infection DC mature and alter their chemokine receptor repertoire allowing migration to the draining lymph nodes where DC induce T cell responses. Based on this knowledge, chemokines are considered to have a key role during DC development and function [169]. Several chemokine receptors are expressed selectively on the surface of DC and one of the major functions of chemokines is to navigate DC from the periphery to the secondary lymphoid organs both during steady state and in response to infection [164]. Upon antigen recognition, DC mature and express CCR7 [170] which make them responsive to CCL19 and CCL21. CCR7 and its chemokine ligands are crucial components in the migration of DC to the draining lymph nodes [171, 172]. In contrast, immature DC express CCR1, CCR5 and CXCR2 that make them responsive to CCL3, CCL5 and CXCL8, which are produced by tissue macrophages upon infection. Expression of those chemokines will recruit DC to the site of infection [173-175]. It has been suggested that migration of DC precursors and mature DC from blood into peripheral tissue in response to infection is regulated by CCR2 and its ligand CCL2 [165, 176, 177].

Table 2. Chemokines and their receptors divided in either constitutive or inflammatory based on the chemokine expression

Receptor	Ligand(s)
CXCR1	CXCL8, CXCL6
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3A	CXCL9, CXCL10, CXCL11
CXCR3B	CXCL9, CXCL10, CXCL11, CXCL4
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL12, CXCL11
CCR1	CCL3, CCL3L1, CCL5, CCL7, CCL14, CCL15, CCL16
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16
CCR3	CCL5, CCL7, CCL11, CCL13, CCL28, CCL3L1, CCL15
CCR4	CCL17, CCL22
CCR5	CCL3, CCL4, CCL5, CCL3L1, CCL4L1, CCL16
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, CCL28
CCR11	CCL25
XCR1	XCL1, XCL2
CX_3CR1	CX_3CL1
Unknown	CXCL14, CXCL17, CCL18

Table 2. Schematic representation of chemokines and their receptor superfamily. Chemokines that have been shown to be induced upon inflammation and considered ”inflammatory” are highlighted in red. Chemokines that are expressed under steady state conditions and considered ”constitutive” are highlighted in blue and those that have been observed to display both of the functions, constitutive/inflammatory, are shown in green. The ”atypical” receptor family is not included in this figure.

DC precursors have also been shown to express CXCR4 and respond to its ligand CXCL12, which suggests involvement of this chemokine for the entry of DC precursor into tissue [178].

It has also become evident that DC are producers of several chemokines. Immature DC can express a wide range of chemokines upon stimulation in tissue. this includes inflammatory chemokines such as CXCL8, CXCL10, CCL3, CCL4 and CCL5, which are believed to enhance the recruitment of neutrophils and monocytes to the infection site [179-181]. Other chemokines that are produced by DC under homeostatic conditions in specific tissues, include for example, the chemokine CCL17/TARC [182], CCL18/PARC [183, 184], CCL22/MDC [185], CCL25 [186] and CCL19 [187]. CCL18 is constitutively and highly expressed in peripheral tissue (i.e., lung) but at

lower levels in lymphoid tissues such as thymus and lymph nodes [184]. Monocytes, macrophages and DC are the main producers of CCL18 and CCL18 production by DC acts on the recruitment of naive T cells [183, 184] and immature DC [188]. In addition, CCL18 can attract Th2 cells, basophils [189] and skin-homing memory T cells [190]. This suggests that CCL18 is involved in both primary and secondary immune responses. Mostly CCL18 has been associated with anti-inflammatory or Th2 immune responses as it is induced by IL-10, IL-4 and IL-13 and downregulated by IFN-γ [188, 191]. In addition, overexpression of CCL18 has been associated with allergic diseases such as atopic dermatitis [190, 192], rheumatoid arthritis [193] and asthma [189]. Recently, CCL18 has also been shown to support differentiation of regulatory T cells from $CD4^+$ memory T cells [194] as well as to differentiate DC into tolerogenic cells [195]. There is no CCL18 homologue in rodents [196] and as of yet no CCL18 receptor has been identified.

The chemokines CCL17 and CCL22 are highly expressed in thymus but are weakly expressed in peripheral tissues such as lung, spleen, colon and intestine under steady state conditions [182, 185]. In contrast, both CCL17 and CCL22 are more easily detected in inflamed peripheral tissue and therefore considered inflammatory chemokines. Mainly myeloid cells including macrophages and monocyte-derived DC express these two chemokines [182, 185] that signal through the receptor, CCR4. CCL22 attracts DC, NK-cells and monocytes, while CCL17 attracts mainly T lymphocytes. Studies have suggested that CCR4 is associated with a Th2 type immune response [197]. In addition, high levels of CCL17 and CCL22 are associated with allergic diseases such as pulmonary fibrosis [198] and asthma [199].

Given the strategic positions of DC in most tissue and their importance in orchestrating immune responses locally [3, 200], interfering with DC is therefore thought to provide an important strategy in the clinical management of several human diseases. However, most studies of DC biology have been performed in 2D cultures on plastic surfaces or in mouse experimental models, which may not capture many important aspects of human DC behaviour. Therefore, there is an increased demand for the development of new approaches allowing the exploration of human DC in more physiological relevant milieus. Thus, organotypic models, we believe, provide good tools recapitulating the 3D structures of human tissue where DC normally act. Using such 3D tissue models will allow modelling of immunological responses that occur in human tissue, under next to *in vivo* setting.

3 STUDY DESIGN

In this chapter I have summarized the hypotheses and methods, which are the basis for this thesis. The first section focuses on the experimental set up of the lung tissue model with DC and the second section focuses on how the experiments on HSPC regulation by stromal cells were performed.

3.1 THE ORGANOTYPIC LUNG TISSUE MODEL PROJECTS

The majority of DC subsets are derived from the bone marrow and a common hematopoietic progenitor cell that proceed into precursor DC seeding the blood. In blood precursor DC are considered brief transients that traffic to peripheral organs and tissue [30]. The local tissue microenvironment further supports differentiation of precursor DC into different DC subsets, thereby contributing to DC heterogeneity [13]. Emerging evidence also indicates that DC residing in tissue receive instructions from ECM components and non-immune cells of the tissue, i.e. fibroblasts and epithelial cells, shaping their phenotype and function locally and this is believed to be essential for the maintenance of tissue homeostasis and the regulation of local immune responses [12, 82, 104]. In this respect, we believe that reductionist approaches, in which only one or a couple of cellular components are studied at a time, are generally, insufficient and sometimes misleading. Instead, experimental designs owes to move towards a more comprehensive and quantitative understanding of how the countless interrelated components form networks that exert a particular function, including shaping DC functions in tissue. Hence, *in vitro* human organotypic tissue models combining DC with tissue-specific cells can be useful tools to capture molecular events that determine DC functions as well as to provide a potential bridge between human cell-based research and patient studies (Figure 4). Therefore, we have been focusing on the development of a tissue model of human lung mucosa combining DC with fibroblasts and epithelial cells cultured in a 3D microenvironment. The use of *in vitro* tissue models will contribute to the increase of our knowledge in mechanisms regulating tissue immune responses under homeostasis and during inflammation.

3.1.1 Our hypotheses

Local tissue microenvironments, such as lung, gut and intestine are recognized to regulate DC differentiation and function. Therefore, we first hypothesized that implanting DC into an organotypic model of human lung, composed of lung fibroblasts and epithelial cells, should impact on DC function mimicking real tissue.

In lung tissue, DC and epithelial cells are the first cells to encounter inhaled antigens and they are well equipped with pattern recognition receptors such as TLRs. By responding to TLRs they orchestrate the outcome of immune responses to pathogens. Therefore, in the second study we hypothesised that stimulation of the lung tissue model with TLR-ligands or chemokine should induce DC migration that can be monitored in real time.

In the third study, we hypothesized that the tumour microenvironment in lung tissue will have the capacity to modulate DC functional properties promoting immunological abnormalities thriving in lung cancer.

Figure 4

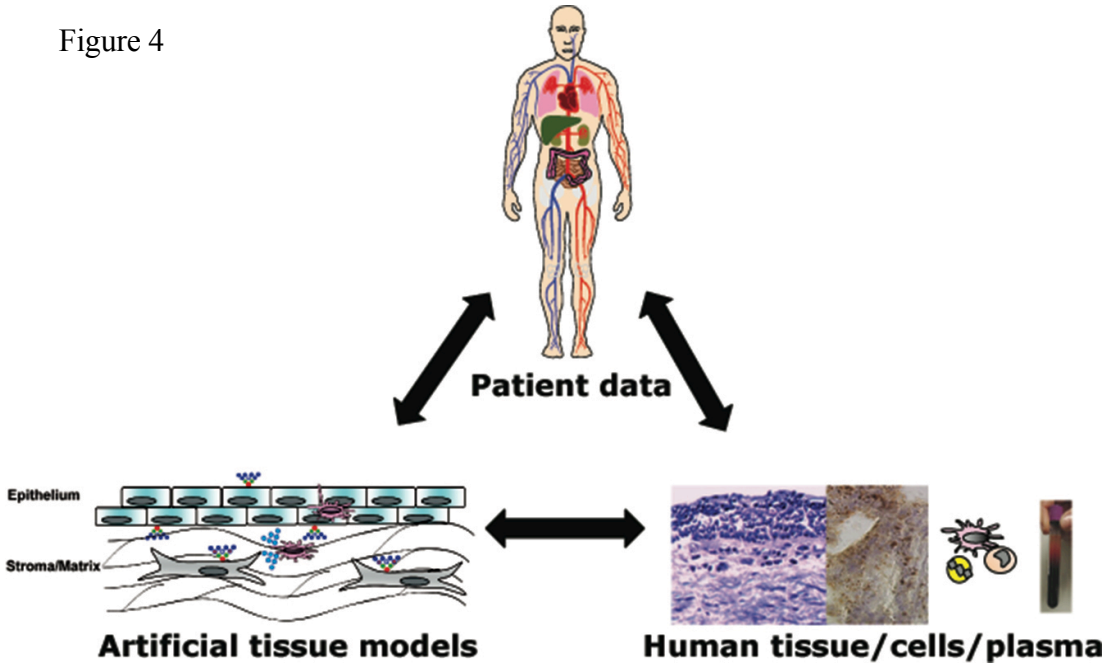

Figure 4. This illustration places the 3D human tissue model as a potential bridge between human cell-based research and patient studies.

3.1.2 Experimental design to test our hypotheses

The studies in this thesis are based on advanced cell culture techniques with different human cell populations to create a 3D tissue model system, which resembles the tissue microenvironment *in vivo*. To test our hypotheses, I developed a 3D lung tissue model with DC. To answer the question if the microenvironment in this model has the ability to influence DC function mimicking real lung tissue, I investigated if this model can support DC survival without addition of exogenous growth factors and how the lung tissue model influences chemokine expression by DC under homeostatic condition. To test if DC in the tissue model respond to TLR-ligands and chemokine, I first established a lung tissue model with fluorescent expressing cells and then used quantitative 4D (x, y, z, time) fluorescence imaging analyses to monitor DC migration in the tissue model in response to stimulation. To investigate if DC function is influenced by the tumour microenvironment in lung tissue, we further developed the 3D lung tissue model with DC and incorporated epithelial tumour spheroids, mimicking micro-tumours in tissue. We then investigated if DC were recruited to the tumour area in the tissue model and if DC engulf tumour cells more readily than “normal” cells. In the following sections I will give a description of the design and set up of the lung tissue model and thereafter I will describe the research methods that were used to apply our experimental design.

3.1.3 Design and set up of human lung tissue model with dendritic cells

3.1.3.1 The choice of cells for the model set up

To generate a highly reproducible tissue model system, we choose to use MRC-5 lung fibroblasts, 16HBE14o- (16HBE) lung epithelial cells and human monocyte-derived DC. MRC-5 is a primary cell line derived from normal lung tissue of a 14-week-old male fetus [201]. It is relatively easy to culture and can be passaged up to 42 to 46 times before senescence. The epithelial cell line 16HBE14o- originates from primary bronchial epithelial cells immortalized with the large SV40 T-antigen. The 16HBE cell culture exhibits similar characteristics as primary epithelial cells by maintaining essential transport mechanisms as well as expression of tight junctions and structures resembling cilia. To culture the 16HBE cells we pre-coat tissue culture flasks with fibronectin, which promote the cells to maintain their original differentiated phenotype [202]. Dendritic cells used in the tissue model were generated from human blood monocytes cultured in CSF-2 and IL-4. As mentioned in the background chapter, MoDC have been widely used as a model system for studying human DC because they are readily accessible and can be generated in large numbers. The *in vitro* generated MoDC are thought to resemble the DC that are differentiated from monocytes *in vivo* primarly during inflammation [80, 203, 204].

3.1.3.2 The set up of a functional three-dimensional tissue culture

To culture the tissue model, I used a Transwell system where the tissue model is grown on a permeable membrane with a pore size of 3 μm in a six-well insert. During air-exposure the cells in the model receive nutrition from the medium in the outer chamber, therefore, it is important to have a relatively large pore size of the insert membrane in order to obtain an optimal diffusion of the medium. I have also tried with a pore size of 0.45 μm, but then models did not grow appropriately, which may depend on insufficient diffusion of nutritions. Also, six-well inserts have an appropriate size to use for practical reasons, but smaller inserts may be used.

A crucial component in the development of 3D tissue cultures is the scaffold that supports the cells in the model. In this setting, I have used bovine type I collagen from Organogenesis which provides a good and reproducible collagen matrix [139]. To create, *in vitro*, a functional microenvironment, MRC-5 fibroblasts were mixed and cultured in bovine type I collagen for seven days in the inserts. During this time, the fibroblasts reorganized and contracted the collagen matrix to form the structural framework, the basis, of the tissue model [83]. The next cellular component implanted was the DC, which were generated from human blood monocytes cultured in CSF-2 and IL-4 for six days before being implanted into the model. Dendritic cells were seeded onto the fibroblast matrix before seeding of epithelial cells, which were then seeded onto the DC-fibroblast matrix. After epithelial cells had formed a confluent layer, the culture was air exposed (Figure 5). The air-exposure is important for the epithelial cells to form a stratified and polarized multilayer that recapitulates the *in vivo* epithelial barrier architecture [83]. The culture procedure takes approximately 16-20 days including the time of air-exposure, which usually is for 4-7 days.

Figure 5

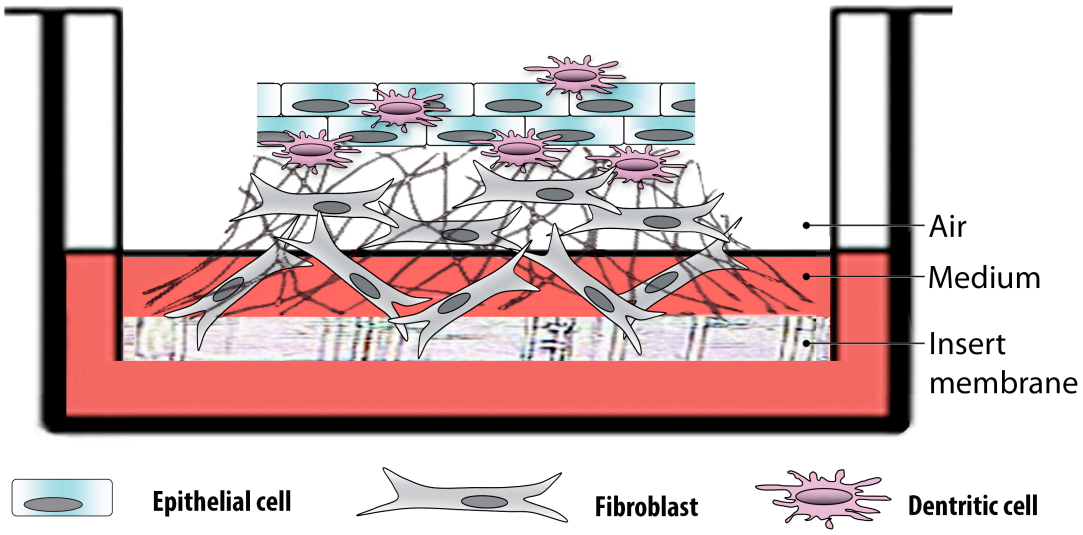

Figure 5. This illustration depicts a complete 3D lung tissue model of fibroblasts in collagen matrix and DC that are located close to the stratified epithelial layer. The model is cultured under air-exposure on a permeable membrane and receives nutrition through diffusion of the medium from beneath.

3.1.4 Selection of research methods

I have used several research methods to analyse cellular responses in the tissue model. These methods include immunohistological analysis of the tissue model using immunohistochemistry and immunofluorescence staining of tissue sections, as well as performing RNA analysis using real time QRT-PCR, protein analysis using ELISA and collagenase digestion of the tissue model to isolate cells for flow cytometric analysis. In addition, one key method in this study has involved assessment of DC migratory behaviour in the tissue model using quantitative fluorescence live imaging microscopy. In the following sections, I will give a brief description of the methods used. I distinguish between methods that require "invasive" techniques for analysis of the lung tissue models, such as sectioning, lysis of the model for RNA extraction and collagenase digestion, and those that are "non-invasive" techniques, which include live cell imaging of the models.

3.1.4.1 "Invasive" methods

After completion of the lung tissue model set up, models were removed from the culture inserts and either frozen for cryostat sectioning or lysed directly for mRNA analysis. The supernatant in the outer chamber can be collected for protein analysis. Models can also be digested using collagenase and the cells recovered can be used for flow cytometric analysis.

Cryostat sectioning of the lung tissue model

To visualize the structure of the 3D lung tissue model, cryostat sectioning of the model was performed following hematoxylin and eosin staining of the tissue sections. Sectioning of the lung tissue model can be difficult, because the model is very thin (approximately 500 μm) and fragile. To obtain optimal sectioning conditions, the model was treated in 2M sucrose for one hour following snap freezing in liquid nitrogen and storage in -80 °C until sectioned. Models were sectioned with a thickness of 8 μm/section, which we have experienced being an optimal thickness for immunohistological stainings. The sections were then fixed in either icecold acetone for 2 minutes or paraformaldehyde for 12 minutes, dependent on the antibodies used in the staining. The fixed sections can be stored in PBS in 4 degree for up to one week, but staining directly after sectioning is preferred for reproducibility reasons. After the first sections were cut I always checked that model structures were properly formed using hematoxylin staining.

