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

STUDIES OF HUMAN DENDRITIC CELLS IN THE SKIN AFTER ANTIGEN EXPOSURE

Emily Bond

Karolinska Institutet

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To my mother Monique and my grandmother Ebba,
who both loved to read, and passed that love on to me.

ABSTRACT

Dendritic cells (DCs) act at the interface of innate and adaptive immunity. They are capable of inducing strong effector immune responses to invading pathogens, but also exert important functions in the maintenance of tolerance. The DCs of the skin are strategically located to be amongst the first cells to react to exposure to foreign substances and initiate immune responses, and increased understanding of these cells is critical for the development of new treatments of infections, allergic reactions and autoimmune disorders, as well as of new vaccine delivery strategies, targeting the skin. Studies of skin DC subsets are held back by technical difficulties in isolating the cells. In **paper I** of this thesis, we therefore aimed to develop a new DC purification method, using a skin graft mesher. This device is used clinically for expansion of skin graft tissue for transplantation. We found that by using the skin graft mesher the processing of skin was significantly accelerated, which had both practical and biological advantages. The DCs isolated from epidermis consisted of a uniform $CD1a^+$ $Langerin^+$ Langerhans cell population with high antigen uptake capacity. The DCs from dermis showed varied antigen uptake capacity and could be divided into three distinct populations based on CD1a expression. In addition, a skin explant model to study antigen uptake by DC subsets *in situ* was developed. We found that antigen injected into the skin explants was rapidly taken up, mainly by DCs in the dermis. This model can be a useful tool to study immediate responses to antigen exposure. However, since no recruitment of new cells into the tissue can occur, skin explants cannot illustrate all the components of innate immune responses. This prompted for the studies in **paper II** and **III**, where we took the approach to study infiltration of DCs to sites of antigen exposure in the skin *in vivo*. By using skin punch biopsies collected from standard skin antigen test sites like the tuberculin skin test (TST), we quantified recruited immune cells by immunohistochemical stainings of cryosections. We found that several DC subsets including plasmacytoid DCs (pDCs) accumulated in the dermis in the indurations induced in individuals with positive TSTs. In contrast, donor-matched saline injected skin did not show any DC recruitment, nor did TST sites of individuals lacking induration. The positive TST induration tissue was also associated with cell death and high expression of the antimicrobial peptide LL37, which together can provide a means for pDC activation and IFNα production. In line with this, IFN-inducible MxA was highly induced at the positive TST sites. By *in vitro* experiments, we found that pDCs were not as efficient as myeloid DC subsets to take up the TST antigens. Further, IFNα containing supernatants from pDC activated with LL37-DNA complexes reduced antigen uptake in myeloid DCs as well as decreased their capacity to activate T cells. Infiltrating pDCs in the TST reaction may thus have a regulatory effect upon the antigen processing and presentation functions of surrounding DC subsets, to limit potentially detrimental and excessive immune stimulation. TST and other antigen skin tests are used to monitor the integrity of the cellular immune system in HIV-1 infected individuals. To evaluate whether mobilization of DCs in response to antigen skin tests was impaired in HIV-1 infected individuals, we analyzed skin punch biopsies from skin test sites using mumps virus, *Candida albicans* or TST. Control skin biopsies showed that there was no difference in the number of skin-resident DCs between healthy and HIV-1 infected individuals. Also, multiple DC subsets infiltrated the dermis in response to injection with all antigens. However, the levels of DC infiltration correlated with the generally lower levels of memory T cells that accumulated at the antigen sites. A depleted or deficient T cell compartment could therefore lead to compromised DC recruitment and insufficient antigen presentation to T cells. Taken together, these studies contribute with new methods and data on the function and phenotypes of skin DCs, and their response to antigen administration in the skin in health and HIV-1 infection.

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscript, which will be referred to in the text by their Roman numerals:

I. **Emily Bond**, William C. Adams, Anna Smed-Sörensen, Kerrie J. Sandgren, Leif Perbeck, Anette Hofmann, Jan Andersson, Karin Loré. Techniques for time-efficient isolation of human skin dendritic cell subsets and assessment of their antigen uptake capacity.
Journal of Immunological Methods, 2009 Aug 31;348(1-2):42-56.

II. **Emily Bond***, Frank Liang*, Kerrie J. Sandgren, Anna Smed-Sörensen, Peter Bergman, Susanna Brighenti, William C. Adams, Senait A. Betemariam, Molebogeng X. Rangaka, Christoph Lange, Robert J. Wilkinson, Jan Andersson, Karin Loré. *shared first authorship. Plasmacytoid dendritic cells infiltrate the skin in positive tuberculin skin test indurations.
Journal of Investigative Dermatology 2012 Jan;132(1):114-23.

III. Frank Liang, **Emily Bond**, Kerrie J. Sandgren, Anna Smed-Sörensen, Molebogeng X. Rangaka, Christoph Lange, Richard A. Koup, Grace A. McComsey, Michael M. Lederman, Robert J. Wilkinson, Jan Andersson, Karin Loré. Recruitment of dendritic cells to antigen injection into the skin of HIV-1 infected individuals is dependent on T cell infiltration.
Manuscript in revision.

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LIST OF ABBREVIATIONS

Ab	antibody
AIDS	acquired immunodeficiency syndrome
Ag	antigen
AMP	antimicrobial peptide
APC	antigen presenting cell
BDCA	blood dendritic cell antigen
BG	Birbeck granules
CD	cluster of differentiation (designation)
CLR	C-type lectin receptor
CLA	cutaneous lymphocyte antigen
CTL	cytotoxic lymphocyte
DAMP	danger associated molecular pattern
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
dDC	dermal dendritic cell
DNA	deoxyribonucleic acid
ds	double stranded (as in DNA)
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorter
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	flouroisothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
HAART	highly active anti-retroviral therapy
HEV	high endothelial venule
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMGB-1	high-mobility group protein B1
IDEC	inflammatory dendritic epithelial cells
IFN	interferon

IL	interleukin
LC	Langerhans cell
mDC	myeloid dendritic cell
MDDC	monocyte derived dendritic cell
MHC	major histocompatibility complex
MR	mannose receptor
NK cell	natural killer cell
NKT cell	natural killer T cell
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PPD	purified protein derivate
PRR	pattern recognition receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
ss	single stranded (as in RNA)
TB	tuberculosis
TCR	T cell receptor
TLR	toll like receptor
TNF	tumour necrosis factor
TST	tuberculin skin test

1 INTRODUCTION

Understanding the immune system is at the very heart of understanding human physiology and pathology. The delicate balance of the immune system is absolutely crucial to our survival, from the very beginning of life by allowing attachment of the fertilized egg to the uterine wall, through growth processes, destruction of defective cells that could turn cancerous, healing of wounds and broken bones, to the most obvious task of protecting us from foreign invasion in the shape of various microbes. While many questions about human immunology have been answered, many remain. Increased knowledge of key components of the immune system is crucial to understand, prevent and treat infectious diseases and autoimmune disorders. The work presented here focuses on a subset of immune cells called dendritic cells (DCs).

DCs are at the interface of innate and adaptive immunity and are key cells for initiating immune responses. Understanding more about these cells, including how they collect antigens from pathogens or vaccine formulations, could help us manipulate their subsequent stimulation of effector cells in the desired direction i.e. toward antigens derived from threatening infections, but away from self antigens and autoimmune disease. For the scientific community to reach this level of understanding, many issues regarding the study of DCs remain. These relate to challenges in both methodology and sample material. The studies included in this thesis involve refinement of methods to isolate DC subsets in human skin, which also includes characterization of some of the phenotypical and functional differences of these subsets. Following on from this, *in situ* investigation of recruitment of DC subsets to sites of antigen administration in the skin were performed using unique skin biopsy material. This enabled studies of how DCs infiltrate the skin after antigen exposure and identification of factors in the environment that could contribute to cell activation. Finally, by using similar skin biopsies from HIV-1 infected individuals, alterations in DC recruitment in response to antigen exposure could be examined. All studies in this thesis were performed using human cells and skin and I only refer to human cells in the text, unless otherwise specified.

The first part of this thesis is dedicated to provide a background for our studies. A general introduction to the human immune system, with the main focus on innate immunity, is followed by a description on specific immune cells and processes in the skin. The various subsets of DCs and their properties are then introduced. To provide a background for our studies on HIV-1 infected individuals in the final paper, a short introduction to HIV-1 and DCs in HIV-1 infection is also provided. The second part of the thesis includes a presentation of the materials and methods used in our studies, and the results presented together with a discussion of our findings. Included at the end are the three papers on which this thesis is based.