Immunohistochemistry and computerized image analysis

Immunohistochemistry (IHC) staining was used to visualize tissue morphology and cell distribution in the lung tissue model. In this method, specific antibodies are used to detect antigens in tissue sections [205]. The antigen-antibody binding can be demonstrated with a colored histochemical reaction that is visible by light microscopy [206]. This method provides a powerful detection technique to examine protein expression within cells or tissue. In addition, I have used immunohistochemistry in combination with computerized image analysis to analyse DC marker expression in the tissue model. Computerized image analysis enables semi-quantitative analysis of protein expression at the single cell level in cryopreserved tissue. This analysis uses a highly sensitive computerized analysis software (Leica Qwin) that can distinguish between a wide range of colours and provide a detailed assessment of different proteins. Analysis of positive immunostaining was performed by transferring digital images of the stained tissue, acquired by a light microscopy, to a computerized Quantimet 5501W image analyzer [207]. Single-positive stained cells were quantified in 25 high-power fields, and protein expression was determined as the percent positive area of the total relevant cell area using the Qwin 550 software program (Leica Imaging Systems). The total cell area was defined as the nucleated and cytoplasmic area within the tissue. Tissue sections stained with secondary antibodies only were used as negative controls. This technique is well established in our laboratory and has been widely used to study expression of different proteins in human as well as in animal tissues.

Immunofluorescence analysis

Immunofluorescence was used to characterize structural proteins and cellular markers in the tissue model. This method is complementary to the immunohistochemistry analyses as it enables visualization of several proteins and colocalization of proteins in the same section. This technique uses specific antibodies as for IHC to bind antigens in the cells or the tissue. Secondary antibodies direct conjugated with different fluorescent dyes of choice were used to detect the primary antibody binding. The fluorescent dyes were then visualized with fluorescence or confocal microscopy.

Real time reverse transcription polymerase chain reaction, RT-PCR

This method provides a sensitive and powerful tool for analysing RNA that compares the relative transcriptional abundance in tissue and cells. For comparison of gene expression between different samples, I used the mRNA analysis as complement to the protein analysis and also as a screening method to investigate multiple markers before analysing protein expression of interest. The method is based on quantitative measurement of the amplified DNA using fluorescent probes. The RNA is first converted to complementary DNA using a reverse transcriptase and the DNA is used as a template for amplification using PCR. This method gives the most sensitive detection of RNA and can detect transcript of principally any genes [208, 209]. In this study, I used relative RT-PCR to compare gene expression between different samples. For this purpose, an internal control that was not effected by the experimental treatment, was monitored in the cells at the same time as the gene of interest and was used to normalize the samples. Relative amounts of the gene of interest were calculated using the comparative threshold cycle method [210]. The threshold correlates to the cycle number where there is sufficient amplified product to give a detectable reading, and, if the threshold is not attained after 35–40 cycles, the mRNA is considered undetectable.

Enzyme-linked immunosorbent assay, ELISA

To identify proteins secreted from the 3D tissue models and single cell culture, ELISA was used. ELISA provides a highly sensitive quantitative measurement of protein levels in culture supernatants, and is based on the use of capture and detection antibody pairs that bind specifically to the protein of interest in the supernatant forming a "sandwich" complex with the antigen. The detection antibody is then linked to an enzyme that is developed by adding an enzymatic substrate to produce a colour change that can be measured using a spectrophotometer [211, 212].

Collagenase digestion and flow cytometry

Collagenase digestion has enabled extraction of the cellular components from the tissue model and following extraction the cells were stained with fluorescently conjugated antibodies directed against specific surface markers. To analyse surface marker expression, flow cytometry was used. This method allows us to characterize the different cell types in the tissue model based on their expression of specific cell surface markers, size, and granularity. Flow cytometry is a useful method for qualitative measurement of cell surface marker expression as well as protein expression in the cells [213, 214]. This technique is based on detection of the fluorescent-labelled cells that are passing through a laser beam, cell by cell.

3.1.4.2 "Non-invasive" method - live cell imaging analysis

Live cell imaging, we consider a "non-invasive" method that enables studies on cellular structures, localization of molecules and dynamic processes in real time. The technique also allows visualization and quantification of cell trafficking, enzyme activity and signal transduction as well as monitoring cellular processes within live tissue. In this thesis work, I have used live cell imaging to study DC migration in the tissue model stimulated with different inflammatory stimuli and chemokine. For this purpose, we have generated fluorescent fibroblasts (orange) and epithelial cells (GFP), using a

retroviral transduction system to obtain stable transduction of the cells. Dendritic cells as well as epithelial cells used for spheroid formation were labelled with a cell tracker dye before implanting into the model. Before live imaging experiments, models were stimulated with different stimuli and were then removed from the culture inserts and mounted onto a glass bottom culture six well plate suitable for live imaging. Then an inverted confocal microscope was used for live imaging of six models simultaneously. Models were imaged over a time period of 12-16 hours with 20 minutes intervals and images were captured with a z-dimension of 120-150 um. The deep penetration of the laser into the models was possible due to the fact that the tissue models, we believe, are much more transparent than real tissue, which reduces the light scattering.

The main critical aspect to consider during live cell imaging experiments is the maintenance of the conditions supporting cell survival and the well being of cells during acquisition. This requires keeping the temperature, pH and humidity in the microscope chamber constant. Other important aspects to consider include the choice of appropriate fluorophores, such that they should have low cytotoxicity and allow a minimum of laser exposure time. Also, physical vibrations should be kept at a minimum [215]. We used a confocal laser microscope system, A1R, from Nikon. The A1R has a resonant scanner system that allows ultra high-speed imaging and enables simultaneous photo-activation. This system enables us to image several models simultaneously using multi-colour imaging and a *z* projection of 150 mm at an interval of 20 minutes. In addition, we have equipped the instrument with an incubator that automatically control temperature, CO_2 and humidity. Furthermore, the microscope is equipped with a feedback-controlled isolation table that is gas-filled to reduce room and building low-frequency vibrations. In our experiment, DC were labelled with a far red cell tracker dye that is excited using a long wavelength, which minimize phototoxicity for the cells.

The analysis of the images collected requires the appropriate software. It should, preferably, be able to perform automatic data analysis and have the ability to handle large data sets. There are many software programs available, which are open source, such as Fiji and CellProfiler. Another powerful, but rather expensive, live imaging software is Imaris. Cost aside, it is one of the best, we believe, softwares for analysis of 3D and 4D microscopy datasets. For data analysis of DC migration in the tissue model, we have chosen to use the Imaris software, because it can perform automatic tracking of the cells as well as handle large datasets and is user-friendly.

3.2 STROMAL CELL-MEDIATED DEVELOPMENT OF REGULATORY DENDRITIC CELLS

The hematopoietic nische, such as the bone marrow, spleen and fetal liver, in which HSPC and stromal cells are situated, have been shown to exert strong influence on the migration, homing, survival and differentiation of HSPC [2]. Homing and migration of HSPC involves chemokines derived from the stromal cells in the hematopoietic microenvironment [216, 217].

3.2.1 Our hypotheses

There is evidence that during *L. donovani* infection, a rapid mobilization of HSPC into the blood circulation occur. In addition, HSPC are dramatically increased in the spleen and bone marrow of infected mice [102]. Furthermore, studies have shown that splenic stromal cells support the differentiation of HSPC into regulatory DC and this ability is enhanced during *L. donovani* infection [12, 13]. However, in the latter study it was not shown if the enhanced effect to support HSPC differentiation depended on direct infection of the stromal cells or on the inflammation that occurred during infection. Also, the specific stromal cell-derived factors that promote the differentiation of HSPC into regulatory DC at steady state and in chronic infection have not been identified.

In this study, we first hypothesized that bone marrow stromal cells from mice could support HSPC differentiation into regulatory DC by the involvement of chemokines. . We then hypothesized that the direct infection of bone marrow stromal cells with *L. donovani* will alter their chemokine expression, which may lead to enhanced ability of the stroma to recruit HSPC and to support increased development of regulatory DC.

3.2.2 Experimental design to test our hypotheses

To test our hypotheses, we first used a murine bone marrow stromal cell line MBA-1 [218] that was accessible, easy to culture and susceptible to *L. donovani* infection. For this purpose, MBA-1 cells were tested for their ability to recruit HSPC as well as supporting the differentiation of HSPC into regulatory DC both at steady state and during *L. donovani* infection. A macrophage cell line RAW264.7 that did not support hematopoiesis was used as negative control [218]. To analyse differences in gene expression of MBA-1 and RAW264.7 cells, we performed genome-wide analysis of mRNA expression in the cell lines in the presence and absence of infection. Based on the outcome of the genome-wide analysis, hypotheses were tested first *in vitro* using MBA-1 cells and second *in vivo* using a murine model of visceral leishmaniasis.

3.2.3 Selection of research methods

Immunofluorescence analysis

We used immunofluorescence analysis to verify expression of surface markers on DC that differentiated from HSPC cultured on MBA-1 cells. Also, this method was used to visualize *L. donovani* infection in MBA-1 and RAW264.7 cells as well as chemokine expression by MBA-1 cells and in mouse splenic tissue.

Flow cytometry

This method was used to examine the different DC population that developed from HSPC cultured on MBA-1 cells as well as examine the number of HSPC that migrated during different culture conditions in a Transwell migration assay.

Allogeneic Mixed leukocyte reaction

This assay was used to investigate if DC derived from HSPC displayed regulatory properties by examining their ability to inhibit T cell proliferation induced by CSF-2-derived LPS-stimulated DC. Mixed leukocyte reaction (MLR) is a reaction that occurs when leukocytes from two genetically differing individuals are cultured together [219]. Leukocytes in the culture will stimulate each other to proliferate and the proliferation

can be measured by tritiated thymidine uptake. In a one-way MLR test, one donor serves as responder and the other as stimulator. The stimulator cells (DC) are inactivated by radiation or by mitomycin C to allow only the responder cells (T cells) to proliferate in response to foreign histocompatibility antigens. In this way, this assay can measure the ability of DC to induce T cell proliferation.

Transwell migration assay

This assay was used to study the migratory response of HSPC towards MBA-1 cells. This assay is also known as the Boyden chamber assay [220]. The assay procedure was performed as follows: HSPC were placed on the permeable membrane in the upper chamber (insert) of a Transwell systems and the MBA-1 cells or culture supernatants from the MBA-1 cells were placed in the lower chamber. Following incubation (3-18 hours), the cells that migrated through the membrane were stained and counted using flow cytometry.

Microarray analysis

This method was used to analyse gene expression profiling in MBA-1 cells and RAW264.7 cells by measure the expression of thousands of genes at once to provide an overall view of the transcriptional activity and to display the status of the cells in response to a particular treatment. This assay is based on hybridization of total mRNA to thousands of different synthetic oligonucleotides of define sequence and serve to probe the composition of the RNA content [221]. The oligonucleotides contain pairs of probes for each of the RNAs of interest. Before hybridization, fluorescently labelled ribonucleotides were incorporated into the target RNA following randomly fragmenting of the RNA that was hybridized to arrays. Fluorescence images, also called heat maps, of the array were obtained using a scanning confocal microscope that revealed data of individual values represented as colours, for example high gene expression was represented by red colour while lack of gene expression was represented by blue colour.

Real time quantitative RT-PCR

Since high throughput microarray lack the quantitative accuracy, we used real time QRT-PCR to validate and semi-quantify candidate chemokine genes of interest from the microarray analysis in MBA-1 cells and RAW264.7 cells, as well as splenic tissue. A more detailed description of the method can be found in section 3.1.4.

Immunohistochemistry

This method was used to investigate chemokine protein expression in splenic tissue of uninfected mice and mice infected with *L. donovani*. A more detailed description of the method can be found section 3.1.4.

Enzyme-linked immunosorbent assay, ELISA

This assay was used to measure the protein levels of chemokines in the culture supernatants of splenic stromal cells isolated from uninfected and infected mice with *L. donovani*. A more detailed description of the method can be found section 3.1.4.

4 AIMS OF THE THESIS

The overall aims of this thesis were to study tissue regulation of DC function, migration and chemokine production as well as stromal cell-derived chemokine regulation of regulatory DC development following *L. donovani* infection. The specific aims were:

1. To establish a human organotypic model of human lung with DC and study DC survival and chemokine production in the tissue model under homeostatic conditions.

2. To further develop the organotypic model for live imaging analysis and investigate DC migration in response to TLR-ligands and chemokine.

3. To generate an organotypic-based epithelial spheroid model of human non-small lung cancer and study DC interaction with cancer cells.

4. To investigate the influence of stromal cell-derived chemokines on HSPC migration and differentiation into regulatory DC under steady state and in response to *L. donovani* infection.

5 THE RESULTS OBTAINED IN OUR STUDIES

This chapter presents the results achieved in the thesis work. The first section describes the establishment of a 3D lung tissue model with DC and the examination of chemokine expression by DC in the lung tissue model. In the second section, the further development of the lung tissue model for live cell imaging analysis is described, as well as the investigation of DC migration in response to inflammatory stimuli in the tissue model. The third section focuses on the development of the lung tissue model to study DC interaction with tumor cells, and finally, the fourth section concentrates on how murine stromal cell-derived chemokines support the development of regulatory DC during homeostasis and in an experimental model of *L. donovani* infection.

5.1 DEVELOPMENT OF A THREE-DIMENSIONAL TISSUE MODEL OF HUMAN LUNG MUCOSA WITH DENDRITIC CELLS

Despite the many important progresses in the field of DC biology, little is known about the function of DC within the complex microenvironment of human tissue. To investigate DC function and behaviour in the lung microenvironment and to reveal pathways by which human DC interact with tissue-specific cells and may orchestrate lung tissue homeostasis and regulate immune responses against pathogens, we sought to develop a 3D tissue model of human lung mucosa. This model composes human DC embedded in a physiological microenvironment made of lung epithelial cells and lung fibroblasts, which produce extracellular proteins and proteins essential for epithelial barrier functions.

5.1.1 Architecture and extracellular matrix composition of the model

The human airway epithelial tissue comprises of a stratified epithelial layer supported by a basement membrane and an underlying extracellular stroma matrix layer containing extracellular matrix proteins, fibroblasts, lymphatic vessels and immune cells such as lymphocytes and DC. Our results from hematoxylin and eosin (H&E) histological analysis of the lung tissue model revealed a stratified and polarized structure of the epithelial cells lining an extracellular matrix layer of fibroblasts (Figure 1D, **paper I**). Furthermore H&E staining of the tissue model indicated a basement membrane-like structure separating the epithelium from the fibroblast compartment (Figure 1D, **paper I**). Therefore, we investigated the expression of essential extracellular matrix proteins and proteins associated with basement membranes in the tissue model, such as tropoelastin, collagen IV and laminin-5. Immunofluorescence staining revealed expression of the ECM proteins by both epithelial cells and fibroblasts in the model. Deposition of these proteins by the cells in the model displayed a continuous basement membrane at the boundary between the epithelial layer and the underlying fibroblast matrix (Figure 2A, B and D, **paper I**). In addition, fibroblasts in the model were positively stained for vimentin, which is the major cytoskeletal component of fibroblasts and mesenchymal cells [222] (Figure 2C, **paper I**). Furthermore, epithelial cells in the model expressed and deposited tight and adherence junctional proteins, including E-cadherin, occludin and claudin (Figure 3A

and B, **paper I**), which have been shown to be essential for the formation of a functional epithelial cell barrier [223, 224]. These data revealed that our 3D lung tissue model comprises a fibroblast collagen matrix rich in extracellular matrix proteins and a tightly joined stratified epithelium on top of a continuous basement membrane. Thus, our 3D lung tissue model displays morphological and functional properties that mimic normal lung epithelial tissue (Figure 1E, **paper I**).

5.1.2 Survival and distribution of dendritic cells in the lung tissue model

DC are found throughout the airway mucosa and are strategically situated in close association with the epithelium where they act as sensors sampling antigens in the airway [3, 103]. Immunohistochemistry staining of HLA-DR on tissue model sections revealed that DC were located close to the epithelial layer (Figure A and B, **paper I**). To investigate whether or not DC survived equally well without growth factors, models were cultured either with or without CSF-2 and IL-4 for 11 days. Quantitative computerized image analysis of HLA-DR immunostaining indicated that DC survived equally well in models cultured with or without exogenous growth factors (Figure 4D, **paper I**). Although we observed that DC survived in the absence of exogenous growth factors, we were interested in knowing how well DC survived over time in the tissue model. For this purpose, DC were labelled with a far red cell tracker dye before being implanted into the model. Live confocal microscopy was used to monitor the quantity of DC over time. Images were captured at day 1, 3, 5, and 8 after implantation of DC into the tissue model (Figure 5A and B, **paper I**). The number of DC in each of the acquired confocal images was calculated and the results revealed a significant decrease in DC quantity at day 8 compared to day 1 (Figure 5C, **paper I**). Although, our data demonstrated that the lung tissue model support survival of DC in the absence of exogenous growth factors there was a loss of DC overtime.

To further identify and investigate the location of DC in the lung tissue model, tissue sections were stained for well-defined DC markers such as HLR-DR, DC-SIGN and CD11c. The immunofluorescence staining revealed HLA-DR^+ DC located within the epithelium (Figure 6A, **paper I**) and HLA-DR^+DC-$SIGN^+$ DC could be detected in the basolateral space beneath the epithelial layer (Figure 6B, **paper I**). Interestingly, we could also detect $CD11c^+$DC-$SIGN^+$ DC distributed on the apical side of the epithelium (Figure 6C, **paper I**). In lung tissue, it has been suggested that the chemokine CX_3CL1, the ligand for CX_3CR1, is important for DC interaction with the epithelial layer. CX_3CL1 has been reported to be produced mainly by epithelial cells, whereas the receptor CX_3CR1 expression is restricted to leukocytes [225]. To investigate the expression of CX_3CL1 in the lung tissue model, we performed mRNA analysis of CX_3CL1 expression in lung tissue model with and without DC and compared this to the expression of CX_3CL1 in DC cultured in medium. Interestingly, the lung tissue model with DC showed the highest expression of CX_3CL1 among the three conditions (Figure 4E, **paper I**). In addition, immunofluorescence staining of CX_3CL1 revealed that the chemokine is highly expressed by epithelial cells in the lung tissue model, (unpublished observation). Therefore we sought to confirm the expression of the CX_3CL1 receptor, CX_3CR1, in the lung tissue model. As shown in Figure 6D, **paper I**, immunofluorescence staining revealed CX_3CR1 positive cells situated close to the epithelium in the lung tissue model. To prove that the CX_3CR1 positive cells indeed are DC we will need to perform double staining and include DC specific markers.

In summary, DC in our lung tissue model are strategically located underneath, within, and at the apical side of the epithelium consistent with a previous report [226]. Furthermore, the model supports DC survival without exogenous growth factors for at least eleven days and DC survival can be monitored over time. Together, these results indicate that our 3D lung tissue model composed of human epithelial cells, fibroblasts and DC form a functional physiological microenvironment that recapitulates key anatomical and functional features of lung mucosal tissue.

5.1.3 Regulation of dendritic cell chemokine producing capacities by the microenvironment

To investigate whether our lung tissue model regulates chemokine production by DC under physiological conditions, we investigated the expression of CCL18, CCL17 and CCL22 mRNA and protein levels in the lung tissue model using real time QRT-PCR and ELISA. CCL18 is constitutively expressed in lungs, whereas CCL17 and CCL22 have low expression in lungs under homeostatic conditions but can be induced upon inflammation. Interestingly, these analyses revealed that CCL18 was highly expressed in the tissue model with DC, both at mRNA and protein levels, compared to DC cultured in medium only. In contrast, lung tissue models without DC showed no expression of CCL18 (Figure 7A and B, **paper I**). In addition, low levels of CCL17 and CCL22 mRNA and protein expression were detected in lung tissue models with and without DC compared to DC cultured in medium (Figure 7C and F, **paper I**). These data show that the lung tissue model has the ability to regulate chemokine production by DC, which involves enhanced expression of CCL18 and a reduced expression of CCL17 and CCL22. In order to investigate whether or not the induction of CCL18 production in DC depends on soluble factors secreted by the tissue model, DC were conditioned with supernatant from the lung tissue model. As shown in Figure 8A and B, **paper I**, DC conditioned with supernatant from the lung tissue model had enhanced expression of CCL18 mRNA and CCL18 protein secretion compared to DC cultured in medium only. As secreted compounds from the lung tissue model regulated CCL18 induction in DC, we were interested investigating if this regulation was dependent on the composition of the epithelial cells and fibroblasts that were grown together under air-exposure in a 3D structure. DC were conditioned with supernatants from air-exposed epithelial cells, fibroblasts or a submerged mixed monolayer of epithelial cells and fibroblasts. The data from mRNA analysis revealed that only supernatant from the lung tissue model could robustly induce CCL18 production by DC compared to DC cultured in medium only (Figure 8C, **paper I**). These data indicate that only the complete lung tissue model is required to efficiently induce enhanced CCL18 expression in DC.

In this study, we have developed a 3D human lung tissue model that comprises three parts: a stratified and polarized epithelium that is grown on top of a basement membrane, an underlying fibroblast matrix layer rich in extracellular matrix proteins as well as DC that are closely distributed to the epithelial layer. Our lung tissue model recapitulates the structure of normal lung mucosal tissue and is fully functional in the deposition of extracellular matrix proteins and production of tight and adherence junctions. Furthermore, the lung tissue model supports DC survival for at least eleven days without addition of exogenous growth factors and we could also observe that DC

production of the chemokines CCL17, CCL18 and CCL22 is regulated by the lung tissue microenvironment in ways resembling physiological conditions.

5.2 THE LUNG TISSUE MODEL ENABLES LIVE IMAGING ANALYSIS OF HUMAN DENDRITIC CELLS IN A PHYSIOLOGICAL MILIEU

In the previous section, we demonstrated the establishment of our 3D lung tissue model that allows studies of human DC in a physiological relevant milieu. Here, we demonstrate how this tissue model can be used for live imaging analysis of DC migratory behaviour in response to inflammatory stimuli and chemokine.

Little is known about local tissue-specific mechanisms regulating human DC functions, including activation and migratory behavior. Studies of human DC activation and migration in tissues, especially in lung tissue, are difficult to perform, and we need to improve human tissue models that allow analysis of DC in a microenvironment that mimics *in vivo* situations. The aim of this project was to further develop our human 3D lung tissue model to enable live imaging analysis of DC and investigate DC activation and migratory behaviour in response to inflammatory stimuli and chemokines.

5.2.1 The lung tissue model enables quantitative live imaging analysis of human dendritic cells in the microenvironment

To establish the lung tissue model for live imaging analysis, lung epithelial cells and fibroblasts were transduced with fluorescent proteins using a retroviral vector system that enable stable transduction of the cells. Epithelial cells and fibroblasts were transduced with green and orange fluorescent protein, respectively. DC were labeled with a far red cell tracker dye before being implanted into the model. The lung tissue model was generated according to Figure 1B, **paper II.** After the completion of the model set up, models were removed and mounted in a six-well glass bottom culture plate for live imaging (Figure 1C-L, **paper II**). Confocal image analysis of the lung tissue model revealed that tissue architectures comprised a dense epithelial layer (green) on top of a collagen matrix of fibroblasts (red). Dendritic cells, were located close to the epithelial layer (Figure 2A and B and supplementary video 1, **paper II**), as we have observed before (**paper I**). The confocal images also revealed that the epithelial cells were well separated from the underlying fibroblast layer. This raised the question regarding whether or not there are interactions going on between the two layers since interactions between cells within the microenvironment have been reported to be essential for cell function such as differentiation, proliferation, adhesion and migration [84, 227, 228]. We therefore analyzed in more detail the connection between the epithelial cells and the underlying fibroblast matrix by excluding the upper epithelial layer in the image analysis. Interestingly, at the epithelial-fibroblast boundary, we observed elongated epithelial cells forming a network of fibers that stretched down into the fibroblast matrix underneath (Figure 2C and D, supplementary videos 2 and 3, **paper II**). Together, these data indicated that our lung tissue model display a well-defined tissue structure and is well suited to study DC activation and migration in a physiological milieu mimicking human lung tissue.

5.2.2 Relocation of dendritic cells towards the epithelial layer in response to TLR-1/2 and CCL2

To explore DC migratory behaviour in response to inflammatory stimuli and chemokine in a physiological milieu, the tissue model was stimulated with TLR-4 ligand, TLR-1/2 ligands and recombinant human CCL2. Confocal image analysis for 4 hours of stimulation with TLR-1/2 revealed that DC in the stimulated model were distributed closer to the epithelial layer compared to the unstimulated model (Figure 3A and B, **paper II**). Quantitative analysis comparing the distance of each DC in relation to the center of the epithelial layer revealed that TLR-1/2 and CCL2, but not TLR4, induced relocation of DC resulting in DC being distributed closer to the center of the epithelial layer. These data demonstrate that the response of DC to different inflammatory stimuli and the pathways involved in regulating DC migration to different stimuli in lung tissue may differ.

5.2.3 Stimulation of TLR-ligands and CCL2 induces dendritic cell motility in the lung tissue model

To further assess the role TLRs and chemokines have on DC migration, we performed live imaging experiments on lung tissue models stimulated with TLR-4 ligand, TLR-1/2 ligands or CCL2 over a time period of 16 hours. Interestingly, DC in the stimulated models explored a wider territory in x and y plane as shown in the track displacement graphs (Figure 4A-D, **paper II**). Furthermore, analysis of automatic tracking of the different stimulation revealed that DC migrated a longer distance and increased their mean velocity over time compared to DC in unstimulated models (Figure 5C-D, **paper II**). In addition, we could also observe that DC in stimulated models exhibited a less spherical shape (Figure 5E, **paper II**), which correlated with an increase in DC mean velocity (Figure 5F-I, **paper II**). These data demonstrate that DC in the tissue model respond to inflammatory stimuli and chemokines by relocating in relation to the epithelial layer and by displaying a more exploratory phenotype. Stimulations also induced changes in DC morphology correlating to increased velocity and longer distances of migration. Thus, our data demonstrate that our human lung tissue model is well-suited for quantitative 4D (x, y, z and time) fluorescence imaging of DC migratory behaviour in response to inflammatory stimuli and chemokines in real time, under next to *in vivo* settings.

5.3 DENDRITIC CELL-TUMOUR INTERACTION IN THE THREE-DIMENSIONAL TISSUE MODEL

The identification and testing of new targetable steps aimed at preventing cancers from altering the microenvironment and immune cell functions, including the regulation of human DC and non-hematopoietic tissue specific cells, such as fibroblasts, are generally difficult to recapitulate *in vitro.* The aim of this project was to generate micro-tumours in the form of NSCLC, which could be implanted into our human lung tissue model containing DC, and investigate DC-tumour interaction in the tissue model.

5.3.1 Generation of micro-tumour spheroids for implantation of tumor epithelial cells in the lung tissue model

To approach the challenge of creating a model for studying DC interactions with NSCLC cells, we decided trying to generate tumour spheroids, which could be implanted into the lung tissue model, and monitored by live imaging analysis. For this purpose we used the A549 adenocarcinoma lung epithelial cell line and the normal 16HBE epithelial cell line that we labeled with a red cell tracker dye before creating the spheroids. To create the spheroids we used a hanging drop system, which generated spheroids of well-defined structure and a certain number of cells. First, the fluorescent A549 and 16HBE cells were mixed with a viscous carboxymethylcellulose solution and 25 µl droplets containing 500 cells each were placed up side down in a petri dish and incubated at 37°C for 48 hours (Figure 1A-C, **paper III**). The spheroids made of 16HBE (data not shown) or A549 cells (Figure 1D) were then implanted in the tissue model at the same day of air-exposure. After four days of air-exposure, models were removed from the six-well inserts and mounted for live imaging experiments. Confocal image analysis of a tumor spheroid model revealed distinct microepithelial environments (red) that were separated from the normal epithelial layer (green), while DC distributed both inside the tumor area and in the normal epithelial layer (Figure 1F, **paper III**). These data indicate that the tumour spheroids enable implantation of tumour cells that form well-define tumour areas in the lung tissue model and that can be monitored in real time.

5.3.2 Migration of dendritic cells in the tumour microenvironment

Next, we investigated the impact on DC migratory behaviour in the presence of NSCLC. Images were acquired of models with A549 (Figure 2A, **paper III**) and 16HBE spheroids (data not shown). Each spheroid area was identified (Figure 2B) and DC distribution was then quantified in the "tumour" area and the "healthy" area. Quantification of DC distribution revealed that DC were more frequently located within the tumour spheroids as compared to implanted 16HBE spheroids in the control model, (Figure 2C, **paper III**). This indicates that DC are attracted to the tumours and that this may depend on differentially secreted chemokines from the tumor cells compared to healthy epithelial cells.

To elucidate the influence of tumour cells on DC behaviour and function, we investigated DC's ability to take up tumour cells. For this purpose, confocal images of the spheroid areas with A549 (Figure 3A, **paper III**) or 16HBE (data not shown) were extracted to visualize DC located in the epithelial cell spheroids only (Figure 3B, **paper III**). Rendered images were further segmented using the Imaris software to identify DC that had engulfed epithelial cells within the spheroid areas of implanted 16HBE (Figure 3C) or A549 (Figure 3D) cells. Quantification of DC colocalization to implanted epithelial cells revealed that DC were more frequently associated with tumour cells and also engulfed A549 cells more efficiently as compared to 16HBE cells, (Figure 3E, **paper III**). Thus, NSCLC tumour spheroids can be created for implantation in our lung tissue model, and this enables functional studies of DC interactions with cancer cells in a 3D-tumour microenvironment of NSCLC, previously not achievable.

5.4 STROMAL CELLS SUPPORT INCREASED DEVELOPMENT OF REGULATORY DENDRITIC CELLS FOLLOWING *LEISHMANIA* INFECTION

Stromal cells have been recognized for their function to regulate the differentiation of HSPC into terminally differentiated blood cells. The stromal cells niches in the bone marrow and the spleen control homing, migration and differentiation of hematopoietic progenitor cells under steady state conditions. The aim of this study was to investigate the role of stromal cell-derived chemokines in the differentiation of hematopoietic progenitor cells into regulatory DC in the spleen of mice during homeostasis and in response to *L. donovani* infection.

5.4.1 Stromal cell guided hematopoietic progenitor cell differentiation into regulatory dendritic cells

In a previous study, Svensson *et al.* [12], demonstrated that freshly isolated splenic stromal cells from mice have the capacity to support HSPC differentiation into regulatory DC. As a follow up study, we sought to investigate if the fibroblast-like bone marrow stromal cell line MBA-1 [218] also had the ability to support HSPC differentiation into regulatory DC. MBA-1 cells have shown to have the ability to stimulate hematopoiesis and reduce rejection of mismatched allograft transplantation [229]. As shown in Figure 1A and B, **paper IV**, $BMLin^{-}CD117^{+}$ HSPC developed to $CD11c^{+}MHC\text{-}II^{+}$ DC in cocultured with MBA-1 cells for 6 days. These DC expressed a heterogeneous level of CD11c and high levels of CD11b (Figure 1C, **paper IV**). To determine the regulatory capacity of these cells, CD11c-purified DC, (Figure D, **paper IV**), derived on MBA-1 cells were cultured with $CD4^{+}$ T cells from BALB/c mice and CSF-2-derived LPS stimulated DC in an MLR reaction. This revealed that $CD11c^{+}$ DC that developed on stromal cells could inhibit the MLR induced by CSF-2-derived DC by 90%, (Figure 1E, **paper IV**). In addition, we also observed that the $CD11c^{-}$ fraction also suppressed the MLR induced by CSF-2 derived DC by 70% (Figure 1F, **paper I**). These data indicated that MBA-1 stromal cells have the capacity to support HSPC differentiation into DC with regulatory properties.

Chemokines secreted by stromal cells are important for HSPC migration and homing to hematopoietic niches [230, 231]. However, the role of chemokines in HSPC differentiation into regulatory DC has not been reported. Therefore, we sought to investigate if the development of regulatory DC from HSPC was influenced by stromal cell-derived chemokines. We used a transwell system in which MBA-1 cells were seeded in the lower chamber and $BMLin^{-}CD117^{+}$ HSPC were seeded in the upper chamber. We could detect $CD45^{+}H2Kb^{+}$ cells in the lower chamber after 3 hours, (Figure 2A) and 16 hours, (Figure 2B), of coculture, indicating that HSPC migrated towards MBA-1 cells in the lower chamber. After 6 day of coculture, we collected $CD11c^{+}$ and $CD11c^{-}$ cells expressing CD11b in the lower chamber of the transwell system, Figure 2C, **paper IV**. This demonstrated that HSPC that had migrated towards the MBA-1 cells differentiated into $CD11c^{+}CD11b^{+}$ and $CD11c^{-}CD11b^{+}$ cells. In contrast, HSPC remaining in the insert differentiated only into $CD11c^{-}CD11b^{+}$ cells (Figure 2C, **paper IV**). These observations indicate that soluble factors from MBA-1 cells are sufficient to support differentiation of $CD11c^{-}CD11b^{+}$ cells, whereas differentiation of $CD11c^{+}CD11b^{+}$ cells required contact with stromal cells. We also confirmed that DC generated from the migrated HSPC in the Transwell system have

the capacity to suppress an MLR reaction induced by CSF-2 derived DC (Figure 2D, **paper IV**).

5.4.2 *L. donovani* infection enhanced hematopoietic stem and progenitor cell differentiation into regulatory dendritic cells

L. donovani is a protozoan parasite that can infect bone marrow [232] and splenic stromal cells [12]. *L. donovani* infection enhance the capacity of splenic stromal cells to support HSPC differentiation into regulatory DC [12]. However, it was never ascertained if this was due to direct infection of the stromal cells by the parasite or if it could be influenced by inflammatory cytokines at the site of infection. To address this question, we infected MBA-1 cells and RAW264.7 macrophages with *L. donovani* amastigotes (Figure 3A, **paper IV**) and investigated if the infected cells had enhanced capacity to support HSPC differentiation into regulatory DC. The analysis revealed that HSPC differentiation into $CD11c^+$ DC was increased in coculture with infected MBA-1 cells compared to uninfected cells. However, coculture with RAW264.7 cells did not support HSPC differentiation into $CD11c^+$ DC (Figure 3A, **paper IV**). As shown in figure 3C, **paper IV**, DC derived on infected MBA-1 cells also had regulatory capacity. This finding indicates that direct infection of stromal cells enhances their capacity to support HSPC differentiation into regulatory DC.