2 AIMS OF THESIS

The studies in this thesis have focused on the isolation and identification of specific dendritic cell subsets in human skin, and their response to antigen exposure in various settings. The more specific objectives were:

Paper I: To develop an efficient isolation protocol for human dendritic cells from skin, and to phenotypically and functionally characterize the cells with a focus on antigen uptake capacity.

Paper II: To study the recruitment and function of dendritic cells in the positive tuberculin skin test (TST).

Paper III: To compare recruitment of dendritic cells in response to skin antigen tests in healthy versus HIV-1 infected individuals.

3 THE HUMAN IMMUNE SYSTEM

3.1 WHAT'S IN A NAME?

The immune system is subdivided into innate and adaptive immunity. As suggested by the very name, the innate immunity is what we were born with. The capacities of this branch of the immune system are genetically predetermined, encoded in our germ-line (1). The advantage of this is that it benefits from evolution through natural selection to be as efficient as possible in defining and annihilating invasive microorganisms. The disadvantage is that we can only carry so much genetic load; hence the innate immune system cannot carry memory of all possible pathogenic structures. It therefore needs completion by the almost infinite variety of specificities of the cells of the adaptive immune system. While these cells are naturally also inborn, their respective specificities are not. The greatness of their diversity has the drawback of not being germ-line encoded; hence their lessons have to be relearned by every generation (2). Another major difference between the branches is that it takes several days for a complete activation of the adaptive immune response, time that we do not have when exposed to an infection. In contrast, the innate immune response is instantaneous and can hold the fort while clonal expansion and maturation of the adaptive immune cells takes place, forming a specific response that in most cases eventually clears the infection. The capacity of the adaptive immune system to form immunological memory, thereby preventing reinfection with the same pathogens and making vaccination strategies possible, is another great ability of this branch (3). In conclusion, the innate and adaptive branches of immunity have intrinsic qualities that complement each other.

3.2 INNATE IMMUNITY

Innate immunity is usually described as including all the non-specific cells and components of our immune system. Even our physical barriers to the outside world, the skin and mucosal surfaces, and the tight junctions holding them together, as well as mucus, tears and the cilia of the nasal cavity and respiratory tract are often included (4). Several other non-cellular components, like the proteins of the complement activation cascade, contribute to the initiation of inflammation and the clearing of invading pathogens. Natural killer cells (NK-cells), clearing both infected and cancerous cells from the body, are considered part of the innate immune response since their response is instant and non-specific. They have, however, recently been suggested to be able to form memory-like responses (5), a capacity previously thought to be characteristic for cells for the adaptive immune system. In the studies included in this thesis, some parts of the innate immune response have been studied more closely.

3.2.1 Antimicrobial peptides

The antimicrobial peptides (AMPs) constitute an evolutionarily ancient part of the innate immune system (6), and their importance and relevance to many pathologic conditions is being increasingly appreciated (7). They are characterized by being small, less than 100 amino acids long, and are able to kill both gram-negative and gram-

positive bacteria, fungi and enveloped viruses (8, 9). They are produced by a wide range of cells, including epithelial cells, neutrophils, macrophages, platelets, lymphocytes, DCs, and mast cells (4). Some are constitutively expressed and others are induced by inflammatory cytokines. They are bactericidal, immune-regulatory, and also seem to have important roles in wound healing and angiogenesis (10). The main three classes of AMPs in humans are defensins, cathelicidins and histatins. The defensins lyse bacteria, fungi and some enveloped viruses, by inserting a hydrophobic region into the lipid membrane of the microbe, disrupting the integrity of the membrane. Apart from the capacity to directly kill invaders, the defensins act as chemoattractants for other immune cells, and can have stimulatory effects on DCs (11). There is only one human cathelicidin, an 18 kDa cationic protein named hCAP18. It is produced mainly by leukocytes, epithelial and mucosal cells. When activated, a cationic carboxy-terminal is cleaved off to form LL-37, a peptide with a wide range of properties. Apart from, like the defensins, being able to directly lyse microbial cell membranes, LL-37 is chemotactic, induces histamine release and angiogenesis. It is also upregulated in wound healing, and is suggested to play a role in the pathogenesis of autoimmune disease, as well as by its immune-regulatory properties affect our ability to respond to cancers (8, 9, 12, 13). Both defensins and LL-37 are secreted by, and interact with, keratinocytes, and are therefore of great interest in cutaneous immunobiology (10). The third class of human antimicrobial peptides, the histatins, are mainly expressed in the oral cavity and have antifungal effects (14).

3.2.2 Phagocytes

As ever, somebody has to clean up, and in the body it is the phagocytes. Apart from the physical engulfing of non-desired material (namely live or dead pathogens, and apoptotic or necrotic cells from our own body), they act on a wider plane sounding the alarm, alerting components of both the adaptive and innate immune response, as well as non-immune cells, to the danger at hand (1). They do this via release of pro-inflammatory cytokines and other substances. There are three major classes of phagocytes; macrophages and monocytes, granulocytes and DCs. Macrophages are tissue resident or can mature from circulating monocytes being recruited to inflamed tissues, and are highly phagocytic (15). Macrophages in different tissues have been assigned different names, e.g. microglia in the central nervous system or Kupffer cells in the liver, but they all belong to the same class (1). DCs, discussed in detail in section 5 of this introduction, can belong to this class but will much more often be referred to as constituting their own. The granulocytes share the feature of distinct cytoplasmic granules consisting of molecules and enzymes ready for release to limit infection, and include neutrophils, eosinophils, basophils and mast cells. They are important early responders to infection, but are also important in the pathogenesis of allergic disorders (16).

3.2.3 Immune recognition

The cells of the innate immune system recognize pathogens by identifying pathogen associated molecular patterns (PAMPs) on their surface. These patterns are highly conserved regions that large groups of microbes share. The patterns are recognized by pattern recognition receptors (PRRs) which can be soluble, like Mannan-binding lectin

(MBL) – specifically binding terminal mannose residues present on many microorganisms, and C-reactive protein (CRP) or ficolins that are able to activate the complement cascade when encountering their ligands (17, 18), or cellular. There are many types of cellular PRRs, some are found on the cell surface, others in the cytoplasm. One of the main functions of the cell surface C-type lectin family of receptors is pattern recognition, with ligation triggering internalization of the antigens. Macrophage mannose receptor (MMR, or CD206) recognises a wide range of bacterial, viral and fungal pathogens and stimulates their phagocytosis (19), and DEC-205 (CD205) seems to have similar functions (17). DC-SIGN (CD209) detects a wide range of pathogens, and has importantly been shown to detect gp120 from HIV-1 and mediate its uptake to promote *trans*-infection of the virus (20). Langerin (CD207) has been shown to bind fungi, mycobacteria and viruses, including HIV-1 (21). The role of the receptor in transmission of HIV-1 is however not clear, with some conflicting evidence as to its function (22). Another example of an endocytic PRR is the macrophage scavenger receptor (MSR) which recognises viral double-stranded RNA, as well as lipopolysaccharide (LPS) and lipoteionic acid (LTA) from bacterial cell walls (23). In the cytosol, NOD-like receptors (NLRs) NOD and NALP have been described, which are activated in response to exposure to bacterial peptidoglycans, and in the case of NALP also to DAMPs (24). Nalp3 has been identified as the receptor activated by aluminium adjuvants (alum), the most commonly used vaccine adjuvant (25). Retinoic acid-inducible gene I (RIG-I)-like receptors are also found in the cytosol, and respond to viral RNA (24).