5.4.3 Expression of stromal cell-derived chemokines is modulated by *Leishmania donovani* infection

Next, we investigated chemokines that are expressed in MBA-1 cells but not in RAW264.7 cells before and after infection of *L. donovani* based on the observation that RAW264.7 did not support HSPC differentiation in the Transwell assay. For this purpose, we performed genome-wide mRNA expression profiling of uninfected and infected MBA-1 and RAW264.7 cells. As a result of this we identified a variety of genes with changed expression in response to infection in both cell lines but we focused our analysis primarily on changes in the chemokine expression. This analyses revealed eight chemokine genes CCL7, CCL8, CCL27, CXCL1, CXCL3, CXCL5, CXCL12 and CXCL15 that were differentially expressed in MBA-1 cells compared to RAW264.7 cells (Figure 4A, **paper IV**). We got interested in one particular chemokine, CCL8, which was increased in MBA-1 cells in response to infection according to the microarray analysis. In addition, studies have shown that CCL8 was expressed in splenic stromal cells that can support DC development [233]. Based on this observation and the knowledge that CXCL12 is important in regulating HSPC homing and migration [98, 230, 234], we initially focused our studies on CCL8 and CXCL12. First, we confirmed the microarray data with real time QTR-PCR and ELISA analysis that revealed expression of CCL8 and CXCL12 mRNA and protein by MBA-1 cells. In contrast, the chemokine expression was undetectable in RAW264.7 cells (Figure 4B-E, **paper IV**). The real time QRT-PCR analysis also revealed that CCL8 expression was increased in infected MBA-1 cells compared to uninfected cells, whereas CXCL12 was slightly reduced 48 hours post infection (Figure 4F, **paper IV**). Together, the analyses revealed a unique stromal cell chemokine expression pattern that was modulated by *Leishmania donovani* infection.

5.4.4 *L. donovani* infection induced CCL8 expression in splenic stromal cells

The expression of CXCL12 and CCL8 has not been reported during *L. donovani* infection *in vivo*. To investigate the expression of these chemokines in splenic tissue of mice infected with *L. donovani*, we performed real time QRT-PCR analysis and interestingly the results revealed an increased expression of CCL8 by 100-1000 times in infected mice. The expression of CXCL12 was unaltered or minimally reduced in response to infection (Figure 5A, **paper IV**). To investigate the CCL8 expression in splenic stromal cells, we analyzed mRNA level of CCL8 in *ex vivo* enriched splenic stromal cells. The analysis revealed that CCL8 expression was increased 10,000 fold in splenic stromal cells from infected mice (Figure 5A, **paper IV**), thus, indicating a 10-fold enrichment of signal by purification of stromal cells compared with the CCL8 expression seen in the whole spleen. In addition, immunohistology analysis revealed CCL8 protein expression in splenic tissue from mice infected with *L. donovani*, whereas tissue from naive spleen showed no expression of CCL8 (Figure 5B, **paper IV**). CCL8 protein could also be detected in medium conditioned with freshly isolated splenic stromal cells from infected mice (Figure 5C, **paper IV**). Furthermore, CXCL12 protein was still expressed in enriched splenic stromal cells from naive and infected mice (Figure 5F, **paper IV**). These findings indicate that *L. donovani* infection modulates chemokine expression of splenic stromal cells, in particular that of CCL8.

5.4.5 Induction of stromal cell-derived CCL8 during *L.donovani* infection is associated with increased recruitment of hematopoietic stem and progenitor cells

As the expression of CCL8 is increased during *L. donovani* infection, we decided to investigate the role of CXCL12 and CCL8 in the migration of HSPC. We investigated the contribution of these chemokines in the migration of HSPC induced by MBA-1 cells in a transwell migration assay. Neutralizing antibody against CXCL12 revealed efficient blocking of HSPC migration induced by MBA-1 cells (Figure 6A, **paper IV**) to the level that was observed for pertussis toxin, an inhibitor of chemokine receptor signaling (Figure 6B, **paper IV**). This confirms the crucial role of CXCL12 in the induction of HSPC migration that has previously been reported [98, 230]. Interestingly, we also observed that neutralizing antibody against CCL8 had a partial blocking effect against HSPC migration induced by MBA-1 cells (Figure 6C, **paper IV**).

To further investigate the role of CXCL12 and CCL8 in the migration of HSPC, conditioned medium from MBA-1 cells was used in the migration assay. The reason of using conditioned medium instead of the cells was to avoid involvement of other chemokines that may be induced following contact between HSPC and the stromal cells. The analysis revealed that conditioned medium induced a higher migration of HSPC compared to the concentration of recombinant CXCL12 that was detected in MBA-1-conditioned medium (Figure 6D, **paper IV**). This observation indicates that other chemokines may be involved in the migration of HSPC. In addition, using a neutralizing antibody against CCL8 in the MBA-1-conditioned medium, reduced HSPC migration to the level detected with recombinant CXCL12 (Figure 6D, **paper IV**). We also observed that CCL8 alone did not induce HSPC migration, however, in

combination, CXCL12 and CCL8 induced HSPC migration to the levels seen with MBA-1-conditioned medium (Figure 6E, **paper IV**). These data indicate that stromal cell-derived CCL8 synergized with CXCL12 in the recruitment of HSPC.

Next, we sought to determine if the CCL8 induction observed in *L. donovani* infection in mice could affect the recruitment of HSPC by using a transmigration assays with conditioned medium from splenic stromal cells isolated from uninfected and infected mice. The analyses revealed that HSPC migration was induced in cocultures with medium conditioned with splenic stromal cells isolated from infected mice (Figure 6G, **paper IV**). Furthermore, we observed that neutralizing antibodies against CCL8 reduced HSPC migration in medium conditioned with splenic stromal cells from infected mice to the level that was observed for medium conditioned with splenic stromal cells of naive mice (Figure 6G, **paper IV**). The analysis also revealed that neutralizing antibody against CXCL12 in medium conditioned with splenic stromal cells from infected mice blocked HSPC migration to the level that was observed for medium only (Figure 6G, **paper IV**). In this study, we found that the stromal cell-derived chemokines CXCL12 and CCL8 cooperate to recruit hematopoietic progenitors that can differentiate into regulatory DC. In addition, *L. donovani* infection of bone marrow stromal cells showed increased production of CCL8 and enhanced capacity to support the development of regulatory DC. Also, CCL8 production was induced in splenic stromal cells from *L. donovani* infected mice, which may lead to increased capacity to recruit HSPC with the potential of developing into regulatory DC.

6 DISCUSSION OF OUR RESULTS

This chapter is divided into two sections. In the first section, I will discuss the results obtained using the 3D lung tissue model with DC. The second section will focus on the discussion of results obtained from experiments of stromal cell regulation of HSPC differentiation into regulatory DC in response to *L. donovani* infection.

6.1 THE THREE-DIMENSIONAL LUNG TISSUE MODEL WITH DENDRITIC CELLS

Tissue microenvironments, such as gut and lung mucosa, are increasingly recognized as important factors in influencing and coordinating tissue homeostasis and inflammation. This coordination depends on a delicate interaction between immune cells, tissue-specific cells and ECM. Therefore, the establishment of 3D-human tissue models has become increasingly appreciated to study infectious diseases and tissue pathophysiology, as they are likely to better capture the cellular events that occur in real tissue compared to those that occur in monolayer cultures. The establishment of 3D tissue models of human lung, skin and oral mucosa composed of a physiological fibroblast matrix and a stratified epithelial layer has been accomplished [138, 139, 235]. However, *in vitro* models of human lung that include human immune cells are lacking. Therefore, we have developed a 3D tissue model of human lung by implanting human DC in a multicellular microenvironment composed of lung fibroblasts and lung epithelial cells. The findings from this work demonstrate that our 3D lung tissue model can be used to study tissue regulation of DC functional properties in a physiologically relevant environment. We also showed that the tissue model is useful for visualizing the migratory behaviour of DC in response to inflammatory stimuli in real time using live cell imaging confocal microscopy. Furthermore, we demonstrated that generation of tumour spheroids from cancer cells could be implanted in the lung tissue model and enable analysis of DC interaction with tumour cells in the lung tissue microenvironment in real time.

6.1.1 The lung tissue model recapitulates the structure of airway mucosal tissue and supports the survival of dendritic cells

Exposure of cells to the spatial constraints imposed by a 3D milieu determines how cells perceive and interpret biochemical cues from the surrounding microenvironment, e.g., the extracellular matrix, adhesion molecules, growth factors, inflammatory mediators, and metabolites as well as pathogens. It is in this biophysical and biochemical context that cells display bona fide tissue and organ specificity. Even though this is the way we tend to view the situation for non-hematopoietic cells, it should also be applicable to our view on hematopoietic cells when it comes to understanding their functional properties associated to specific tissues. The lung tissue model that we have developed provides a proper tissue microenvironment, although less complex, than real tissue. Nevertheless, it allows DC to perform their functions while embedded in an multicellular microenvironment of fibroblasts, epithelial cells

and extracellular matrix components, all of which are essential for normal tissue function.

In vivo, collagen IV and laminin 5 are produced mainly by the fibroblasts. These proteins are the major components of the basement membrane, which for example is essential for the formation of epithelial cell barriers [86]. Collagen IV and laminin 5 also influence cell differentiation, adhesion and movement [236-238]. Tropoelastin is another important component of the ECM and the basement membrane, and play a key role in the control of tissue elasticity [239]. Epithelial cells not only need the basement membrane to function properly, but they also need to hold together. Epithelial cells *in vivo* are held together by adherence junction proteins such as E-cadherin, that play a key role in epithelial anchoring and adhesion within the tissue. The structural integrity of epithelial cells is crucial for the maintenance of a functional epithelial barrier. To perform this task, the adherence junction proteins are accompanied by tight junction proteins. The latters have a pivotal role in the regulation of epithelial cell permeability and transportation of ions and water. The major components in the tight junctional complex formation are the Occludin and Claudin proteins [240]. Formation of junctional complexes is specific to the epithelium and provides useful markers for recognizing epithelial cells *in vitro*. The findings that our lung tissue model produced and deposited all of the essential proteins mentioned above, indicate that the tissue model has a functional structure that resembles epithelial tissue of real lung.

Further observation of the lung tissue model with DC includes that we identified DC being widely distributed in the tissue model, most of which were located close to the epithelial layer, which is in line with a previous report [226]. It may suggest that DC, are patrolling the epithelial cell barrier being ready to respond to foreign material and tissue damage, followed by their coordinated production of cytokines and chemokines. There is evidence suggesting that DC can express tight junction proteins, such as Claudin and Zonula-2, which enables DC to form tight junctions with the airway epithelial cells. This enables DC to open up the epithelial tight junction barrier without disturbing the epithelial integrity and enter the airway lumen to sample inhaled antigens [241, 242].

To identify DC in the tissue model, we used antibodies specific for HLA-DR, DC-SIGN and CD11c that are well-defined DC markers not expressed by the non-hematopoietic cells. The DC survived in the tissue model for at least eleven days without exogenous growth factors. This number differs substantially from the ones seen when culturing DC in medium only; as monocyte-derived DC normally do not survive in culture without CSF-2 and IL-4 for more than three days. The tissue model is likely to produce cytokines and growth factors that support DC survival. For example, we identified that the tissue model abundantly expresses mRNA for the cytokine CSF-2 but not for IL-4. CSF-2 has been suggested to play a crucial role in the survival of steady state DC in the lung, skin and lamina propria [81]. Furthermore, in lung tissue, DC have a rapid turnover at steady state where resident population are renewed within one week [243, 244]. Nevertheless, at day 8 there are still many DC in the tissue model, which can be monitored upon intervention, and additionally, imaging of DC turnover may be a useful tool for investigating the effect of harmful compounds on DC survival.

Together, these results demonstrate that our 3D lung tissue model supports survival of DC and allows functional studies of human DC in a physiologically relevant milieu previously not achievable. Furthermore, we believe these findings have important

implications for increasing our understanding of tissue-specific regulation of local immune responses in lung tissue in general, and DC function in particular.

6.1.2 Lung tissue model regulates chemokine production by dendritic cells

The secretion of chemokines orchestrates the migration of leukocyte migration between lymphoid and peripheral tissues at steady state and during infection. Dendritic cells are producers of several chemokines and recent studies indicate that the quality of chemokine production in tissue is determined by the local microenvironment. Our study shows that the 3D lung tissue model regulates the ability of DC to produce the chemokines, CCL18, CCL17 and CCL22. At steady state, the chemokine CCL18 is produced at high levels in lungs [184], whereas CCL17 and CCL22 [182, 185] are highly expressed in thymus and lymph nodes, and at relatively low levels in lung. Our results suggest that the lung tissue model supports DC production of CCL18 since DC in the tissue model produce more CCL18 than DC cultured in medium only. In contrast, DC production of CCL17 and CCL22 was reduced when DC were implanted into the tissue model compared to the production by DC cultured in medium. These findings indicate that the microenvironment in the lung tissue model selectively regulates chemokine production by DC, in ways resembling the production of chemokines under physiological conditions in lung tissue. Thus, our results implicate that specific tissue niches in the lung may regulate the outcome of immune responses by influencing functional properties of DC locally. However, the mechanism that controls the expression of CCL18, CCL17 and CCL22 in lung tissue needs further investigations. Furthermore, our study demonstrates that DC expression of CCL18 is enhanced by soluble factors that are secreted by the lung tissue model. However, the mechanisms regulating CCL18 production by DC in lung tissue are still unknown. There are several factors in the lung tissue model that could lead to the induction of CCL18. At present we are investigating two potential signaling pathways in DC that may lead to increased CCL18 expression. One pathway could be mediated by a stromal cell-derived cytokine called thymic stromal lymphopoietin (TSLP), which has been shown to induce CCL18 expression in human monocytes [245]. Thymic stromal lymphopoietin is produced by epithelial cells and fibroblasts and is constitutively expressed by many tissues including skin, lungs, intestines and thymus [246, 247]. The cytokine preferentially stimulates myeloid cells and is a potent regulator of DC function [111]. The signaling pathway of TSLP is mediated via a receptor that is composed of two subunits, TSLP receptor and IL-7Rα (CD127). Receptor ligation leads to phosphorylation of STAT5 and STAT3. Our preliminary results showed that the lung tissue model express TSLP and TSLP was found to be produced by both lung fibroblasts and lung epithelial cells. Currently we are investigating the role of TSLP in inducing CCL18 by DC in our model system. These studies also include analysis of TSLPR receptor expression on DC in the model. Another signaling pathway that may lead to CCL18 expression could be mediated by collagen type I and IV in the tissue model. Collagen type I and IV have been shown to induce CCL18 expression in myeloid cells, such as macrophages, by binding to αβ/integrin, which induces STAT6 signaling cascade that could lead to CCL18 production [248] (Figure 6). Using blocking antibodies against TSLP/TSLPR, as well as integrins and collagens, we will investigate the role of such molecules in the regulation of CCL18 by DC.

Figure 6

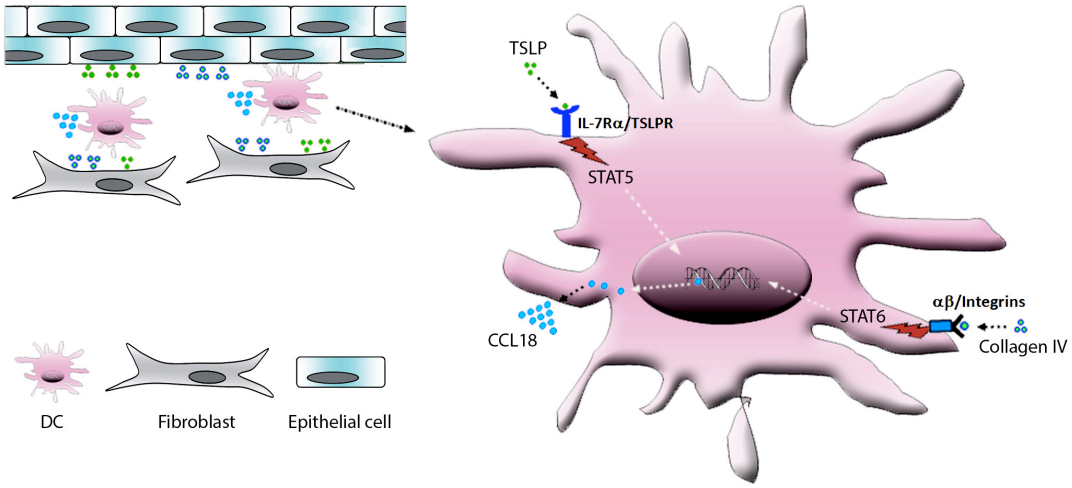

Figure 6. Schematic illustration of potential mechanisms that may lead to CCL18 induction in DC. The cytokine TSLP that is constitutively expressed by fibroblasts and epithelial cells may bind to the TSLP receptor on DC and signal via STAT5 to induce CCL18 production. Another potential signaling pathway may be mediated by collagen IV that binds to the intergrin receptor on DC and signals via STAT6.

CCL18 is a chemokine that is mostly associated with anti-inflammatory and Th2 immune responses[249]. Recent studies have demonstrated a role for CCL18 in the differentiation of DC into tolerogenic cells that have the ability to prime regulatory T cells [195]. This suggests that the constitutive expression of CCL18 observed in lung tissue may play an important role in the maintenance of tolerance to inhaled antigens under homeostatic conditions. Hopefully, it should be possible explore the pathways controlling CCL18 production by DC using the lung tissue model and future observations can then be verified in normal lung tissue.

6.1.3 Live imaging analysis of dendritic cell migration in the lung tissue model

Having established a robust and reliable tissue model system that enables studies of DC functional properties in a physiologically relevant lung microenvironment, we wished to further develop our 3D lung tissue model for live cell imaging analysis, which would allow imaging of DC behaviour under next to *in vivo* settings. This led to the second major outcome of my thesis work and includes that DC migratory behaviour in response to inflammation and chemokines can be studied in the lung tissue model using a live cell imaging technique. Here, we showed that lung epithelial cells and fibroblasts are responsible for the formation of intricate cellular networks, which allows for DC distribution in a multicellular microenvironment. This model set up enables studies of human DC activation and migration in response to inflammatory stimuli and chemokines in live tissue. Our results demonstrate that the lung tissue model established for live imaging shares similar characteristics to typical epithelial lung tissue, such as the formation of a well-defined stratified epithelium

that is well separated from the underlying fibroblast matrix layer. The separation between the two layers depends on the formation of a basement membrane that is made up of collagen, laminin, fibronectin and proteoglycans. The basement membrane is usually a joint product of the epithelium and the underlying stroma [83]. Furthermore, our data demonstrated that epithelial cells located closest to the boundary between the fibroblast and epithelial cell layer displayed a more elongated morphology and formed a network of fibers that connects them to the underlying fibroblast layer. The interaction between epithelial cells and other non-hematopoietic cells has also been proposed to be regulated by diffusible factors, rather than via direct contact between the cells [83]. However, the structure of the epithelium is most likely dynamic and there may be constant communication between the epithelial cells and the underlying extracellular matrix, providing a proper microenvironment for DC to migrate in.

In lung, activation of DC is driven by their encounter with inhaled antigens. To perform their immune surveillance and regulation of immune responses in tissues, DC express a variety of pattern recognizing receptors, amongst them the TLRs [250]. Using our established lung tissue model for live cell imaging analysis, we showed that DC in the tissue model responded to TLR-ligands stimulation and the chemokine CCL2, which resulted in a relocation of DC towards the epithelial layer. The chemokine CCL2 is known to mediate DC migration and maturation. It has also been shown that CCL2 plays an important role in the recruitment of DC to the lung during acute allergen challenge [177]. It is possible that the relocation of DC to the epithelial layer mediated by TLR-ligands does not solely depend on direct TLR-stimulation of DC. It could also depend on the release of chemokines by epithelial cells, which may contribute to the recruitment and redistribution of DC. There is evidence suggesting that airway epithelial cells also express TLRs and upon TLR-stimulation epithelial cells release chemokines and cytokines, such as CCL20 and CSF-2, that may mediate the recruitment of DC to the epithelial tissue [108, 251]. Using the live imaging set up of the lung tissue model system, we could show that DC are attracted to the epithelial layer in response to TLR-1/2 stimulation and CCL2, but not in response to TLR-4. The differences observed between TLR1/2 and TLR-4 ligand may be due to the fact that the concentration of TLR-4 ligand used in the experiment was not sufficient to induce chemokine expression by epithelial cells that could recruit DC to the epithelial layer. However, we showed that DC in the tissue model explored a wider territory in the x and y plane and moved longer distances in response to TLR-1/2, TLR-4 and CCL2, suggesting that DC responded in one way or another to all stimuli applied. The results also revealed that DC in the tissue model moved faster in response to the stimuli. The rapid movement by DC, that was observed, in response to TLR-ligands and CCL2 may be a necessary mechanism for DC to scan the microenvironment in the search for foreign antigens. How this migratory behaviour of DC is induced in response to TLR stimulation and which tissue specific-derived factors that are involved in this regulation remains to be determined. One possible theory could be that the surrounding tissue-specific cells secrete chemokines, which are deposited or possibly transcytosed to build gradients of chemokines that DC can migrate along. Also, adhesion molecules are likely to support and guide the migration of DC in the tissue microenvironment. For example, bronchial epithelial cells have the ability to express chemokines that mediate both leukocyte migration and adhesion. CX_3CL1 is a chemokine that exerts dual function and can act as adhesion molecules through their

membrane-bound form or as soluble chemotactic factors. Our data indicated that CX_3CL1 is highly expressed by the lung tissue model and that CX_3CR1, the receptor for CX_3CL1, is expressed by DC in the model, which suggests that CX_3CR1 and its ligand may contribute to the epithelial organization of DC observed in the lung tissue model.

Our findings demonstrate that the lung tissue model enables studies on DC distribution and migration in response to different inflammatory stimuli and chemokines. The quantification of DC migration and distribution in the tissue model can provide a powerful tool to investigate the mechanistic actions of adjuvants or demonstrate effects of noxious compounds as well as testing reagents aiming at blocking DC activation and migration locally.

6.1.4 Exploring dendritic cell behaviour and function in an organotypic-based epithelial spheroid model of non-small cell lung cancer

There is evidence supporting that cancers affect the immune system by imposing deregulated immune responses. However, the mechanisms that mediate interactions between tumours and human hematopoietic cells within the tumour tissue are poorly defined. In the tumour microenvironment, the surrounding tissue-specific cells and ECM are also likely to play an important role in the regulation of immune cell differentiation and function during tumour development [119, 252]. However, most studies on cancer have been performed using one cell component at a time and may not capture the complexity of numerous cellular processes that occur *in vivo*. Therefore, we believe that tissue model systems that recapitulate many key anatomical and functional features of the *in vivo* parental tissue are indispensable to study cancer. To allow studies of DC-tumour cell interactions in a 3D milieu, we developed our 3D tissue model to also include cancer cells. Using a hanging drop system we generated microtumours in the form of epithelial spheroids and implanted these as micro tumours. The use of epithelial spheroids enables us to implant a fixed number of cells into the tissue model. In this study, A549 was used as a model system for NSCLC and 16HBE epithelial cells were used as a control for "healthy" cells. The two epithelial cell lines formed a well-defined spheroid structure when cultured in a viscose cellulose solution using the hanging drop system. The advantage of using spheroids for studying tumour biology is that spheroids grown from tumour cell lines show growth kinetics similar to those of *in vivo* tumours [121]. The implantation of spheroids in the lung tissue model revealed formation of distinct microtumour environments in the epithelial layer. The microtumours in the tissue model are well separated from the rest of the "healthy" epithelial layer and are likely held together by surface membranes, extracellular matrix and junctional complexes. Implantation of tumour epithelial spheroids and normal epithelial cells revealed that the tumour cells attracted DC to the microtumour area much more efficient than the normal epithelial cells. This may be due to tumour-derived chemokines or adjacent fibroblasts and epithelial cells in the tissue model that secrete chemokines, attracting DC to the tumour area. In preliminary experiments we have been able to detect chemokine production in the lung tissue model with micro-tumours. We are currently investigating the differences between chemokine production in models with tumour spheroids and in models with normal epithelial cell spheroids. We will also isolate the different cell populations from both models to compare activation markers of DC and

investigate chemokine expression in the different cell types from the lung tissue model with and without tumours.

In tumour tissues, DC can engulf necrotic tumour cells, which induces the maturation of DC that have the ability to activate $CD4^+$ and $CD8^+$ T cells [253]. In addition, we observed that DC engulf the tumour cells more readily, which may indicate the presence of molecular signals displayed or secreted by necrotic cells, leading to enhanced engulfment of the adenocarcinoma cells by DC. Furthermore, we have shown that the tissue model can be digested and DC and tissue specific cells in the model can be isolated. This method will enable us to investigate phenotypic markers of DC as well as cytokine and chemokine expression secreted by tumour cells in more detail.

We believe, our newly developed lung tumour model system provides a powerful tool to investigate the mechanisms behind the attraction and modulating of DC functional properties by NSCLC and capture early events in the course of tumour progression in lung tissue. For example, the role of certain chemokines in the tumour tissue model could be examined by gene manipulation to alter chemokine expression in A549 cells or by using blocking reagents against candidate chemokines and chemokine receptors. Alternatively, gene expression can be manipulated in 16HBE cells to perform gain of function experiments. It will also be interesting to follow up on and elucidate the molecular signals that lead to enhanced engulfment of cancer cells by DC in the tumour microenvironment. This may be of importance in the context of DC-uptake-processing and presentation of tumour associated antigens to T cells.

For this part of the thesis work, I have developed a human 3D lung tissue model, which comprises bronchial epithelial cells, fibroblasts and DC. This tissue model is highly reproducible and although quite laborsome, it is a relatively inexpensive model system that provides a novel platform for studies on tissue-specific regulation of human DC functional properties in a physiological microenvironment that resembles the morphological and functional features of the one found in *in vivo* parental tissues. The cells in this model system can further be modified to express fluorescent proteins that enable live cell imaging studies of DC migration in tissue. Assay of human DC behaviour in live tissue is difficult to perform, but this difficulties may be overcome by the use of tissue model systems as described here. This is important because there is evidence demonstrating that DC migration is largely influenced by the tissue origin, degree of maturity and the 3D-structure of the microenvironment [254]. Even though, not every aspect of DC biology can be investigated using 3D tissue models, our model system can provide a powerful tool to explore at least some important mechanisms of DC regulation in tissue, some of which include pathways dictating DC anchoring and migrating behaviour as well as chemokine producing and immune stimulatory capacities. As our lung tissue model recapitulates many key functional and anatomical features of lung tissue and it is well suited for a wide range of studies including those on DC interaction with cancer cells and DC immune function in the tumour microenvironment.

6.2 STROMAL CELL-DERIVED CHEMOKINES SUPPORT HEMATOPOIETIC PROGENITOR CELL DIFFERENTIATION INTO REGULATORY DENDRITIC CELLS

As discussed in the previous sections, chemokines play an important role for the migration and functional properties of terminally differentiated hematopoietic cells in peripheral tissues. In addition, they also play a crucial role in the homing and migration of HSPC in the bone marrow and spleen [216, 230] [231]. Here, I will discuss our results regarding the role of stromal cell-derived chemokines in the regulation of hematopoietic progenitor cell differentiation into regulatory DC and how parasite infection can modulate this process, which may be one of the mechanisms in which the parasite influences the stromal microenvironment and promotes persistent infection.

Stromal cells are increasingly recognized for their role in influencing immune cell differentiation and survival during inflammation and in response to infection [11, 13, 255]. In this study, we found that the bone marrow-derived stromal cell line, MBA-1, attracted HSPC by producing CXCL12 and CCL8. The chemokine CXCL12 is known to have a crucial role in the regulation of HSPC migration, survival and differentiation [98, 256]. Recent studies indicate that CXCL12 cooperates with other cytokines such as CSF-2, thrombopoietin and IFN-γ to enhance the survival capabilities of HSPC, [257, 258]. It has also been shown that HSPC migration in short-term culture is synergistically enhanced by the interaction between CXCL12 and Flt3 ligand and their receptors [259]. This suggests that CXCL12 may contribute to HSPC survival and migration by itself as well as in combination with other cytokines. Synergistic interaction between CXCL12 and other chemokines such as CCL2 has also been investigated. Gouwy *et al.* demonstrated that CXCL12 and CCL2 synergized to enhance monocyte migration [260]. However, synergisms between CXCL12 and other chemokines leading to altered capacity to support HSPC migration and differentiation are not known. Here, we showed for the first time the cooperation of CXCL12 and CCL8 in guiding migration of HSPC that have the ability to differentiate into regulatory DC. It has been suggested that the synergistic effect evoked by chemokines depends on receptor dimerization [261]. Moreover, Sohy *et al.* showed that the receptor CXCR4 and CCR2 formed dimers with each other. CXCR4 is the receptor for CXCL12 and CCR2 is one of the receptors for CCL8. Both receptors have been observed to exist as constitutive homo- and heterodimers. It was also shown that using specific antagonists of one receptor inhibits the binding of chemokines to the other receptor, both in recombinant cell lines and primary leukocytes. In our experiments, using neutralizing antibodies against CXCL12 and CCL8 it was revealed that anti-CXCL12 could inhibit HSPC migration to the levels seen with pertussis toxin, an inhibitor of G-protein couple receptors, while anti-CCL8 could inhibit HSPC migration partially. This suggests that the CXCL12 signaling pathway is required to induce migration of HSPC and CCL8 synergizes together with CXCL12 to enhance HSPC migration. However, the mechanisms of the chemokine receptor signaling that may cooperate in regulating enhanced HSPC migration needs further investigations.

The second major observation in this study is that infection with *L. donovani* altered chemokine expression in MBA-1 cells and splenic stromal cells isolated from mice, leading to increased production of CCL8 and a slight reduction of CXCL12

expression. Although CXCL12 was reduced to some extent, the expression of CXCL12 mRNA was still highly abundant in the MBA-1 cells and the splenic stromal cells of mice infected with *L. donovani*. Our findings indicate that CCL8 is induced upon infection and may contributes to the enhanced recruitment of HSPC to the spleen [102] and the increased number of regulatory DC that has been observed during *L. donovani* infection [12, 262]. In addition, we suggest that the cooperation of CXCL12 and CCL8 in regulating hematopoiesis provides evidence for additional pathways regulating hematopoietic activity that can be targeted by intracellular pathogens. However, the role of CCL8 in the development and maintenance of chronic infection needs further investigations and depends on the development of tools that can block CCL8 *in vivo* or to generate conditional CCL8 knockout mice. Although CXCL12 and CXCR4 knock out mice exist, they die *in utero* and are defective in vascular development, hematopoiesis and cardiogenesis [263]. Another possibility could be to use CCR2 knockout mice, but one disadvantage is that CCR2 binds to several chemokines such as CCL2, CCL7 and CCL13, which may have important functions in responses to pathogens; it was previously shown that CCR2 knockout mice were susceptible to *Mycobacterium tuberculosis* infection which was associated with impaired recruitment of immune cells to the site of infection [176, 264, 265]. Also, it has recently been shown that CCL8 binds to CCR8 rather than to CCR2 in mouse skin [266]. CCR8 knockout mice exist, but they are shown to have aberrant Th2 cell response and impaired recruitment of eosinophils [267].

7 CONCLUSIONS

7.1 THE STUDIES ON THE THREE-DIMENSIONAL LUNG TISSUE MODEL

The studies comprised in this thesis contribute to the knowledge of establishing a physiologically relevant tissue model system for studies on DC functional properties in human lung tissue under next to *in vivo* conditions. This multicellular modelling approach that uses an advanced cell culture technique provides a powerful tool that, in combination with animal models and human clinical data, can increase our understanding of the cellular mechanisms that underlie tissue pathology caused by infectious agents, tumour progression and aberrant inflammatory reactions to harmful reagents. The results demonstrate that our lung tissue model supports the survival of DC as well as DC production of homeostatic chemokines and therefore enables functional studies of human DC in a physiological lung mucosal environment. This 3D tissue model provides a flexible and high throughput experimental platform that allows for interventions of various kinds as well as manipulation of gene expression in tissue-specific cells. It enables us to visualize processes of human DC function and migration with 4D fluorescence live imaging technique in models mimicking real tissue. The finding that DC migratory behaviour in the tissue model is influenced by TLR-ligands and chemokines provides new possibilities to explore in detail the mechanisms of tissue communication that underlie DC anchoring and migrating behavior in the microenvironment. In addition, the quantification of DC migration and distribution in the tissue model can provide a powerful tool to investigate the mechanistic actions of adjuvants or demonstrate effects of noxious compounds as well as testing reagents aimed at blocking DC activation and migration locally.

Our newly established lung tissue model represents a physiologically relevant assay system that can be used for a wide range of studies and it has an exciting potential to reveal pathogenic processes in infectious and inflammatory diseases. In this regard, we aim to make this model accessible for others to show the usefulness of the model in other diseases. Currently, we are collaborating with other groups to provide new insights into the pathogenesis of acute severe Staphylococcal infections, tuberculosis and Hantavirus infection using our lung tissue model. Future research using 3D tissue model systems, in combination with patient samples and clinical data may provide a more complete understanding of the interactions that occur between the host and pathogens, which might lead to potential strategies for preventing and treating infectious diseases and cancers affecting lung tissue.

7.2 THE STUDY ON STROMAL CELL-MEDIATED DEVELOPMENT OF REGULATORY DENDRITIC CELLS

This study in the thesis work contributes to an increased understanding of altered hematopoietic capacity of stromal cells in response to *L. donovani* infection and in which ways an altered stromal cell chemokine production capacity affects HSPC migration and differentiation. Our results emphasize the cooperation between CXCL12 and CCL8 leading to enhanced recruitment of HSPC that have the ability to develop into regulatory DC. Moreover, we found that infection with *L. donovani* induced CCL8 expression in a bone marrow stromal cell line and freshly isolated murine splenic stromal cells. Our finding that CXCL12 and CCL8 cooperate in regulating hematopoiesis provides evidence for additional pathways regulating hematopoietic activity that can be targeted by intracellular pathogens.

The ability of the local tissue niches to shape the functional properties and repertoire of DC provides important mechanisms for tissue regulation of homeostasis as well as responses to inflammation and infection. However, in chronic infections, such as leishmaniasis, tuberculosis and malaria, the observation of altered stromal cell function leading to the development of DC and T cells with regulatory function, may be an important mechanism for preventing immune-mediated pathology that is exploited by the pathogens. By identifying in detail, the mechanisms that contribute to shaping the DC repertoire and functionality, this may allow us to invent new targets therapies to manipulate DC, aiming at resolving chronic infections, modulate overt inflammatory reactions and restore DC functions.

8 ACKNOWLEDGEMENTS

The studies presented in this thesis were conducted at the Center for Infectious Medicine, Department of Medicine, Karolinska Institutet. It has been an exciting and memorable journey and I would like to thank all the people who have contributed to my thesis work.

I would especially like to thank my supervisor, **Mattias Svensson,** for all his support and guidance through the years. I have learned so much from you, both scientifically and personal characteristics. You have influenced and inspired me with your passion for research, enthusiasm, energy and wealth of knowledge. I am very grateful that you always took the time to discuss ideas, experiments and results as well as guide me in the scientific presentations and writings. One of the most important things I have learned from you is that one should always strive to achieve the utmost accomplishment. This characteristic has yielded rewarding results, as evidence by the completion of my thesis writing during these seven weeks that I had to accomplish this task. Thank you for believing in me and for your support during all this time. I also want to thank you for all the wonderful dinners, all the good times at conferences and the journey to York.

I would also like to say "thank you" to the following people who, in one way or another, have helped me reach my goals:

My co-supervisors **Susanna Brighenti** and **Jan Andersson,** for their support, scientific discussions and rewarding collaborations as well as all the great dinners and workshops.

Hans-Gustaf Ljunggren for your great support during these years and for creating an open and inspiring research environment that provided great opportunities for conducting excellent research.

All the co-authors for successful collaborations: **Puran Chen** for your great contribution to all three papers in this thesis; **Julius Juarez and Lidija Bosnjak** for your help and expertise in 3D tissue models; **Mark Coles and Patty Sachamitr** for your generous hospitality and taking care of me and showing me around when I visited York; **Alf Grandien and Kari Högstrand** for your help and expertise in viral transduction; **John Lock** for your help and expertise in live imaging analysis; **Barbro Dahlén and Bo Billing** for the lung tissue material and **Paul Kaye, Hao Liu and Naveed Aziz** for all your help and support with the fourth paper in this thesis.