The most well described cellular PRRs are the toll-like receptor (TLR) family. They are expressed on many cell types, including macrophages, DCs, B cells and some epithelial cells (26). Despite the fact that there are relatively few TLR genes, the specificity with which they recognize highly conserved regions (usually essential for survival or pathogenicity) on various classes of microbes, enables them to identify most pathogenic microbes. There are 10 TLRs described in humans, together detecting a wide variety of PAMPs, e.g. lipopolysacceride (TLR4), bacterial lipoproteins and lipoteichoic acids (TLR2), flagellin (TLR5), unmethylated CpG DNA from bacteria and viruses (TLR9) and single-stranded viral RNA (TLR7) (26). While most TLRs are found on the cell surface, TLR3 and 7-9 are instead situated in endosomal compartments. All TLRs share the common characteristic of being trans-membrane proteins with an extracellular region composed of leucine-rich repeats that creates a horse-shoe shape protein that is adaptable for ligand binding (27). The correct ligand will cross-link the ectodomains of two TLRs (either two of the same kind, or heterodimers of TLR1/2 or TLR 2/6), making their intracellular domains come together which in turn will activate different pathways of intracellular signalling, depending on the specificity of the TLR (17, 27, 28). This allows the different TLRs to steer the resulting cytokine production to either a strong antiviral, or antibacterial response, depending on the predominant ligands the cells was exposed to. A common characteristic of the patterns recognized by the TLRs is that they are considered specific for pathogenic structures. This makes the innate immune response specific for infection, and since the adaptive immune response has to be activated by the antigen presenting cells (APCs) of the innate immune system, this is a vital step to prevent autoimmune reactions. While the APCs presents both self and microbial antigen to the T cells, a co-stimulatory signal, induced by TLR ligation, is needed to activate the T

cells. No rule without an exception though - here it is the capacity of TLR7 and TLR9 to bind nucleic acids, which is a component of healthy mammalian cells as well as certain viruses. The protection mechanism here is that ssRNA is normally not present in endosomes, where TLR7 and TLR9 are found (26), but it has been shown that this is not enough – the signalling of host ssRNA through TLR7 and 9 seems to be involved in the pathogenesis of autoimmune disease (discussed in detail in **paper II**) (29, 30). Important from a cutaneous perspective is the fact that activation of most TLRs drives activation of $CD4^+$ T cells to a T_H1 phenotype, protecting from atopic reactions in the skin (31). The use of synthetic TLR ligands for modulation of the immune response *in vitro* or in clinical settings is a promising field both when it comes to treatment of disease and development of new vaccine strategies. For example, the TLR7 ligand Imiquimod is already in clinical use to treat condylomas, and in pre-clinical testing as vaccine adjuvant.

3.3 ADAPTIVE IMMUNITY

As opposed to the cells of the innate immune system, the major effector cells of the adaptive immune system, T cells and B cells, have an almost infinite variety of specificity of their receptors to recognise invading pathogens. These receptors are generated during development of the cells, by random gene rearrangements of germline encoded segments called variable (V), diversity (D) and junction (J). In addition to the random combination of the genes, the junctions at which they are combined also varies, and all this provides almost endless variations of the T cell receptor (TCR) and the immunoglobulin receptor of the B cell (32). The fact that the receptors are created in this random fashion means that the genetic code for the receptors is not transferred between generations, but every individual has to build their own adaptive immunity to every pathogen they are exposed to, a fact painfully obvious to all parents of young children. Another effect of the receptors being created randomly is that many of them will be reactive to self-Ag. To prevent these self-reactive receptors from ever being activated and thereby causing autoimmune reactions, there are many check points to pass before a B or T cell receptor is allowed to activate its host cell, both in the bone marrow and thymus and in the periphery. One of the most important functions of the DCs is the initiation of adaptive immune responses by presentation of antigen to T cells, a process that in the absence of activation signals is also important for maintaining tolerance (33).

3.3.1 Antigen presentation

For T cells to be activated and develop into strong effector cells, they need to be presented to antigens on MHC class I or MHC class II. Both receptors are produced in the ER, and consist of an α and a β chain, arranged differently on the receptors, with the antibody binding cleft of the MHC class I being formed by α chain segments only, and the MHC class II being formed by segments of both α and β chains (1). MHC class I is expressed on all cells in the body, except red blood cells, and presents intracellular antigens mainly to $CD8^+$ T cells. MHC class II on the other hand is only present on APCs, the most potent being the DCs, and will display exogenous antigen that has been taken up by endocytosis or macropinocytosis and processed within the cell. The MHC II/antigen complex is mainly recognised by $CD4^+$ T cells. Activation of $CD8^+$ T cells

will lead to lysis of the cell presenting the antigen, thus killing the intracellular pathogen along with the cell. $CD4^+$ T cell activation by the MCH class II, on the other hand, will save the cell, but through the effects of the $CD4^+$ T cells alert surrounding cells to the fact that there are pathogens in the extracellular space (34). Hence, there is a very functional basis to which T cells respond to which MHC. To prevent MHC class II from taking up intracellular proteins on its way from the ER, the antigen binding cleft is covered by an invariant chain, that will be cleaved of in the acidic milieu of the endosome taking the molecule to the cell surface (1). In some instances though, MHC class I can present extracellular antigens, after these have been taken in by endocytosis, in a process called cross-presentation. In this process, APCs are able to induce cytotoxic T cell responses to microbes that do not infect the APC itself by taking up extracellular antigens and presenting them on MHC class I. Cross-presentation is more common in some DC subsets than others (discussed in 5.4.1), and is enhanced by TLR ligand stimulation of the DCs (35, 36).
Lipid antigens can also be presented on CD1 molecules. In humans CD1a-d have been defined, and are expressed on some dendritic cells and monocytes (37). Glycolipids are non-covalently bound to a hydrophobic cleft of the molecule and the complex can be recognised by the TCR of various T cell subsets (38).

3.3.2 T cells

As described above, the T cell receptor (TCR) is greatly variable; it is estimated there is roughly a billion unique TCRs in the naïve T cell population (39). To prevent variants that are reactive to self-antigens, the T cells go through rigorous control in the thymus, where they mature from their lymphoid progenitors (hence the T), before they are allowed out into the body (3). If a self-reactive T cell slips through the thymus tests and binds a MHC-self antigen-complex, the cell presenting the complex will not give the T cell any secondary activating signal (as an infected, or by other means activated, cell would), which will rendered the T cell anergic and incapable of further maturation to an effector cell (33). In case of a T cell encountering a non-self-MHC-complex for which it is specific, and receiving co-stimulatory signals from the activated APCs, it will be activated to undergo clonal expansion, and to in turn exert activating effects on B cells and other surrounding immune cells.

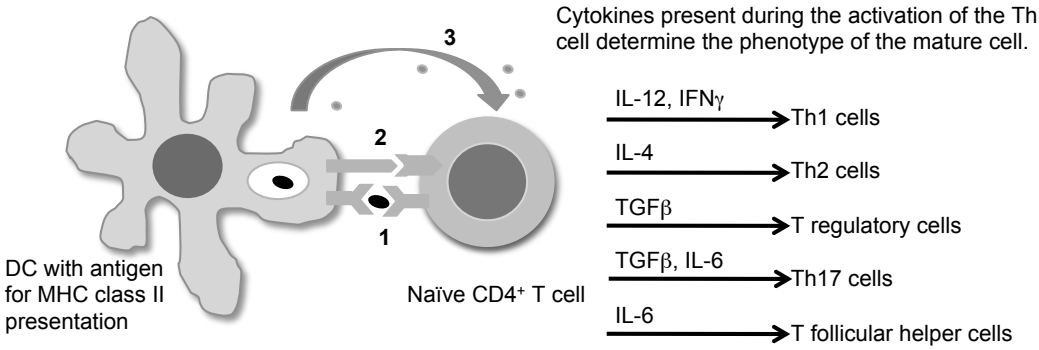

Figure 1. CD4 T cell activation by dendritic cells. The activation signal is provided in three steps: 1) Presentation of antigen on MHC class II, recognized by the TCR. 2) Binding of co-stimulatory molecules of the B7 family upregulated on activated DCs to CD40 on the T cell surface. In the absence of this signal, the T cell will be rendered anergic. 3) Cytokines from the DC and the local environment provide the final signal, and also determine the phenotype of the mature T cell.

Cells of the $CD4^+$ phenotype are also referred to as T helper (T_H) cells, and can be further subdivided into T_H1 type or T_H2 type. T_H1 are major controllers of cell-mediated adaptive immunity, with capacity to help $CD8^+$ T cell precursors differentiate, activate macrophages, boost the cytotoxic effects of NK cells and activate B cells. T_H2 cells induce humoral immunity to extracellular pathogens, like extracellular bacteria, fungi or parasites. They stimulate antibody production, and activate eosinophils, basophils and mast cells (32). T_H1-T_H2 balance is often discussed in models of autoimmune disease and atopic/allergic disease, with the T_H2 phenotype often predominating in patients with such conditions (32). If the CD4 is exposed to IL-6 or TGFβ during activation, it can take on a T_H17 phenotype, described to be efficient in defence against certain extracellular bacteria, but also to mediate autoimmune disease and chronic allergic processes (3). Another T cell type with important immune-regulatory function is the regulatory $CD25^+CD4^+$ T cell. It secretes immunosuppressive cytokines, and downregulates both T_H1 and T_H2 T cell activity. $CD8^+$ T cells recognise MHC class I:antigen complexes, and when activated by co-stimulatory receptors assume a cytotoxic effector phenotype capable of lysing the target cell by granzymes and perforin, which form a complex that perforates the cell membrane of the target cell so that is undergoes apoptosis. Like their $CD4^+$ counterpart, $CD8^+$ T cells also produce multiple cytokines to affect surrounding tissues and immune cells. Natural Killer T cells (NKT) are a subset of cells with a TCR, but also with NK cell lineage markers. They recognise lipid antigen on CD1 and seem to inhibit autoimmunity (32).