My former and present collaborators in the project of:

Tuberculosis - **Jagadeesh** for your generous and brilliant contribution to the CCL18 project; **Senait** for a rewarding collaboration on the tuberculosis project; **Sayma** for introducing me to the world of immunohistochemistry; **Joshua and Anders** for your help with real time RT-PCR; **Maria Lerm, Ramana, Jubayer and Maria** for a great collaboration and generous support.

Staphylococcus aureus - **Anna Norrby-Teglund** for a successful collaboration and great scientific discussions; **Srikanth** for being a great collaborator and for your enthusiasm and energy in this project; **Axana** for introducing me to the research field of Staphylococcus aureus and for your friendship and extraordinary advices.

Hantavirus - **Karin Sundström** for your wonderful energy and enthusiasm; **Jonas Klingström, Shawon Gupta and Clas Ahlm** for rewarding collaboration and scientific discussions.

Anquan Liu for your generous help with the viral transduction for the CCL18-TSLP project.

The members of the **Svensson lab: Julius** for introducing me to the research field of 3D tissue models, for all the discussions, ideas and all the good times during these years, **Magda** for your encouragement, friendship and support, **Puran, Sofia and Egle** for all the good times and wonderful friendship.

Aenni Ali for your help with the development of the lung tissue model for live imaging analysis; **Sylvie Le Guyader** for the help with the two photon imaging and Imaris analysis; **Salah** for your help with the photos in paper II and III.

Linda, for your friendship, positive attitude and lovely smile, **Senait, Sayma, Erika and Axana** for the pleasant dinners that I hope we will continue having.

Annette for all the fun times at the DC conferences and for being a great friend.

Malin for your generosity and kindness with the office space, which has helped me a lot during the thesis writing. It was a pleasure to share the office with you!

Anna-Lena Spetz and Karin Loré for your help with the skin and lung fibroblasts.

Lena for all the help with many things in the lab; **Anette** for introducing me to the world of confocal microscopy; **Elisabeth** for the help with the lung tissue material for paper I and **Hernan** for your good company.

Carina, Ann, Berit and Eva for great help with the administration during these years.

Eva Hellström-Lindberg, Christian and Martin for your invaluable support and friendship.

All the former and present members of CIM for making it such a nice place to work at: **Nikolai** for great discussions, ideas, support and friendship; **Pablo** for your pleasant company, **Sandra, Martin, David and Monika** for all the help with the Fortessa; **Oscar, Sanna N, Katharina, Su, Martha, Steph, Sam, Heinrich, Kim, Sofia, Venkat, Pär, Steve, Markus, Robban, Emma, Virginia, Terezia, Renata, Mirko, Anna L, Nicole, Margit, Jenny, Johanna, Vivien, Lisa, Andreas, Eliisa, Adil, Eva, Steffi, Moni, Terry, Vero, Joana, Michal, Monica, Stella, Cattis, Ulrika, Mattias C,**

Cyril, Carlotta, Yenan, Niklas, Frank, Emily, Will, Cornelia, Kerrie, Valentina, Edwin L, Dominic, Helena, Michael H, Jacob M, Jacob T, Kalle, Lilian, Barbro, Johan, Adnane, Antonio, Jessica, Polya, Claudia, Mark, Benedict, Edwin H, Sushil and Peter B.

I would also like to thank my friends at the KI research school, **Karin, Eva, Christine, Jeanette, Sofia, Jingwen, Masako, Annelie and Evgenia** for the wonderful company and support during these years. I hope that I will have more time now to catch up all the activities that I have missed.

I would like to thank all my friends and relatives outside the lab for wonderful support and encouragement during these years.

At last but not least, I would like to thank my family for being the rock of my life: **Mom and Dad** for your love and that you always have been there for me. Thank you for all the help with Minh and that I could stay at your place during my thesis writing; **Nhan,** my wonderful brother who has helped me with the language correction of the text in this thesis; **Quynh and Mai,** my loving sisters for have been so helpful with everything.

Tan, my loving husband, thank you for your patience and understanding, for your wonderful help with the thesis layout and the figures; **Minh,** for being the joy of my life. Heaven is a place on earth with you!

9 REFERENCES

1. Ding L, Morrison SJ: **Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches**. *Nature* 2013, **495**(7440):231-235.

2. Adams GB, Scadden DT: **The hematopoietic stem cell in its place**. *Nat Immunol* 2006, **7**(4):333-337.

3. Hammad H, Lambrecht BN: **Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma**. *Nat Rev Immunol* 2008, **8**(3):193-204.

4. Kitamura H, Cambier S, Somanath S, Barker T, Minagawa S, Markovics J, Goodsell A, Publicover J, Reichardt L, Jablons D *et al*: **Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin αvβ8–mediated activation of TGF-β**. *The Journal of Clinical Investigation* 2011, **121**(7):2863-2875.

5. Iwasaki A: **Mucosal dendritic cells**. *Annu Rev Immunol* 2007, **25**:381-418.

6. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**(6673):245-252.

7. Steinman RM, Hawiger D, Nussenzweig MC: **Tolerogenic dendritic cells**. *Annu Rev Immunol* 2003, **21**:685-711.

8. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K: **Immunobiology of dendritic cells**. *Annu Rev Immunol* 2000, **18**:767-811.

9. Lu N, Wang YH, Wang YH, Arima K, Hanabuchi S, Liu YJ: **TSLP and IL-7 use two different mechanisms to regulate human CD4+ T cell homeostasis**. *J Exp Med* 2009, **206**(10):2111-2119.

10. Trumpp A, Essers M, Wilson A: **Awakening dormant haematopoietic stem cells**. *Nat Rev Immunol* 2010, **10**(3):201-209.

11. Roozendaal R, Mebius RE: **Stromal cell-immune cell interactions**. *Annu Rev Immunol* 2011, **29**:23-43.

12. Svensson M, Maroof A, Ato M, Kaye PM: **Stromal cells direct local differentiation of regulatory dendritic cells**. *Immunity* 2004, **21**(6):805-816.

13. Svensson M, Kaye PM: **Stromal-cell regulation of dendritic-cell differentiation and function**. *Trends Immunol* 2006, **27**(12):580-587.

14. Sponaas AM, Cadman ET, Voisine C, Harrison V, Boonstra A, O'Garra A, Langhorne J: **Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells**. *J Exp Med* 2006, **203**(6):1427-1433.

15. Steinman RM, Cohn ZA: **Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution**. *J Exp Med* 1973, **137**(5):1142-1162.

16. Nobel AKI: **The gatekeepers of the immune system.** *2011 Nobel Prize in Physiology or Medicine: Popular Information* 2011:8 pp.

17. Steinman RM, Hemmi H: **Dendritic cells: translating innate to adaptive immunity**. *Current topics in microbiology and immunology* 2006, **311**:17-58.

18. Kapsenberg ML: **Dendritic-cell control of pathogen-driven T-cell polarization**. *Nat Rev Immunol* 2003, **3**(12):984-993.

19. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SA, Kapsenberg ML, de Jong EC: **Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells**. *Immunity* 2007, **27**(4):660-669.

20. Palucka K, Banchereau J: **Cancer immunotherapy via dendritic cells**. *Nat Rev Cancer* 2012, **12**(4):265-277.

21. Sela U, Olds P, Park A, Schlesinger SJ, Steinman RM: **Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice**. *J Exp Med* 2011, **208**(12):2489-2496.

22. Idoyaga J, Fiorese C, Zbytnuik L, Lubkin A, Miller J, Malissen B, Mucida D, Merad M, Steinman RM: **Specialized role of migratory dendritic cells in peripheral tolerance induction**. *J Clin Invest* 2013, **123**(2):844-854.

23. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG: **A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis**. *Nature* 1997, **389**(6652):737-742.

24. Weiner HL: **Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells**. *Microbes Infect* 2001, **3**(11):947-954.

25. Weiner HL: **Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells**. *Immunol Rev* 2001, **182**:207-214.

26. Klein L, Hinterberger M, Wirnsberger G, Kyewski B: **Antigen presentation in the thymus for positive selection and central tolerance induction**. *Nat Rev Immunol* 2009, **9**(12):833-844.

27. Tokoyoda K, Hauser AE, Nakayama T, Radbruch A: **Organization of immunological memory by bone marrow stroma**. *Nat Rev Immunol* 2010, **10**(3):193-200.

28. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA: **Specific Migratory Dendritic Cells Rapidly Transport Antigen from the Airways to the Thoracic Lymph Nodes**. *The Journal of experimental medicine* 2001, **193**(1):51-60.

29. Huang F-P, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG: **A Discrete Subpopulation of Dendritic Cells Transports Apoptotic Intestinal Epithelial Cells to T Cell Areas of Mesenteric Lymph Nodes**. *The Journal of experimental medicine* 2000, **191**(3):435-444.

30. Merad M, Sathe P, Helft J, Miller J, Mortha A: **The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting**. *Annu Rev Immunol* 2013, **31**:563-604.

31. Shortman K, Heath WR: **The CD8+ dendritic cell subset**. *Immunol Rev* 2010, **234**(1):18-31.

32. Poulin LF, Henri S, de Bovis B, Devilard E, Kissenpfennig A, Malissen B: **The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells**. *The Journal of experimental medicine* 2007, **204**(13):3119-3131.

33. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, Moon C, Albring JC, Ise W, Michael DG *et al*: **Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells**. *J Exp Med* 2010, **207**(4):823-836.

34. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG *et al*: **Differential antigen processing by dendritic cell subsets in vivo**. *Science* 2007, **315**(5808):107-111.

35. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, Allan RS, Wojtasiak M, Shortman K, Carbone FR *et al*: **Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells**. *Nat Immunol* 2009, **10**(5):488-495.

36. Merad M, Ginhoux F, Collin M: **Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells**. *Nat Rev Immunol* 2008, **8**(12):935-947.

37. Seneschal J, Clark RA, Gehad A, Baecher-Allan CM, Kupper TS: **Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells**. *Immunity* 2012, **36**(5):873-884.

38. Igyarto BZ, Haley K, Ortner D, Bobr A, Gerami-Nejad M, Edelson BT, Zurawski SM, Malissen B, Zurawski G, Berman J *et al*: **Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses**. *Immunity* 2011, **35**(2):260-272.

39. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V: **Plasmacytoid dendritic cells: recent progress and open questions**. *Annu Rev Immunol* 2011, **29**:163-183.

40. Serbina NV, Jia T, Hohl TM, Pamer EG: **Monocyte-Mediated Defense Against Microbial Pathogens**. *Annual Review of Immunology* 2008, **26**(1):421-452.

41. Takizawa H, Boettcher S, Manz MG: **Demand-adapted regulation of early hematopoiesis in infection and inflammation**. *Blood* 2012, **119**(13):2991-3002.

42. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Takatsu K, Kincade PW: **Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment**. *Immunity* 2006, **24**(6):801-812.

43. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, Jeffrey KL, Anthony RM, Kluger C, Nchinda G *et al*: **Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas**. *Cell* 2010, **143**(3):416-429.

44. Hespel C, Moser M: **Role of inflammatory dendritic cells in innate and adaptive immunity**. *European Journal of Immunology* 2012, **42**(10):2535-2543.

45. O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM: **Human blood contains two subsets of dendritic cells, one**

immunologically mature and the other immature. *Immunology* 1994, **82**(3):487-493.

46. Collin M, Bigley V, Haniffa M, Hambleton S: **Human dendritic cell deficiency: the missing ID?** *Nat Rev Immunol* 2011, **11**(9):575-583.

47. Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, Buck DW, Schmitz J: **BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood**. *J Immunol* 2000, **165**(11):6037-6046.

48. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN: **Characterization of human blood dendritic cell subsets**. *Blood* 2002, **100**(13):4512-4520.

49. Nestle FO, Zheng XG, Thompson CB, Turka LA, Nickoloff BJ: **Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets**. *J Immunol* 1993, **151**(11):6535-6545.

50. Lenz A, Heine M, Schuler G, Romani N: **Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization**. *J Clin Invest* 1993, **92**(6):2587-2596.

51. Zaba LC, Fuentes-Duculan J, Steinman RM, Krueger JG, Lowes MA: **Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+ macrophages**. *J Clin Invest* 2007, **117**(9):2517-2525.

52. Poulin LF, Reyal Y, Uronen-Hansson H, Schraml BU, Sancho D, Murphy KM, Hakansson UK, Moita LF, Agace WW, Bonnet D *et al*: **DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues**. *Blood* 2012, **119**(25):6052-6062.

53. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, Keller AM, Joffre O, Zelenay S, Nye E *et al*: **Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells**. *J Exp Med* 2010, **207**(6):1261-1271.

54. Lindstedt M, Lundberg K, Borrebaeck CA: **Gene family clustering identifies functionally associated subsets of human in vivo blood and tonsillar dendritic cells**. *J Immunol* 2005, **175**(8):4839-4846.

55. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, Xu H, Vivier E, Sellars M, Pierre P *et al*: **Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling**. *Genome biology* 2008, **9**(1):R17.

56. Valladeau J, Duvert-Frances V, Pin JJ, Dezutter-Dambuyant C, Vincent C, Massacrier C, Vincent J, Yoneda K, Banchereau J, Caux C *et al*: **The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface**. *Eur J Immunol* 1999, **29**(9):2695-2704.

57. Fithian E, Kung P, Goldstein G, Rubenfeld M, Fenoglio C, Edelson R: **Reactivity of Langerhans cells with hybridoma antibody**. *Proc Natl Acad Sci U S A* 1981, **78**(4):2541-2544.

58. Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, Dalod M, Soumelis V, Amigorena S: **Human inflammatory dendritic cells induce Th17 cell differentiation**. *Immunity* 2013, **38**(2):336-348.

59. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH: **Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells**. *J Exp Med* 2000, **192**(9):1213-1222.

60. Lutz MB, Kukutsch NA, Menges M, Rossner S, Schuler G: **Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy in vitro**. *Eur J Immunol* 2000, **30**(4):1048-1052.

61. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N: **Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells**. *J Exp Med* 2001, **193**(2):233-238.

62. Johansson-Lindbom B, Svensson M, Pabst O, Palmqvist C, Marquez G, Förster R, Agace WW: **Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing**. *The Journal of experimental medicine* 2005, **202**(8):1063-1073.

63. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo CV, Hall J, Sun C-M, Belkaid Y, Powrie F: **A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β– and retinoic acid–dependent mechanism**. *The Journal of experimental medicine* 2007, **204**(8):1757-1764.

64. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg P-L, Davidsson T, Powrie F, Johansson-Lindbom B *et al*: **Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans**. *The Journal of experimental medicine* 2008, **205**(9):2139-2149.

65. Matta BM, Raimondi G, Rosborough BR, Sumpter TL, Thomson AW: **IL-27 production and STAT3-dependent upregulation of B7-H1 mediate immune regulatory functions of liver plasmacytoid dendritic cells**. *J Immunol* 2012, **188**(11):5227-5237.

66. Akbari O, DeKruyff RH, Umetsu DT: **Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen**. *Nat Immunol* 2001, **2**(8):725-731.

67. Bakdash G, Sittig SP, van Dijk T, Figdor CG, de Vries IJ: **The nature of activatory and tolerogenic dendritic cell-derived signal II**. *Frontiers in immunology* 2013, **4**:53.

68. Kautz-Neu K, Noordegraaf M, Dinges S, Bennett CL, John D, Clausen BE, von Stebut E: **Langerhans cells are negative regulators of the anti-Leishmania response**. *J Exp Med* 2011, **208**(5):885-891.

69. Nguyen Hoang AT, Liu H, Juarez J, Aziz N, Kaye PM, Svensson M: **Stromal cell-derived CXCL12 and CCL8 cooperate to support increased development of regulatory dendritic cells following Leishmania infection**. *J Immunol* 2010, **185**(4):2360-2371.

70. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA: **The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults**. *Cell* 1996, **86**(6):973-983.

71. Poltorak A, He X, Smirnova I, Liu M-Y, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C *et al*: **Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene**. *Science* 1998, **282**(5396):2085-2088.

72. Iwasaki A, Medzhitov R: **Toll-like receptor control of the adaptive immune responses**. *Nat Immunol* 2004, **5**(10):987-995.

73. Takeuchi O, Akira S: **Pattern Recognition Receptors and Inflammation**. *Cell* 2010, **140**(6):805-820.

74. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: **Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8**. *Science* 2004, **303**(5663):1526-1529.

75. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C: **Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA**. *Science* 2004, **303**(5663):1529-1531.

76. Akira S: **TLR signaling**. *Current topics in microbiology and immunology* 2006, **311**:1-16.

77. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG: **TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection**. *Immunity* 2003, **19**(1):59-70.

78. De Trez C, Magez S, Akira S, Ryffel B, Carlier Y, Muraille E: **iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice**. *PLoS pathogens* 2009, **5**(6):e1000494.

79. Copin R, De Baetselier P, Carlier Y, Letesson JJ, Muraille E: **MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during Brucella melitensis infection**. *J Immunol* 2007, **178**(8):5182-5191.

80. Sallusto F, Lanzavecchia A: **Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha**. *J Exp Med* 1994, **179**(4):1109-1118.

81. Greter M, Helft J, Chow A, Hashimoto D, Mortha A, Agudo-Cantero J, Bogunovic M, Gautier EL, Miller J, Leboeuf M *et al*: **GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells**. *Immunity* 2012, **36**(6):1031-1046.

82. Wrenshall L: **Role of the microenvironment in immune responses to transplantation**. *Springer seminars in immunopathology* 2003, **25**(2):199-213.

83. Freshney RI: **Introduction**. In: *Culture of Epithelial Cells.* edn.: John Wiley & Sons, Inc.; 2002: 1-30.

84. Baker BM, Chen CS: **Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues**. *J Cell Sci* 2012, **125**(Pt 13):3015-3024.

85. Dennis JE, Charbord P: **Origin and Differentiation of Human and Murine Stroma**. *STEM CELLS* 2002, **20**(3):205-214.

86. Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO: **Diversity, topographic differentiation, and positional memory in human fibroblasts**. *Proc Natl Acad Sci U S A* 2002, **99**(20):12877-12882.

87. Black AF, Bouez C, Perrier E, Schlotmann K, Chapuis F, Damour O: **Optimization and characterization of an engineered human skin equivalent**. *Tissue Eng* 2005, **11**(5-6):723-733.

88. Choe MM, Sporn PH, Swartz MA: **Extracellular matrix remodeling by dynamic strain in a three-dimensional tissue-engineered human airway wall model**. *Am J Respir Cell Mol Biol* 2006, **35**(3):306-313.

89. Fusenig N: **Epithelial-mesenchymal interactions regulate keratinocyte growth and differentiation in vitro. In Leigh IM, Lane EB WattFM, (eds.) ''The Keratinocyte Handbook.''**. *Cambridge: Cambridge University Press* 1994:71-94.

90. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: **Myofibroblasts and mechano-regulation of connective tissue remodelling**. *Nat Rev Mol Cell Biol* 2002, **3**(5):349-363.

91. Weiss L, Geduldig U: **Barrier cells: stromal regulation of hematopoiesis and blood cell release in normal and stressed murine bone marrow**. *Blood* 1991, **78**(4):975-990.

92. Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, Gherardi RK: **Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth**. *The Journal of cell biology* 2003, **163**(5):1133-1143.

93. Hasthorpe S, Bogdanovski M, Rogerson J, Radley JM: **Characterization of endothelial cells in murine long-term marrow culture. Implication for hemopoietic regulation**. *Experimental hematology* 1992, **20**(4):476-481.

94. Morris L, Crocker PR, Fraser I, Hill M, Gordon S: **Expression of a divalent cation-dependent erythroblast adhesion receptor by stromal macrophages from murine bone marrow**. *J Cell Sci* 1991, **99 (Pt 1)**:141-147.

95. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV: **Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo**. *Transplantation* 1974, **17**(4):331-340.

96. Wright DE, Bowman EP, Wagers AJ, Butcher EC, Weissman IL: **Hematopoietic Stem Cells Are Uniquely Selective in Their Migratory Response to Chemokines**. *The Journal of experimental medicine* 2002, **195**(9):1145-1154.

97. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL: **Physiological Migration of Hematopoietic Stem and Progenitor Cells**. *Science* 2001, **294**(5548):1933-1936.

98. Sugiyama T, Kohara H, Noda M, Nagasawa T: **Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches**. *Immunity* 2006, **25**(6):977-988.

99. Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB *et al*: **Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues**. *Cell* 2007, **131**(5):994-1008.

100. Abkowitz JL, Robinson AE, Kale S, Long MW, Chen J: **Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure**. *Blood* 2003, **102**(4):1249-1253.

101. Bensinger W, DiPersio JF, McCarty JM: **Improving stem cell mobilization strategies: future directions**. *Bone Marrow Transplant* 2009, **43**(3):181-195.

102. Cotterell SE, Engwerda CR, Kaye PM: **Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with Leishmania donovani**. *Infect Immun* 2000, **68**(4):1840-1848.

103. Schon-Hegrad MA, Oliver J, McMenamin PG, Holt PG: **Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways**. *The Journal of experimental medicine* 1991, **173**(6):1345-1356.

104. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN: **House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells**. *Nat Med* 2009, **15**(4):410-416.

105. Condon TV, Sawyer RT, Fenton MJ, Riches DWH: **Lung dendritic cells at the innate-adaptive immune interface**. *Journal of leukocyte biology* 2011, **90**(5):883-895.

106. Saito T, Yamamoto T, Kazawa T, Gejyo H, Naito M: **Expression of toll-like receptor 2 and 4 in lipopolysaccharide-induced lung injury in mouse**. *Cell and tissue research* 2005, **321**(1):75-88.

107. Berndt A, Derksen FJ, Venta PJ, Ewart S, Yuzbasiyan-Gurkan V, Robinson NE: **Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction**. *Am J Physiol Lung Cell Mol Physiol* 2007, **292**(4):L936-943.

108. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP: **Activation of airway epithelial cells by toll-like receptor agonists**. *Am J Respir Cell Mol Biol* 2004, **31**(3):358-364.

109. Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP, Xing Z, Jordana M: **GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice**. *J Clin Invest* 1998, **102**(9):1704-1714.

110. Ziegler SF, Liu YJ: **Thymic stromal lymphopoietin in normal and pathogenic T cell development and function**. *Nat Immunol* 2006, **7**(7):709-714.

111. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A *et al*: **Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP**. *Nat Immunol* 2002, **3**(7):673-680.

112. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, Qin FX, Yao Z, Cao W, Liu YJ: **TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand**. *J Exp Med* 2005, **202**(9):1213-1223.

113. Hanahan D, Weinberg RA: **The Hallmarks of Cancer**. *Cell* 2000, **100**(1):57-70.

114. Pardoll D: **Does the immune system see tumors as foreign or self?** *Annu Rev Immunol* 2003, **21**:807-839.

115. Blattman JN, Greenberg PD: **Cancer immunotherapy: a treatment for the masses**. *Science* 2004, **305**(5681):200-205.

116. Gabrilovich D: **Mechanisms and functional significance of tumour-induced dendritic-cell defects**. *Nat Rev Immunol* 2004, **4**(12):941-952.

117. Zou W: **Immunosuppressive networks in the tumour environment and their therapeutic relevance**. *Nat Rev Cancer* 2005, **5**(4):263-274.

118. Balkwill F, Mantovani A: **Inflammation and cancer: back to Virchow?** *Lancet* 2001, **357**(9255):539-545.

119. Lin EY, Pollard JW: **Role of infiltrated leucocytes in tumour growth and spread**. *Br J Cancer* 2004, **90**(11):2053-2058.

120. Nathan C: **Points of control in inflammation**. *Nature* 2002, **420**(6917):846-852.

121. Sutherland RM: **Cell and environment interactions in tumor microregions: the multicell spheroid model**. *Science* 1988, **240**(4849):177-184.

122. Shurin GV, Ouellette CE, Shurin MR: **Regulatory dendritic cells in the tumor immunoenvironment**. *Cancer immunology, immunotherapy : CII* 2012, **61**(2):223-230.

123. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, Peri F, Wilson SW, Ruhrberg C: **Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction**. *Blood* 2010, **116**(5):829-840.

124. Pflicke H, Sixt M: **Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels**. *J Exp Med* 2009, **206**(13):2925-2935.

125. Birkness KA, Deslauriers M, Bartlett JH, White EH, King CH, Quinn FD: **An in vitro tissue culture bilayer model to examine early events in Mycobacterium tuberculosis infection**. *Infect Immun* 1999, **67**(2):653-658.

126. Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, Varga G, Roth J, Kahl BC, Proctor RA, Peters G: **Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils**. *PLoS pathogens* 2010, **6**(1):e1000715.

127. Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, Lupu R, Bissell MJ: **Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology**. *Proc Natl Acad Sci U S A* 1998, **95**(25):14821-14826.

128. Cukierman E, Pankov R, Stevens DR, Yamada KM: **Taking Cell-Matrix Adhesions to the Third Dimension**. *Science* 2001, **294**(5547):1708-1712.

129. Lee GY, Kenny PA, Lee EH, Bissell MJ: **Three-dimensional culture models of normal and malignant breast epithelial cells**. *Nature methods* 2007, **4**(4):359-365.

130. Griffith LG, Swartz MA: **Capturing complex 3D tissue physiology in vitro**. *Nat Rev Mol Cell Biol* 2006, **7**(3):211-224.

131. Yamada KM, Cukierman E: **Modeling tissue morphogenesis and cancer in 3D**. *Cell* 2007, **130**(4):601-610.

132. Barrila J, Radtke AL, Crabbe A, Sarker SF, Herbst-Kralovetz MM, Ott CM, Nickerson CA: **Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions**. *Nat Rev Microbiol* 2010, **8**(11):791-801.

133. Badylak SF: **The extracellular matrix as a scaffold for tissue reconstruction**. *Cell & developmental biology* 2002, **13**:pp. 377–383.

134. Badylak SF: **Modification of natural polymers: Collagen. In ''Methods of Tissue Engineering'' (A. Atala and R. P. Lanza, Eds.).** *Academic Press, San Diego, CA* 2002:pp. 505–514.

135. Rosner BI, Hang T, Tranquillo RT: **Schwann cell behavior in three-dimensional collagen gels: evidence for differential mechano-transduction and the influence of TGF-beta 1 in morphological polarization and differentiation**. *Experimental neurology* 2005, **195**(1):81-91.

136. Hurley BP, McCormick BA: **Translating tissue culture results into animal models: the case of Salmonella typhimurium**. *Trends in microbiology* 2003, **11**(12):562-569.

137. Dvorak A, Tilley AE, Shaykhiev R, Wang R, Crystal RG: **Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome?** *Am J Respir Cell Mol Biol* 2011, **44**(4):465-473.

138. Gangatirkar P, Paquet-Fifield S, Li A, Rossi R, Kaur P: **Establishment of 3D organotypic cultures using human neonatal epidermal cells**. *Nat Protocols* 2007, **2**(1):178-186.

139. Dongari-Bagtzoglou A, Kashleva H: **Development of a highly reproducible three-dimensional organotypic model of the oral mucosa**. *Nat Protocols* 2006, **1**(4):2012-2018.

140. Choe MM, Tomei AA, Swartz MA: **Physiological 3D tissue model of the airway wall and mucosa**. *Nat Protocols* 2006, **1**(1):357-362.

141. Dezutter-Dambuyant C, Black A, Bechetoille N, Bouez C, Marechal S, Auxenfans C, Cenizo V, Pascal P, Perrier E, Damour O: **Evolutive skin reconstructions: from the dermal collagen-glycosaminoglycan-chitosane substrate to an immunocompetent reconstructed skin**. *Bio-medical materials and engineering* 2006, **16**(4 Suppl):S85-94.

142. Bechetoille N, Dezutter-Dambuyant C, Damour O, Andre V, Orly I, Perrier E: **Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells**. *Tissue Eng* 2007, **13**(11):2667-2679.

143. Bachmann MF, Kopf M, Marsland BJ: **Chemokines: more than just road signs**. *Nat Rev Immunol* 2006, **6**(2):159-164.

144. Mueller SN, Hosiawa-Meagher KA, Konieczny BT, Sullivan BM, Bachmann MF, Locksley RM, Ahmed R, Matloubian M: **Regulation of Homeostatic Chemokine Expression and Cell Trafficking During Immune Responses**. *Science* 2007, **317**(5838):670-674.

145. Qin S, Fallert Junecko BA, Trichel AM, Tarwater PM, Murphey-Corb MA, Kirschner DE, Reinhart TA: **Simian immunodeficiency virus infection alters**

chemokine networks in lung tissues of cynomolgus macaques: association with Pneumocystis carinii infection. *Am J Pathol* 2010, **177**(3):1274-1285.

146. Reinhart TA: **Chemokine induction by HIV-1: recruitment to the cause**. *Trends Immunol* 2003, **24**(7):351-353.

147. Ying S, Robinson DS, Meng Q, Rottman J, Kennedy R, Ringler DJ, Mackay CR, Daugherty BL, Springer MS, Durham SR *et al*: **Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells**. *Eur J Immunol* 1997, **27**(12):3507-3516.

148. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR: **The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions**. *J Clin Invest* 1998, **101**(4):746-754.

149. Balashov KE, Rottman JB, Weiner HL, Hancock WW: **CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions**. *Proc Natl Acad Sci U S A* 1999, **96**(12):6873-6878.

150. Zlotnik A, Yoshie O: **Chemokines: A new classification system and their role in immunity**. *Immunity* 2000, **12**(2):121-127.

151. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA: **International union of pharmacology. XXII. Nomenclature for chemokine receptors**. *Pharmacological Reviews* 2000, **52**(1):145-176.

152. Zlotnik A, Yoshie O, Nomiyama H: **The chemokine and chemokine receptor superfamilies and their molecular evolution**. *Genome biology* 2006, **7**(12):243.

153. Broxmeyer HE: **Regulation of hematopoiesis by chemokine family members**. *International journal of hematology* 2001, **74**(1):9-17.

154. Szekanecz Z, Koch AE: **Chemokines and angiogenesis**. *Current opinion in rheumatology* 2001, **13**(3):202-208.

155. Mackay CR: **Chemokines: immunology's high impact factors**. *Nat Immunol* 2001, **2**(2):95-101.

156. Sallusto F, Baggiolini M: **Chemokines and leukocyte traffic**. *Nat Immunol* 2008, **9**(9):949-952.

157. Sallusto F, Mackay CR, Lanzavecchia A: **The role of chemokine receptors in primary, effector, and memory immune responses**. *Annu Rev Immunol* 2000, **18**:593-620.

158. Comerford I, McColl SR: **Mini-review series: focus on chemokines**. *Immunol Cell Biol* 2011, **89**(2):183-184.

159. Muller G, Reiterer P, Hopken UE, Golfier S, Lipp M: **Role of homeostatic chemokine and sphingosine-1-phosphate receptors in the organization of lymphoid tissue**. *Ann N Y Acad Sci* 2003, **987**:107-116.

160. Zlotnik A, Burkhardt AM, Homey B: **Homeostatic chemokine receptors and organ-specific metastasis**. *Nat Rev Immunol* 2011, **11**(9):597-606.

161. Hoshino A, Ueha S, Hanada S, Imai T, Ito M, Yamamoto K, Matsushima K, Yamaguchi A, Iimura T: **Roles of chemokine receptor CX3CR1 in maintaining murine bone homeostasis through the regulation of both osteoblasts and osteoclasts**. *J Cell Sci* 2013, **126**(Pt 4):1032-1045.

162. Raman D, Sobolik-Delmaire T, Richmond A: **Chemokines in health and disease**. *Experimental cell research* 2011, **317**(5):575-589.

163. Cardona SM, Garcia JA, Cardona AE: **The fine balance of chemokines during disease: trafficking, inflammation, and homeostasis**. *Methods Mol Biol* 2013, **1013**:1-16.

164. Ransohoff RM: **Chemokines and Chemokine Receptors: Standing at the Crossroads of Immunobiology and Neurobiology**. *Immunity* 2009, **31**(5):711-721.

165. Charo IF, Ransohoff RM: **The many roles of chemokines and chemokine receptors in inflammation**. *The New England journal of medicine* 2006, **354**(6):610-621.

166. Lipp M, Muller G: **Shaping up adaptive immunity: the impact of CCR7 and CXCR5 on lymphocyte trafficking**. *Verhandlungen der Deutschen Gesellschaft fur Pathologie* 2003, **87**:90-101.

167. Koelink PJ, Overbeek SA, Braber S, de Kruijf P, Folkerts G, Smit MJ, Kraneveld AD: **Targeting chemokine receptors in chronic inflammatory diseases: an extensive review**. *Pharmacology & therapeutics* 2012, **133**(1):1-18.

168. Hansell CA, Hurson CE, Nibbs RJ: **DARC and D6: silent partners in chemokine regulation?** *Immunol Cell Biol* 2011, **89**(2):197-206.

169. McColl SR: **Chemokines and dendritic cells: A crucial alliance**. *Immunol Cell Biol* 2002, **80**(5):489-496.

170. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, Qin S, Lanzavecchia A: **Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation**. *European Journal of Immunology* 1998, **28**(9):2760-2769.

171. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, Lipp M: **CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs**. *Cell* 1999, **99**(1):23-33.

172. Kellermann SA, Hudak S, Oldham ER, Liu YJ, McEvoy LM: **The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3β are potent chemoattractants for in vitro- and in vivo-derived dendritic cells**. *Journal of Immunology* 1999, **162**(7):3859-3864.

173. Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, Briere F, Zlotnik A, Lebecque S, Caux C: **Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites**. *J Exp Med* 1998, **188**(2):373-386.

174. Vecchi A, Massimiliano L, Ramponi S, Luini W, Bernasconi S, Bonecchi R, Allavena P, Parmentier M, Mantovani A, Sozzani S: **Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells**. *J Leukoc Biol* 1999, **66**(3):489-494.

175. Stumbles PA, Strickland DH, Pimm CL, Proksch SF, Marsh AM, McWilliam AS, Bosco A, Tobagus I, Thomas JA, Napoli S *et al*: **Regulation of dendritic cell recruitment into resting and inflamed airway epithelium: use of alternative chemokine receptors as a function of inducing stimulus**. *J Immunol* 2001, **167**(1):228-234.

176. Kurihara T, Warr G, Loy J, Bravo R: **Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor**. *J Exp Med* 1997, **186**(10):1757-1762.

177. Robays LJ, Maes T, Lebecque S, Lira SA, Kuziel WA, Brusselle GG, Joos GF, Vermaelen KV: **Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation**. *J Immunol* 2007, **178**(8):5305-5311.

178. Zou W, Machelon V, Coulomb-L'Hermin A, Borvak J, Nome F, Isaeva T, Wei S, Krzysiek R, Durand-Gasselin I, Gordon A *et al*: **Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells**. *Nat Med* 2001, **7**(12):1339-1346.

179. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A: **Distinct patterns and kinetics of chemokine production regulate dendritic cell function**. *Eur J Immunol* 1999, **29**(5):1617-1625.

180. Foti M, Granucci F, Aggujaro D, Liboi E, Luini W, Minardi S, Mantovani A, Sozzani S, Ricciardi-Castagnoli P: **Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site**. *Int Immunol* 1999, **11**(6):979-986.

181. Padovan E, Spagnoli GC, Ferrantini M, Heberer M: **IFN-alpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8+ effector T cells**. *J Leukoc Biol* 2002, **71**(4):669-676.

182. Imai T, Yoshida T, Baba M, Nishimura M, Kakizaki M, Yoshie O: **Molecular Cloning of a Novel T Cell-directed CC Chemokine Expressed in Thymus by Signal Sequence Trap Using Epstein-Barr Virus Vector**. *Journal of Biological Chemistry* 1996, **271**(35):21514-21521.