4 THE SKIN AS AN IMMUNE ORGAN

4.1 ANATOMY OF THE SKIN

The skin is the largest organ of the body and, along with the mucosal surfaces, is what separates us from the outside world. The protection by the skin from bacteria, viruses, fungi and parasites is physical and chemical as well as immunological. The skin also helps regulate body temperature by the sweat glands capable of releasing water to cool the body, and the hair follicles capable of creating air pockets between hairs to warm the skin – both features involved in regulating the effects of pyrogenic cytokines in infectious disease. The most prominent layers of the skin are the epidermis and the underlying dermis (Figure 2), separated by a basement membrane. While the epidermis is avascular, the dermis harbours fine capillary networks and lymphatic plexus. The epidermis consists mainly of keratinocytes, sealed by tight junctions. The keratinocytes proliferate from the base of the epidermis, and loose their nuclei as they are being pushed up, by new proliferating keratinocytes, to become part of the dead cornified epithelium, or stratum corneum – the outermost layer of the skin. This consists of cross-linked dead keratinocytes (referred to as corneocytes) embedded in a lipid matrix with ceramides, cholesterol and antibacterial free fatty acids. Interspersed in the epidermis are, importantly, the Langerhans cells, and also the melanin producing melanocytes, which protect us against UV radiation. The dermis is less cell-dense than the epidermis. It has a collagen structure, and is home to fibroblasts, capillary networks, c-fibre nerve endings, as well as dermal DCs (dDCs), macrophages, memory T cells and other important actors of the immune system (40). Interestingly, the expanding field of neuroimmunology has demonstrated several important functions of the peripheral c-fibre nerve fibres in the regulation of cutaneous immunity (41), again showing how intricate the regulation of immune responses is.

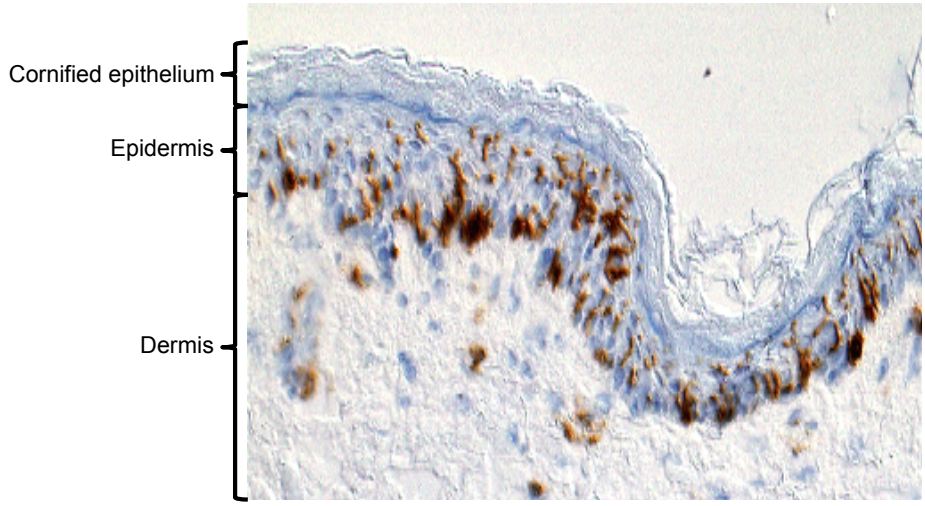

Figure 2. Basic anatomy of the skin. The brown staining represent CD1a^{+} DCs. The cell nuclei are visualized in blue by haematoxylin counterstaining.

4.2 FRIENDLY LANDLORD AND FIERCE DEFENDER

Although the skin is critical for protection of pathogens, it also hosts countless so-called commensals, which are non-pathogenic bacteria. These useful tenants help protect us from pathogenic bacteria by competing with them for nutrients and space. Some even produce antibacterial substances (42). The challenge for the immune system of the skin is hence to balance the protection of the commensal flora with the capacity to respond with immune activation upon encountering pathogenic microbes. The Langerhans cells (LCs) of the epidermis (described in detail in 5.4.2.) are thought to play an important role in maintaining this balance by extending dendrites through the tight junctions of the keratinocytes in the stratum corneum (43) to sample and present antigens from the commensal flora to T cells in a fashion that builds tolerance (44-46). In immune responses to invading pathogens, the skin shares its immune features with most other tissues in the body, but there are some cell types and concepts that are more or less skin specific, or have very defined missions in the skin.

4.3 INNATE IMMUNITY IN THE SKIN

While keratinocytes can be found in other areas of the body, they are uniquely abundant in the skin, where they comprise the principal cell type of the epidermis. Apart from the barrier function described earlier, and their role as scaffolding i.e. providing the actual structure of the epidermis, they have important immune-regulatory properties. They produce a wide range of cytokines, can be activated to present antigens, and also produce antimicrobial peptides; β-defensins and the cathelicidin LL-37, which are packed in lamellar bodies and released into the lipid matrix of the epidermis (10, 47, 48). They also, through cytokines, growth factors and hormones, influence the function and phenotype of the epidermal resident LCs (49). To ensure regeneration of the keratinocytes after abrasions to the skin, hair follicles contain protected repositories of epithelial stem cells capable of regenerating superficial skin layers (50). Another major effector in the skin is the mast cell, a bone marrow derived granulocyte, which is resident in the dermis. Mast cells are normally located perivascularly and respond early to inflammation. They are easily activated and exert rapid and strong inflammatory responses, and urticaria, by the release of leukotriens and histamine (51). As anywhere in the body, the endothelial cells in the skin are highly active in immune responses as well. To allow recruitment and migration of immune cells, activated endothelium increase their permeability by loosening tight junctions and upregulating VCAM-1 and ICAM-1, which binds immune cells in the circulation to slow them down and allow diapedesis (the migration of cells through the junctions between the endothelial cells). Expression of selectins on activated endothelium further enhances migration of lymphocytes (52).

4.4 ADAPTIVE IMMUNITY IN THE SKIN

Adaptive immune responses in the skin are activated by the cells and components of their innate counterparts. There are few resident T cells in the skin, but in cutaneous inflammation E-selectin is specifically upregulated, which attracts $CD45RO^+$ memory T cells expressing cutaneous lymphocyte antigen (CLA) (53) which binds E-selectin. CLA^+ T cells are specific for antigens that were initially presented to them by APCs

(mainly DCs) derived from the skin (54-56). Therefore, specific recruitment of these T cells to the skin facilitates adaptive cellular responses including memory T cells specific for the invading pathogens targeting the skin. In fact, low constitutive expression of E-selectins and ICAM-1 on skin endothelial cells ensures that there are also memory T cells consistently present in the skin, ready for potential invasion (57). If a naïve T cell is activated by a DC that has migrated from the skin to the lymph node, the T cell is prone to upregulate CLA, and also CCR4, CCR6, CCR8 and CCR10, the ligands for which are induced in inflamed skin (52). The activated naïve T cell will then find its way to the skin by interaction of CLA and E-selectin, as does the memory T cell. This process of T cell attraction is referred to as "homing" (58).