183. Adema GJ, Hartgers F, Verstraten R, de Vries E, Marland G, Menon S, Foster J, Xu Y, Nooyen P, McClanahan T *et al*: **A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells**. *Nature* 1997, **387**(6634):713-717.

184. Hieshima K, Imai T, Baba M, Shoudai K, Ishizuka K, Nakagawa T, Tsuruta J, Takeya M, Sakaki Y, Takatsuki K *et al*: **A novel human CC chemokine PARC that is most homologous to macrophage-inflammatory protein-1 alpha/LD78 alpha and chemotactic for T lymphocytes, but not for monocytes**. *The Journal of Immunology* 1997, **159**(3):1140-1149.

185. Godiska R, Chantry D, Raport CJ, Sozzani S, Allavena P, Leviten D, Mantovani A, Gray PW: **Human Macrophage–derived Chemokine (MDC), a Novel Chemoattractant for Monocytes, Monocyte-derived Dendritic Cells, and Natural Killer Cells**. *The Journal of experimental medicine* 1997, **185**(9):1595-1604.

186. Vicari AP, Figueroa DJ, Hedrick JA, Foster JS, Singh KP, Menon S, Copeland NG, Gilbert DJ, Jenkins NA, Bacon KB *et al*: **TECK: A novel CC chemokine**

specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity* 1997, **7**(2):291-301.

187. Ngo VN, Lucy Tang H, Cyster JG: **Epstein-Barr Virus–induced Molecule 1 Ligand Chemokine Is Expressed by Dendritic Cells in Lymphoid Tissues and Strongly Attracts Naive T Cells and Activated B Cells**. *The Journal of experimental medicine* 1998, **188**(1):181-191.

188. Vulcano M, Struyf S, Scapini P, Cassatella M, Bernasconi S, Bonecchi R, Calleri A, Penna G, Adorini L, Luini W *et al*: **Unique regulation of CCL18 production by maturing dendritic cells**. *J Immunol* 2003, **170**(7):3843-3849.

189. Nadaï Pd, Charbonnier A-S, Chenivesse C, Sénéchal S, Fournier C, Gilet J, Vorng H, Chang Y, Gosset P, Wallaert Bt *et al*: **Involvement of CCL18 in Allergic Asthma**. *The Journal of Immunology* 2006, **176**(10):6286-6293.

190. Günther C, Bello-Fernandez C, Kopp T, Kund J, Carballido-Perrig N, Hinteregger S, Fassl S, Schwärzler C, Lametschwandtner G, Stingl G *et al*: **CCL18 Is Expressed in Atopic Dermatitis and Mediates Skin Homing of Human Memory T Cells**. *The Journal of Immunology* 2005, **174**(3):1723-1728.

191. Kodelja V, Müller C, Politz O, Hakij N, Orfanos CE, Goerdt S: **Alternative Macrophage Activation-Associated CC-Chemokine-1, a Novel Structural Homologue of Macrophage Inflammatory Protein-1α with a Th2-Associated Expression Pattern**. *The Journal of Immunology* 1998, **160**(3):1411-1418.

192. Pivarcsi A, Gombert M, Dieu-Nosjean MC, Lauerma A, Kubitza R, Meller S, Rieker J, Muller A, Da Cunha L, Haahtela A *et al*: **CC chemokine ligand 18, an atopic dermatitis-associated and dendritic cell-derived chemokine, is regulated by staphylococcal products and allergen exposure**. *J Immunol* 2004, **173**(9):5810-5817.

193. Radstake TR, van der Voort R, ten Brummelhuis M, de Waal Malefijt M, Looman M, Figdor CG, van den Berg WB, Barrera P, Adema GJ: **Increased expression of CCL18, CCL19, and CCL17 by dendritic cells from patients with rheumatoid arthritis, and regulation by Fc gamma receptors**. *Annals of the rheumatic diseases* 2005, **64**(3):359-367.

194. Chang Y, de Nadai P, Azzaoui I, Morales O, Delhem N, Vorng H, Tomavo S, Ait Yahia S, Zhang G, Wallaert B *et al*: **The chemokine CCL18 generates adaptive regulatory T cells from memory CD4+ T cells of healthy but not allergic subjects**. *The FASEB Journal* 2010, **24**(12):5063-5072.

195. Azzaoui I, Yahia SA, Chang Y, Vorng H, Morales O, Fan Y, Delhem N, Ple C, Tonnel AB, Wallaert B *et al*: **CCL18 differentiates dendritic cells in tolerogenic cells able to prime regulatory T cells in healthy subjects**. *Blood* 2011, **118**(13):3549-3558.

196. Tasaki Y, Fukuda S, Iio M, Miura R, Imai T, Sugano S, Yoshie O, Hughes AL, Nomiyama H: **Chemokine PARC Gene (SCYA18) Generated by Fusion of Two MIP-1α/LD78α-like Genes**. *Genomics* 1999, **55**(3):353-357.

197. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O: **Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine**. *International Immunology* 1999, **11**(1):81-88.

198. Yogo Y, Fujishima S, Inoue T, Saito F, Shiomi T, Yamaguchi K, Ishizaka A: **Macrophage derived chemokine (CCL22), thymus and activation-regulated chemokine (CCL17), and CCR4 in idiopathic pulmonary fibrosis**. *Respir Res* 2009, **10**:80.

199. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H: **Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation**. *Allergy* 2009, **64**(7):995-1002.

200. GeurtsvanKessel CH, Lambrecht BN: **Division of labor between dendritic cell subsets of the lung**. *Mucosal Immunol* 2008, **1**(6):442-450.

201. Jacobs JP, Jones CM, Baille JP: **Characteristics of a human diploid cell designated MRC-5**. *Nature* 1970, **227**(5254):168-170.

202. Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC: **CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells**. *Am J Respir Cell Mol Biol* 1994, **10**(1):38-47.

203. Palucka KA, Taquet N, Sanchez-Chapuis F, Gluckman JC: **Dendritic cells as the terminal stage of monocyte differentiation**. *J Immunol* 1998, **160**(9):4587-4595.

204. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH: **Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking**. *J Immunol* 2007, **179**(11):7577-7584.

205. Coons AH, Creech HJ, Jones RN: **Immunological Properties of an Antibody Containing a Fluorescent Group**. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY)* 1941, **47**(2):200-202.

206. Ramos-Vara JA: **Technical aspects of immunohistochemistry**. *Veterinary pathology* 2005, **42**(4):405-426.

207. Bjork L, Fehniger TE, Andersson U, Andersson J: **Computerized assessment of production of multiple human cytokines at the single-cell level using image analysis**. *J Leukoc Biol* 1996, **59**(2):287-295.

208. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW: **Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods**. *Analytical biochemistry* 2000, **285**(2):194-204.

209. Deepak S, Kottapalli K, Rakwal R, Oros G, Rangappa K, Iwahashi H, Masuo Y, Agrawal G: **Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes**. *Current genomics* 2007, **8**(4):234-251.

210. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF: **High-throughput real-time quantitative reverse transcription PCR**. *Current protocols in molecular biology / edited by Frederick M Ausubel [et al]* 2006, **Chapter 15**:Unit 15 18.

211. Engvall E, Perlmann P: **Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G**. *Immunochemistry* 1971, **8**(9):871-874.

212. Van Weemen BK, Schuurs AH: **Immunoassay using antigen-enzyme conjugates**. *FEBS Lett* 1971, **15**(3):232-236.

213. Gucker FT, Jr., O'Konski CT: **Electronic methods of counting aerosol particles**. *Chemical reviews* 1949, **44**(2):373-388.

214. Hulett HR, Bonner WA, Barrett J, Herzenberg LA: **Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence**. *Science* 1969, **166**(3906):747-749.

215. Frigault MM, Lacoste J, Swift JL, Brown CM: **Live-cell microscopy - tips and tools**. *J Cell Sci* 2009, **122**(Pt 6):753-767.

216. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL: **Physiological migration of hematopoietic stem and progenitor cells**. *Science* 2001, **294**(5548):1933-1936.

217. Wright DE, Bowman EP, Wagers AJ, Butcher EC, Weissman IL: **Hematopoietic stem cells are uniquely selective in their migratory response to chemokines**. *J Exp Med* 2002, **195**(9):1145-1154.

218. Zipori D, Duksin D, Tamir M, Argaman A, Toledo J, Malik Z: **Cultured mouse marrow stromal cell lines. II. Distinct subtypes differing in morphology, collagen types, myelopoietic factors, and leukemic cell growth modulating activities**. *Journal of Cellular Physiology* 1985, **122**(1):81-90.

219. Oppenheim JJ, Whang J, Frei E, 3rd: **The effect of skin homograft rejection on recipient and donor mixed leukocyte cultures**. *J Exp Med* 1965, **122**(4):651-664.

220. Boyden S: **The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes**. *J Exp Med* 1962, **115**:453-466.

221. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H *et al*: **Expression monitoring by hybridization to high-density oligonucleotide arrays**. *Nat Biotechnol* 1996, **14**(13):1675-1680.

222. Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM, Goldman RD: **Introducing intermediate filaments: from discovery to disease**. *J Clin Invest* 2009, **119**(7):1763-1771.

223. Moll R, Cowin P, Kapprell HP, Franke WW: **Desmosomal proteins: new markers for identification and classification of tumors**. *Laboratory investigation; a journal of technical methods and pathology* 1986, **54**(1):4-25.

224. Weinberg RA: **The Biology of Cancer**. *Garland Science* 2006:864.

225. Kim KW, Vallon-Eberhard A, Zigmond E, Farache J, Shezen E, Shakhar G, Ludwig A, Lira SA, Jung S: **In vivo structure/function and expression analysis of the CX3C chemokine fractalkine**. *Blood* 2011, **118**(22):e156-167.

226. Lambrecht BN, Hammad H: **Biology of lung dendritic cells at the origin of asthma**. *Immunity* 2009, **31**(3):412-424.

227. Thery M, Racine V, Piel M, Pepin A, Dimitrov A, Chen Y, Sibarita JB, Bornens M: **Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity**. *Proc Natl Acad Sci U S A* 2006, **103**(52):19771-19776.

228. Xia N, Thodeti CK, Hunt TP, Xu Q, Ho M, Whitesides GM, Westervelt R, Ingber DE: **Directional control of cell motility through focal adhesion positioning and spatial control of Rac activation**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2008, **22**(6):1649-1659.

229. Jacobson S, Kumagai-Braesch M, Tibell A, Svensson M, Flodstrom-Tullberg M: **Co-transplantation of stromal cells interferes with the rejection of allogeneic islet grafts**. *Ann N Y Acad Sci* 2008, **1150**:213-216.

230. Nie Y, Han YC, Zou YR: **CXCR4 is required for the quiescence of primitive hematopoietic cells**. *J Exp Med* 2008, **205**(4):777-783.

231. Broxmeyer HE: **Chemokines in hematopoiesis**. *Current opinion in hematology* 2008, **15**(1):49-58.

232. Cotterell SE, Engwerda CR, Kaye PM: **Leishmania donovani infection of bone marrow stromal macrophages selectively enhances myelopoiesis, by a mechanism involving GM-CSF and TNF-alpha**. *Blood* 2000, **95**(5):1642-1651.

233. Despars G, Ni K, Bouchard A, O'Neill TJ, O'Neill HC: **Molecular definition of an in vitro niche for dendritic cell development**. *Experimental hematology* 2004, **32**(12):1182-1193.

234. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T: **Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1**. *Nature* 1996, **382**(6592):635-638.

235. Choe MM, Tomei AA, Swartz MA: **Physiological 3D tissue model of the airway wall and mucosa**. *Nat Protoc* 2006, **1**(1):357-362.

236. Colognato H, Yurchenco PD: **Form and function: the laminin family of heterotrimers**. *Dev Dyn* 2000, **218**(2):213-234.

237. Li S, Edgar D, Fassler R, Wadsworth W, Yurchenco PD: **The role of laminin in embryonic cell polarization and tissue organization**. *Developmental cell* 2003, **4**(5):613-624.

238. Khoshnoodi J, Pedchenko V, Hudson BG: **Mammalian collagen IV**. *Microscopy research and technique* 2008, **71**(5):357-370.

239. Vrhovski B, Weiss AS: **Biochemistry of tropoelastin**. *Eur J Biochem* 1998, **258**(1):1-18.

240. Hartsock A, Nelson WJ: **Adherens and tight junctions: structure, function and connections to the actin cytoskeleton**. *Biochimica et biophysica acta* 2008, **1778**(3):660-669.

241. Sung SS, Fu SM, Rose CE, Jr., Gaskin F, Ju ST, Beaty SR: **A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins**. *J Immunol* 2006, **176**(4):2161-2172.

242. Jahnsen FL, Strickland DH, Thomas JA, Tobagus IT, Napoli S, Zosky GR, Turner DJ, Sly PD, Stumbles PA, Holt PG: **Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus**. *J Immunol* 2006, **177**(9):5861-5867.

243. Fossum S: **Dendritic leukocytes: features of their in vivo physiology**. *Research in immunology* 1989, **140**(9):883-891; discussion 918-826.

244. Holt PG, Haining S, Nelson DJ, Sedgwick JD: **Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways**. *J Immunol* 1994, **153**(1):256-261.

245. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, Zurawski SM, Johnston J, Liu YJ, Spits H *et al*: **Human thymic stromal lymphopoietin preferentially stimulates myeloid cells**. *J Immunol* 2001, **167**(1):336-343.

246. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, Omori M, Zhou B, Ziegler SF: **TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation**. *Annu Rev Immunol* 2007, **25**:193-219.

247. Ziegler SF, Artis D: **Sensing the outside world: TSLP regulates barrier immunity**. *Nat Immunol* 2010, **11**(4):289-293.

248. Prasse A, Pechkovsky DV, Toews GB, Jungraithmayr W, Kollert F, Goldmann T, Vollmer E, Müller-Quernheim J, Zissel G: **A Vicious Circle of Alveolar Macrophages and Fibroblasts Perpetuates Pulmonary Fibrosis via CCL18**. *American Journal of Respiratory and Critical Care Medicine* 2006, **173**(7):781-792.

249. Schutyser E, Richmond A, Van Damme J: **Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes**. *Journal of leukocyte biology* 2005, **78**(1):14-26.

250. Kaisho T, Akira S: **Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice**. *Trends Immunol* 2001, **22**(2):78-83.

251. Khajah M, Millen B, Cara DC, Waterhouse C, McCafferty D-M: **Granulocyte-macrophage colony-stimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes in vivo**. *Journal of leukocyte biology* 2011, **89**(6):945-953.

252. Friedl P, Alexander S: **Cancer invasion and the microenvironment: plasticity and reciprocity**. *Cell* 2011, **147**(5):992-1009.

253. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N: **Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells**. *J Exp Med* 2000, **191**(3):423-434.

254. Gunzer M, Friedl P, Niggemann B, Brocker EB, Kampgen E, Zanker KS: **Migration of dendritic cells within 3-D collagen lattices is dependent on tissue origin, state of maturation, and matrix structure and is maintained by proinflammatory cytokines**. *J Leukoc Biol* 2000, **67**(5):622-629.

255. Phillips R, Svensson M, Aziz N, Maroof A, Brown N, Beattie L, Signoret N, Kaye PM: **Innate killing of Leishmania donovani by macrophages of the splenic marginal zone requires IRF-7**. *PLoS pathogens* 2010, **6**(3):e1000813.

256. Dar A, Kollet O, Lapidot T: **Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice**. *Experimental hematology* 2006, **34**(8):967-975.

257. Lee Y, Gotoh A, Kwon HJ, You M, Kohli L, Mantel C, Cooper S, Hangoc G, Miyazawa K, Ohyashiki K *et al*: **Enhancement of intracellular signaling associated with hematopoietic progenitor cell survival in response to SDF-1/CXCL12 in synergy with other cytokines**. *Blood* 2002, **99**(12):4307-4317.

258. Hwang JH, Kim SW, Lee HJ, Yun HJ, Kim S, Jo DY: **Interferon gamma has dual potential in inhibiting or promoting survival and growth of hematopoietic progenitors: interactions with stromal cell-derived factor 1**. *International journal of hematology* 2006, **84**(2):143-150.

259. Fukuda S, Broxmeyer HE, Pelus LM: **Flt3 ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1alpha(CXCL12)/CXCR4 axis**. *Blood* 2005, **105**(8):3117-3126.

260. Gouwy M, Struyf S, Noppen S, Schutyser E, Springael JY, Parmentier M, Proost P, Van Damme J: **Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events**. *Mol Pharmacol* 2008, **74**(2):485-495.

261. Rodriguez-Frade JM, Mellado M, Martinez AC: **Chemokine receptor dimerization: two are better than one**. *Trends Immunol* 2001, **22**(11):612-617.

262. Owens BM, Beattie L, Moore JW, Brown N, Mann JL, Dalton JE, Maroof A, Kaye PM: **IL-10-producing Th1 cells and disease progression are regulated by distinct CD11c(+) cell populations during visceral leishmaniasis**. *PLoS pathogens* 2012, **8**(7):e1002827.

263. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S *et al*: **The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract**. *Nature* 1998, **393**(6685):591-594.

264. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV, Jr., Broxmeyer HE, Charo IF: **Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice**. *J Clin Invest* 1997, **100**(10):2552-2561.

265. Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, Maeda N: **Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2**. *Proc Natl Acad Sci U S A* 1997, **94**(22):12053-12058.

266. Islam SA, Chang DS, Colvin RA, Byrne MH, McCully ML, Moser B, Lira SA, Charo IF, Luster AD: **Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells**. *Nat Immunol* 2011, **12**(2):167-177.

267. Chensue SW, Lukacs NW, Yang T-Y, Shang X, Frait KA, Kunkel SL, Kung T, Wiekowski MT, Hedrick JA, Cook DN *et al*: **Aberrant in Vivo T Helper Type 2 Cell Response and Impaired Eosinophil Recruitment in Cc Chemokine Receptor 8 Knockout Mice**. *The Journal of experimental medicine* 2001, **193**(5):573-584.