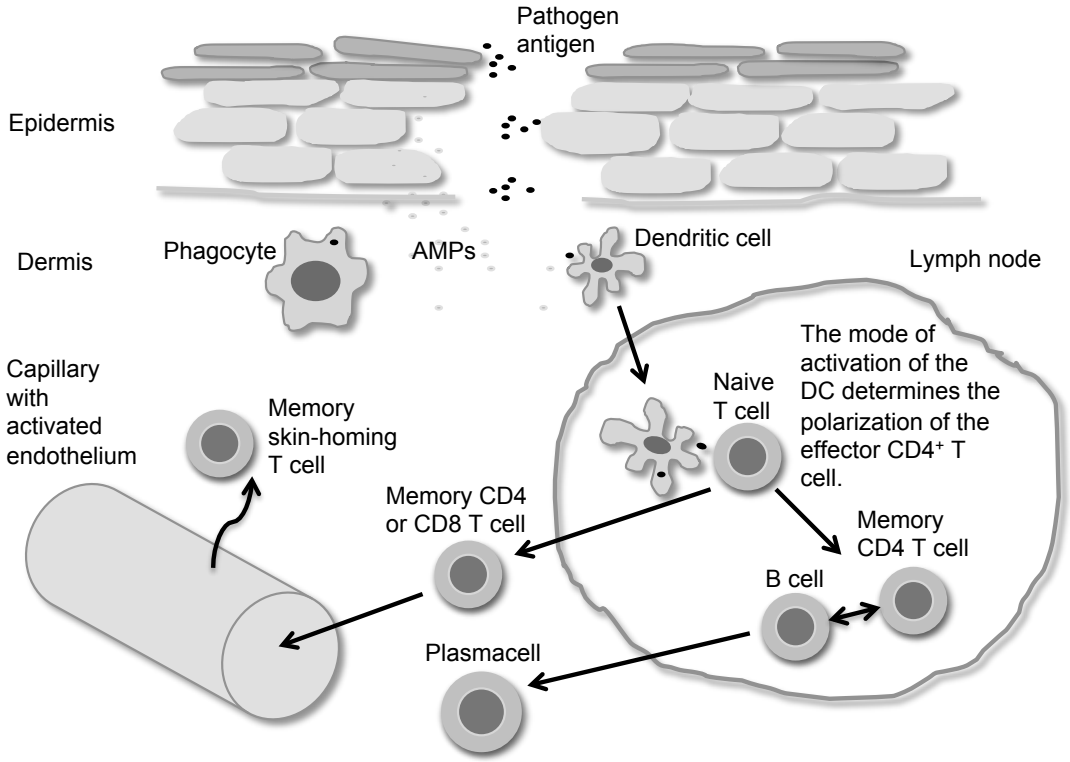

Figure 3. Schematic illustration of the adaptive immune response in skin, inspired by (34).

4.5 DELAYED TYPE HYPERSENSITIVITY SKIN TESTS

Delayed type hypersensitivity (DTH or type IV hypersensitivity) is a T cell-mediated reaction to antigens to which an individual is sensitized i.e. has previously been exposed to. DCs in the skin take up and present antigens to either skin resident or circulating memory T cells (the latter recruited to the site by migratory DCs presenting in lymph nodes). This initializes an inflammatory reaction, including homing of cells to the site of antigen exposure, which usually peaks at 48-72 hours. The classic example of such reactions is the Mantoux text or tuberculin skin text (TST), where purified protein derivate (PPD) from *Mycobacterium tuberculosis* (MTB) is injected intradermally to test for previous exposure to the antigen or its vaccine Bacillus Calmette-Guérin (BCG), or used for monitoring of the disease (59). In addition, the test can be used to evaluate the function of the cellular immune system in immune-compromised individuals. For this purpose other antigens than PPD can also be used,

such as mumps virus or *Candida albicans*, which a large majority of the population has been exposed to through infection or vaccination. These clinical tests consist of an intradermal injection of antigens, and after 48 hours the diameter of the induration that may occur at the site is measured. A test is considered positive at a diameter ≥10 mm according to WHO standards, and sometimes at ≥ 5 mm in clinical settings where tuberculosis exposure would be low. As discussed further in paper III, skin antigen tests have been used to monitor the integrity of the cellular immune responses in HIV infection (60, 61), and the TST has been demonstrated to be an independent predictor of disease progression (61-64).

5 DENDRITIC CELLS

5.1 SENTINELS OF THE IMMUNE SYSTEM

Dendritic cells (DCs) are often thought of as the sentinel guards of the body. Some subpopulations of DCs are situated in the skin and at mucosal linings, sites where we are frequently exposed to pathogens. This enables DCs to quickly respond to pathogen exposure, but likely also to help the body maintain tolerance to the commensal bacteria at theses sites (65). Other DC subsets are found in the bloodstream and in secondary lymphoid organs during steady state but can be recruited to peripheral sites of pathogenic exposure, and/or inflammation, by cytokine/chemokine gradients and selectins. The various DC subsets have different specializations and profiles, but share some key features. Most importantly, they are all antigen presenting cells (APCs). Some DC subsets are more efficient than others to activate $CD4^+$ and $CD8^+$ naïve T cells, and to dictate the subsequent type of immune response elicited by these cells (66). They can also directly activate B cells, NK cells and NKT cells (67) and by release of pro-inflammatory or regulatory cytokines affect multiple other cell types. Since DCs respond quickly to pathogen exposure and have the capacity to present antigen and produce regulatory cytokines, they are key cells for orchestrating the development of immunity.

5.2 ANTIGEN UPTAKE PATHWAYS

DCs take up antigen by two major pathways, phagocytosis and pinocytosis. Phagocytosis means engulfment of large particulate antigens, such as bacteria, yeasts, protozoans and dying cells. Pinocytosis can be subdivided in macropinocytosis and receptor-mediated endocytosis. Macropinocytosis, internalization of soluble antigens, occurs continuously in immature DCs and is non-specific (68). Receptor-mediated endocytosis, on the other hand, requires ligation of antigens to a specific surface receptor such as C-type lectin receptors, scavenger receptors or Fc receptors (recognizing the Fc portion of antibodies) (69, 70). The mode of uptake of an antigen influences the intracellular events following internalization (71, 72). Large particulate antigens taken up by phagocytosis remain in a phagosome, which will become increasingly acidic leading to degradation of the antigen and allows for loading of peptides onto MHC molecules. Macropinocytosed antigen, and antigen taken up by endocytosis via receptors linked to MHC class II-restricted presentation, will be directed to lysosomes for degradation and loading on MHC II. There are also receptors linked to cross-presentation, and antigens taken up after ligation to such receptors will instead be directed to stable early endosomes for loading on MHC class I (36).

5.3 ACTIVATION, MATURATION AND MIGRATION

The antigens taken up by the DCs can contain danger-signals like PAMPs from pathogens, or danger-associated molecular patterns (DAMPs) from self-antigens of distressed cells (e.g. heat shock proteins, HMGB-1, β-defensins and uric acid). These signals are sensed by PRRs, as described earlier. Different subsets of DCs have

different expression repertoires of PRRs (26, 73, 74). Ligation by a danger signal to a PRR activates the cell to undergo a process of maturation. This constitutes a wide range of morphological and functional changes, including; 1) loss of adhesive molecules and the acquisition of high cellular motility – to be able to migrate to lymph nodes and present their antigen, 2) loss of endocytic and phagocytic receptors – to reduce the ability of the DC to take up and present additional antigen while activated which could break the immune tolerance to self antigens, 3) upregulation of co-stimulatory receptors (B7 family) including CD80 and CD86 – enabling the DC to potently activate T cells by interaction with CD28 on the T cell surface, and 4) secretion of a large variety of cytokines that will strongly affect the nature of the immune response elicited (67). In a rapid response to stimulation, even before undergoing complete maturation, the DCs release chemokines, e.g. CXCL1-5 and 9-11, directing other immune cells to the site. The first wave of chemokines produced attracts immune cells such as NK cells, neutrophils, memory T cells and monocytes. This helps create an inflammatory milieu harmful to the intruder and beneficial for recruitment of additional immune cells. As DCs mature they upregulate receptors that will guide them to the secondary lymphoid organs via lymphatic vessels. This enables DCs to interact with and migrate through the high endothelial venule (HEV) walls of the lymph nodes. One of the main receptors is CCR7. Once in the lymph node, DCs will secrete a second wave of chemokines: CXCL13 to attract B cells and T follicular helper cells (specialized to support the development of humoral responses), CCL19 and CCL21 to attract naïve T cell, and CCL22 – attracting T regulatory cells (67). Now in close contact with T and B cells, the DCs can present their antigen and thereby elicit an antigen-specific immune response (discussed in more detail in section 3.3.3). If, in contrast, the DC has taken up an antigen not associated with a PAMP or DAMP motif to activate the DC, the antigen will be presented to T cells in the absence of co-stimulatory molecules. Antigen presentation without accompanying co-stimulatory stimulation will render the T cell anergic and incapable of developing into an effector cell. This is a way to protect the body from self-reactive T cells (75). The antigens presented in this tolerogenic fashion are often host-derived, but may also represent antigens from the commensal flora present in skin and mucosal linings. As mentioned above, LCs at these sites are thought to sample the commensal flora by sampling the outer lining of the skin and mucosa, and present their antigens in a tolerogenic fashion (43, 76). In addition to this; if exposed to the suppressive cytokines IL-10 and TGF-β, immature DCs in lymphoid tissue can regulate immune responses further by inducing unresponsiveness in peripheral T cells (77, 78). DCs also have the capacity to induce regulatory T cells that produce IL-10 (79, 80) or $Foxp3^+$ regulatory T cells (81). Taken together, DCs have been shown to have the capacity to direct the immune system in both activating and tolerogenic directions.

5.4 DENDRITIC CELL SUBSETS

The DC subsets presented below are divided into blood or skin resident DCs. However, it is important to note that DCs are migratory cells and multiple DC subsets can be recruited to inflamed tissues.

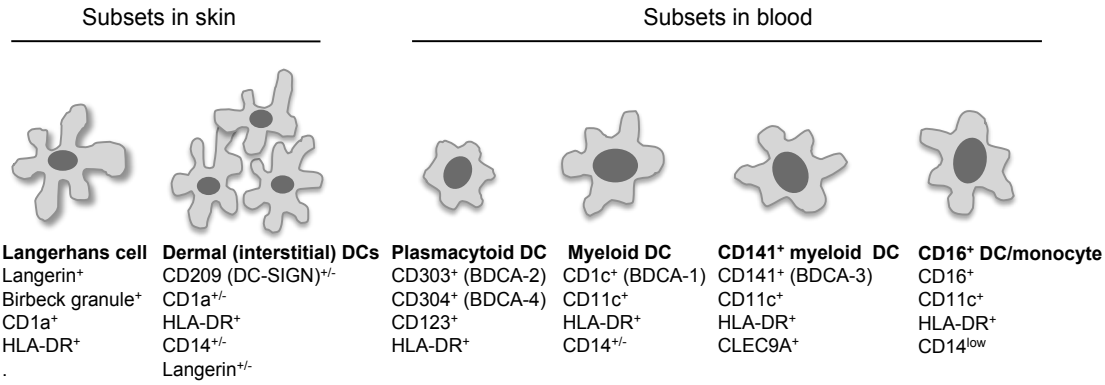

Figure 4. The human DC subsets in skin and blood, and their main phenotypic characteristics.

5.4.1 Subsets in blood

The main DCs subsets in blood are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which can be subdivided based on their expression of blood DC antigens (BDCA). pDCs express BDCA-2 (CD303) and BDCA-4 (CD304), while the mDCs can be further subdivided as BDCA-1 (CD1c) or BDCA-3 (CD141) expressing. The latter subset was only recently described to represent the human counterpart to the mouse $CD8^+$ DCs, known to have superior cross-presenting capacity (82-85). A $CD14^-$ $CD16^+$ DC subset often termed non-classical monocyte, have also recently been identified. This subset shares characteristics with both monocytes and DCs (86-91). The mDCs as a whole have been shown to have a higher capacity for antigen uptake and presentation than pDCs. They are powerful inducers of T_H1 responses by their capacity for IL-12 p70 production, and drive immune responses by secretion of TNF and IL-6 (66, 86, 87, 92-96). Different reports show slightly different data on the specific TLRs expressed on the DC subsets. However, most point to mDCs having some level of expression of TLR receptors 1-8 and 10, thereby being able to respond to a very wide variety of pathogens. In contrast pDCs express mainly TLR 7 and 9 thereby responding specifically to viral infection (97). pDCs have a unique ability to produce high levels of type I interferons (IFNα/β) which among many immune functions are important in anti-viral defenses (98-100). Through secretion of IFNs pDCs enhance the cytotoxicity of NK cells and $CD8^+$ T cells, and also protect surrounding DCs from the cytopathic effects of many viruses (97). pDCs can also secrete IL-12 and IL-6, which in concert with the type I IFN help induce IFNγ production in T cells, and can promote memory B cell differentiation to promote anti-viral Abs (101). All these effects help clear viral infections. Sadly, the potent effects of type I IFN can be turned against the host in a series of autoimmune conditions (102, 103) and graft versus host disease (104), where pDCs are suspected instigators. In contrast, pDCs have also been shown, like mDCs, to be of vital importance in tolerance induction (reviewed by (105)). While mDCs are found throughout the body - in blood, lymphoid tissues and also in skin and mucosa, pDCs are more confined to blood, the

thymus and lymphoid tissue at steady state, but can migrate to sites of antigen exposure in inflammation (102, 106).

5.4.2 DC subsets in the skin

The DC subsets of the skin represent a heterogeneous group of cells, whose definite characteristics and functions are still under debate. The Langerhans cells (LCs) of the epidermis was the first subset of DCs to be identified in skin, and are currently the best characterized. They differ from most other DCs by their distinct expression of the C-type lectin receptor langerin (CD207), and can be defined by unique cytosolic structures referred to as Birbeck granules (BG), where Langerin traffics to after antigen uptake (107). Langerin is also expressed in some dermal DC (dDC) subsets, but it is not clear whether these represent epidermal LCs in transition, or a unique dDC subset. LCs also express MHC class II, CD1a, CD205, and adhesion molecules CD11c and E-cadherin (107). The latter is a key mediator of LC-keratinocyte adhesion (108). When activated, LCs downregulate E-cadherin, becoming free to migrate towards the afferent lymph. While much indicates that LCs develop from a common hematopoietic myeloid progenitor, and are replenished from blood, some studies have evoked questions as to whether LCs actually develop from a skin resident precursor (109-112). The dDCs, in contrast, do appear to be repopulated from blood only (110, 113). Origin is not the only question of debate concerning LCs, their function is also still under investigation. There is definite evidence that LC are strong activators of cellular immunity, by activation of $CD4^+$ T cells in particular (114), but they have also been proven to be key mediators of tolerance (115-117). These functions need not be mutually exclusive, but the conditions under which these opposing function are elicited, and especially how we could replicate these conditions in vaccine strategies and treatments targeting the LCs, still needs to be elucidated. Another DC can be seen in epidermis, but in chronic inflammatory diseases only; the inflammatory dendritic epithelial cell (IDEC). These DCs do not express Langerin, and seem to differentiate from blood monocytes. They represent a more immature DC phenotype than the LCs, with strong antigen presenting capacity (118, 119).

In recent years several DC subsets in the dermis have been identified. Different phenotypical and functional classifications have been suggested, and there is still no definite nomenclature. The classification of both epidermal and dermal subsets is made more difficult by the inherent plasticity of DCs. Since the isolation of skin DCs affects their maturation status (120), and thereby their phenotype and function, different isolation protocols could result in different subsets. The inclusion criteria for DCs also vary and since there is no specific marker for dDCs, different studies often use different markers to identify them. However, dDCs are generally considered to express the C-type lectins MMR (CD206), DEC 205 (CD205) and DC-SIGN (CD209). As discussed earlier, Langerin is expressed on one DC subset in the dermis, but it is not clear whether these cells represent a dermal resident DC, or migratory LC emigrated from the epidermis. Further, dDCs express varying levels of CD14 and CD1a. The dDCs expressing CD14 have been described to exhibit a more immature phenotype, possibly representing a precursor DC population (109). Interestingly, a recent study by Chu et al. introduces the concept of a $CD141^+$ $CD14^+$ $CD1a^-$ $CD11c^{low}$ $CD1c^{low}$ dDC, described as a competent inducer of T regulatory cells, capable of suppressing graft versus host

disease and anti-tumor immunity (121). In mouse models, dDCs have been shown to be able to replace LCs in induction of contact sensitization (122-124), and also to induce cytotoxic T cell responses to herpes simplex virus, and to leishmania, when LCs did not (125-127). This could imply that the LCs main function is tolerogenic, unless strongly stimulated, while dDCs would be more prone to induce immune responses. Given the anatomical location of the subsets, with the LCs at the interface of self and non-self, continuously exposed to the commensal flora of mucosal surfaces and skin, and the dDCs at sites where they should not be exposed to microbial antigens unless there has been a breach of the integrity of the body linings, this division would be fairly logical. But the evidence is still conflicting, and LCs have in other studies, using human DCs, been shown to be superior inducers of effector $CD8^+$ T cell responses (128, 129). Several factors can contribute to the conflicting results. Apart from potential differences between the species, the technical difficulties of *in vitro* studies of DCs might also affect the results.

In the skin, as anywhere else in the body, the delicate balance of immunity and tolerance is sometimes disrupted, resulting in autoimmune disease. With DCs being at the heart of the control of this balance, their role in the pathogenesis, both instigation and propagation of disease, is an intensive field of study. DCs have been shown to migrate to sites of atopic dermatitis (130, 131) systemic lupus erythematosus (SLE) (132, 133), dermatomyositis (134), Sjögren's syndrome (135), graft versus host disease (136), and psoriasis (106, 137), where a suggested mechanism whereby pDCs could drive the pathogenesis has also been published (30).

5.5 DENDRITIC CELLS AS TARGETS AND TOOLS

Since DCs are pivotal for both defense to pathogens and malignancies, and involved in autoimmune diseases, targeting DCs with drugs that can enhance or dampen their function may be a desirable strategy. Protein subunit vaccines contain adjuvants, i.e. substances that enhance and shape the immune responses to the vaccine. To elicit potent immune responses with vaccines to lethal pathogens like HIV, MTB, or *Plasmodium falciparum* (causing malaria), strategies of specifically targeting DCs with adjuvants or antigens, for instance by the use of selected TLR ligands or viral vectors as vaccine adjuvants, are being investigated. Apart from vaccination against microbes, DCs are suggested as potential mediators of cancer treatment through "vaccination". This strategy involves the extraction of monocytes from a cancer patient, which are then differentiated into monocyte-derived DCs (MDDCs) *in vitro,* loaded with antigens from the patients own tumour, and then re-administrated to the patient. Such strategies are already in clinical trials (138). Apart from vaccination strategies, DCs are already indirectly targeted by general immunomodulatory treatments for many infectious and autoimmune conditions.

6 HUMAN IMMUNODEFICIENCY VIRUS

6.1 THE HIV EPIDEMIC

In 1981, an unusual clustering of patients diagnosed with *Pneumocystis carinii* pneumonia was recorded in Los Angeles, USA (139). Additional such cases, and eventually also increasing numbers of previously healthy patients presenting with other unusual diagnoses related to immunodeficiency, led to the unravelling of one of the worst plagues in modern time. Initially being predominantly spread among gay men and intravenous drug users in the western world, the epidemic was eventually fairly well controlled in these populations by intensive preventative work and efficient anti-viral drugs that control the viral load and thereby limit the spread. Tragically, the virus meanwhile decimated Sub-Saharan Africa, where anti-virals are scarce and cultural beliefs limit behavioural interventions, killing millions and wiping out entire generations in some areas. Finally, the spread of HIV is currently decreasing in developing countries, due to improved availability of anti-retroviral drugs. However, there is still an urgent need for a preventative vaccine since no curative treatment is available, and current symptomatic treatments are paired with an array of side effects and are under the constant threat of viral resistance. Additionally, the global epidemic is not showing any signs of declining amongst MSM (men who have sex with men) or commercial sex worker populations (reviewed in (140)).

6.2 HIV-1 AND AIDS PATHOGENESIS, IN SHORT

HIV is a retrovirus and part of the *Lentivirus* genus. As all *retroviridae* HIV has a RNA genome and carries the viral enzyme reverse transcriptase (RT). This enzyme is used by the virus to transcribe its RNA to DNA, which can then incorporate in the host genome. HIV exists in two main forms that infect humans, HIV-1 and HIV-2. The disease progression is slower with HIV-2 and it is also less transmissible. HIV-2 was first reported from West Africa, and is still spread mainly on the African continent (141, 142), while HIV-1 is the by far the most dominant form throughout the world. HIV-1 primarily infect $CD4^+$ T cells by binding to the CD4 receptor and main co-receptors CXCR4 and CCR5, but the virus can infect a great variety of host cells, including DCs (95, 143-148). The infection and severe depletion of $CD4^+$ T cells are the main characteristics in the pathogenesis of HIV/AIDS. As the number of $CD4^+$ T cells in the blood drop, the immune system gets increasingly impaired (149-151). When less than 200 $CD4^+$ T cells/μl blood remains, the patient is diagnosed with acquired immune deficiency syndrome, or AIDS. At this stage multiple opportunistic diseases appear, the course of which is the usual cause of death of infected individuals. In some cases, these infections start to appear earlier, and the patient will receive an AIDS diagnosis once one of a list of several AIDS-defining opportunistic infections (including invasive Candida or CMV-infection, *Pneumocystis carinii* pneumonia, Kaposis sarcoma etc) appear (152). Infection of various other cell types is also of importance for symptoms in advanced disease, as well as factors in transmission and as "hide outs" (viral reservoirs) for the virus (144, 153-155).

6.3 DENDRITIC CELLS IN HIV-1 INFECTION AND AIDS

DCs are resident at mucosal surfaces where primary infection usually takes place, and evidence suggests that they are targeted early in the course of infection (156-158). DCs express the receptors most commonly used by HIV-1 (CD4, CCR5 and CXCR4) and are susceptible to infection (146, 156, 159-165). When productively infected the DCs become a long-term source of infectious virions in the body. Transmission of such *de novo* synthesized virus from DCs is referred to as *cis*-transfer. Additionally, DCs can internalize the virus by CLR-mediated endocytosis without being productively infected, and act as "Trojan horses", bringing the virus from the periphery to lymphocyte-rich areas were it is exocytosed and transmitted to $CD4^+$ T cells (*trans*-infection) (20, 166, 167) DCs can also facilitate infection of T cells by attracting them to sites of HIV exposure after HIV-1 stimulation of DC TLRs (reviewed in (168)), and it has also been suggested that DCs facilitate propagation of infection by tolerogenic effects (169-173). That said, DCs also have to capacity to induce adaptive immune responses to the virus (155, 174-178) and could therefore also have important functions in controlling the infection once established, although some conflicting studies have demonstrated that this capacity is reduced (179-181) compared to healthy individuals. Their ability to induce immune response to the virus could be of great importance in the development of vaccine strategies, but could potentially also be targeted in new treatment strategies.

DCs are directly affected by HIV-1 infection, in that reduced numbers of both mDCs and pDCs are found in blood early in the infection (178, 182-184). While at least some mDC subsets can replenish after HAART, pDCs do not seem to recover as well (185-188). Nonetheless, the levels of both DC subsets remain decreased in comparison to uninfected individuals (185). This might also be true for elite controllers (178) despite the fact that these patients have very low or undetectable viral loads and often unaffected T cell counts. The exact mechanism behind the depletion of DCs is not known. An increased rate of apoptosis (189, 190) and direct infection (179) have been suggested, but it has also been shown that DCs accumulate in lymphoid tissues, which may reflect a redistribution of DCs from the blood (191-194). The reduction of DCs could be important to HIV-1 pathogenesis, as the magnitude of the reduction of blood DCs in primary infection has been suggested to be a prognostic factor for the course of the disease (195). Interestingly, a recent observation indicated that severe pDC depletion in the blood of HIV-2 infected individuals is associated with a more attenuated form of the disease (196). Whether it is the actual depletion of blood DCs, or co-variations of factors contributing to both DC depletion and other manifestations, remains to be proven. Due to the fact that blood DCs are reduced, the question of functionality of DCs in HIV-1 infected individuals has been extensively studied. Some studies have demonstrated that there is reduced IFNα production in blood PBMCs and pDCs of infected individuals (185, 188, 197) while others have shown HIV and HIV-infected cells to be potent inducers of IFN production in pDCs (178, 198-200) and IFN levels to be chronically elevated in HIV infected individuals (201-203). These results might seem contradictory, but they do not have to be. The mode of stimulus of the pDCs in experimental settings, the stage of disease in the subjects, and bystander effects of other cells in the culture might all play a role in shaping the results. It has also been suggested that immune exhaustion of the pDCs *in vivo* due to chronic activation renders them unreceptive to *ex vivo* stimulation, possibly explaining the discrepancy in

the results (204). mDCs have been described to have an abnormal immature phenotype in lymph nodes of HIV-1 infected individuals (172), but also to display hyperfunctionality with increased cytokine production in the blood, which could contribute to the chronic immune activation that is important in HIV-1 pathogenesis (178). The latter study showed the same result for pDCs. In conclusion, the activation status of DCs in HIV-1 seems to be altered, and it is possible that this has an effect on HIV-1 pathogenesis.

7 MATERIALS AND METHODS

7.1 ISOLATION OF HUMAN DENDRITIC CELLS

Presented below is a brief overview of the methods used in the studies included in this thesis. For more comprehensive descriptions, please see respective paper.

7.1.1 Isolation of dendritic cells from skin

In **paper I,** we first developed new methods to be able to study skin DCs, initially by refining previously described skin DC isolation protocols (205, 206). Skin samples of discarded skin from breast surgery were collected from Karolinska University Hospital. The skin was collected immediately after surgery and placed in complete media (RPMI; 10% fetal calf serum (FCS); glutamine; penicillin and streptomycin) with antifungal supplements. The skin was first cleared of subcutaneous fat and then cut in pieces about 3 to 5 cm in size. To allow access of dispase, which enzymatically digests the bindings of epidermis and dermis to enable separation of the layers, the skin has to be cut in thin (1-2 mm) slices. Traditionally, this was performed with a scalpel. To facilitate this very time consuming and laborious step of the protocol, we introduced the use of a skin graft mesher that slices the skin into thin nets, allowing the dispase to access the skin. The nets were incubated in dispase (GIBCO) for 90 min at 37 °C and 5% CO^2. The epidermis could then readily be separated from the dermis using sharp forceps. The epidermal and dermal sheets were washed and then incubated separately in complete media supplemented with Hepes buffer (GIBCO) and GM-CSF (PeproTech) to maintain a pH-stable physiological medium and to enhance migration, respectively. DCs that migrated into the media were harvested by filtering the media through a 70 μm cell strainer to obtain a single cell suspension. The cells were counted and used for experiments or further enriched by centrifugation using Ficoll-Paque Plus (GE Healthcare Biosciences AB, Uppsala, Sweden) density gradient (205). Trypan blue exclusion was used to measure cell viability.

7.1.2 Generation of MDDCs

The ability to produce MDDCs in vitro has been revolutionary in DC research, and lots of data have been generated using these cells. However, it is important to understand potential differences between MDDCs and primary subsets of myeloid DCs. To generate MDDCs for **paper I**, human PBMCs were isolated by Ficoll gradient centrifugation from buffy coats from healthy donor as previously described (205-207). Monocytes were isolated by plastic adhesion and cultured in complete media (as described earlier) supplemented with IL-4 (R&D Systems) and GM-CSF for six days, after which 90% of the cells had downregulated CD14, upregulated CD1a and HLA-DR, and exhibited DC morphology.

7.1.3 Isolation of blood pDCs and mDCs

In **paper II**, primary DCs were purified from blood as described (208, 209). In order to obtain sufficient yields of DCs, high numbers of PBMCs were collected from healthy

donors by aphaeresis (Dept of Transfusion Medicine, Karolinska University Hospital). Lymphocytes and monocytes were then separated based on size and sedimentation by counterflow centrifugation elutriation, and pDCs and mDCs could then be isolated from the elutriated monocytes by anti-BDCA-4 (pDCs) and anti-CD1c microbeads (mDCs) (Miltenyi) and AutoMACS separation (Miltenyi). pDCs and mDCs were cultured in complete medium supplemented with IL-3 (R&D Systems) and GM-CSF, respectively.

7.1.4 Phenotypic characterization by flow cytometry

For identification and phenotyping of the cells in **papers I and II,** the cells were washed and stained for surface or intracellular receptors for 15 min at 4°C and washed as previously described (210). Different combinations of Abs, directly conjugated to the fluorescent dyes FITC, PE, PerCP, Cy5-PE or APC, were used. For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD), according to the manufacturer's instructions, prior to staining. The cells were collected on a FACS Calibur flow cytometer (BD) and data was analyzed using Flow Jo software (Treestar Inc).

7.2 FUNCTIONAL ASSESSMENTS OF DENDRITIC CELLS

7.2.1 Antigen uptake assessment in MDDCs

To study the antigen uptake capacity of various dendritic cell subsets in **paper I**, the model protein ovalbumin (OVA, an egg white protein) was used. OVA is frequently used to study uptake of particulate antigens in mammalian cells. We used OVA conjugated with Alexa 488 (OVA Alexa 488, Molecular Probes) to allow detection by flow cytometry or confocal microscopy. The MDDCs were pulsed with OVA Alexa 488 at 37°C for up to 48 h. To study the affects of maturation on antigen uptake capacity, cells were stimulated by the Toll-like receptor (TLR)-ligand Poly I:C (TLR3), imidazoquinoline compound (TLR7/8), or LPS (TLR 4) or by TNFα, for 24-48 h before antigen exposure to induce maturation. To assess background binding of the antigen, cells were pulsed at 4°C in parallel to the uptake assays performed at 37 °C, since incubation at 4°C will reduce active uptake to a minimum and therefore illustrate how much antigen could be expected to bind to the outside of the cells and not represent true uptake. In addition, the internalization of OVA was confirmed by subsequently treating cells with trypsin (Sigma-Aldrich) to remove cell surface bound OVA. To study the pathways of OVA uptake, cells were pre-incubated with mannan or rottlerin (both Sigma-Aldrich) before exposure to OVA Alexa 488. Cells were analyzed by either flow cytometry or adhered to slides for confocal microscopy analysis. Untreated cells were also incubated with OVA Alexa 488, adhered to slides and stained with anti-EEA-1 (early endosome marker) or anti-LAMP-1 (lysosomal marker) to illustrate their processing in the cell matrix after uptake.

7.2.2 Antigen uptake assessment in skin DCs

(paper I) To assess the antigen uptake capacity of the various DC subsets in skin, OVA Alexa 488 or dextran (Oregon green 488, Molecular Probes), was added to the cells

either during migration or after the cells had completed their migration and been harvested. For antigen exposure during migration, OVA (mainly taken up via CLR-mediated endocytosis) or dextran (known to be taken up by macropinocytosis) was added directly to the media in which the skin nets were incubated. The migrated cells were harvested after 48 h and analyzed for antigen uptake. Uptake could not be assessed at earlier time points since the migration was not complete until after 48 h. However, most of the uptake is likely to occur early after antigen exposure. Alternatively, harvested cells (incubated in media without OVA during their migration) were pulsed with OVA or dextran. These cells would be more mature at the time of antigen exposure. With both exposure procedures, the final concentration of antigen used was 0.1 μg/ml and the total time of antigen exposure 48 h. Antigen uptake was assessed by flow cytometry.

7.2.3 Injection of antigen in skin explants

To illustrate antigen uptake at earlier time points in **paper I**, we developed a skin explant model, where freshly removed skin samples were cleared of subcutaneous fat and cut manually with a scalpel to pieces of an area about 3x3 cm. The skin was then injected with either PBS, OVA Alexa 488 or dextran Oregon Green 488. Each explant was injected several times to make distribution of antigen as even as possible. Injections were made as close to the epidermis as possible to minimize leakage of injected antigen from the tissue into the media through the porous deep dermis. The tissue explants were incubated for 48 h in complete media to allow migration of the cells. The migrated cells were harvested and analyzed for antigen uptake by flow cytometry. For in situ analysis of antigen uptake in intact tissue, OVA Alexa 488 was injected into >1 cm^2 explants. The pieces were incubated for 90 min at 37°C. Biopsies of the injection sites were thereafter taken and immediately put in the freezing media O.C.T. (Sakura Finetek) and frozen at -80 °C to be further analyzed for antigen uptake by confocal microscopy.

7.2.4 *In situ* analysis of antigen uptake in skin explants and characterization of infiltrating cells in skin biopsies

In **paper I-III**, snap frozen skin biopsies were cut to 8 μm sections by cryostat and fixed on slides. Prior to staining, the sections were permeabilized with saponin (Riedel-de Haen). To prevent unspecific signals from the fluorescently labelled streptavidin, endogenous biotin or biotin-binding proteins were blocked using an Avidin-Biotin blocking kit (Vector Laboratories). Primary Abs (raised in mouse, goat or rat) were diluted in EBSS-saponin and applied for incubation overnight. In order to reduce non-specific binding of the secondary biotinylated Abs, the tissue sections were blocked with rabbit or goat serum. The secondary biotinylated Abs (i.e. biotinylated rabbit anti-mouse, rabbit anti-goat or goat anti-mouse) were diluted in EBSS-saponin and applied for 30 min. Finally the sections were incubated with Alexa 488- or Alexa 594-labeled streptavidin (Molecular Probes), diluted in EBSS-saponin, for 30 min. In **paper III**, an Alexa 594 anti-goat Ab was also used in some experiments. All incubations were performed in the dark at RT. After the final incubation, the sections were washed thoroughly with sterile water, dried and mounted using Vectashield mounting medium or SlowFade® Gold antifade regent, both containing DAPI for nuclear staining.

Multiparameter analysis of OVA uptake, cell surface receptor expression and nuclear staining was performed on a laser scanning confocal microscope (TCS SP2, Leica Microsystems) using either 20x/0.7 or 63x/1.3 objectives for sequential image capture z-series of each fluorochrome. Separate analyses of the image data were used to verify co-localization with DC cell surface markers and that the OVA Alexa 488 signals were sequestered in an intracellular compartment and not at the cell surface.
In **paper II** and **III**, the immunoflourescent stainings were preformed only to characterize cells present in the sections, not to visualize antigen uptake. In these papers, the major analysis was performed on sections stained by immunohistochemistry. Primary antibodies were followed by secondary biotinylated antibodies (Abs) as above, but for immunohistochemical stainings the secondary Abs were detected with the peroxidase-based Vectastain Elite ABC kit (Vector), and the reaction developed by diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (Vector Laboratories). Cell nuclei were counterstained with Mayer's Haematoxylin (Histolab Products). The sections were analyzed by a Leica DMR-X microscope (Leica Microsystems) coupled to computerized image analysis (Leica Qwin 5501W, Leica Imaging Systems), as described (211). Quantification of all markers was performed in the dermis except for Langerin, which was done in the epidermis only. Melanin-rich cells at the interface of the epidermis and the dermis were excluded from the analyses.

7.3 STUDIES OF DC AFTER *IN VIVO* ANTIGEN INJECTION IN HUMAN SKIN

7.3.1 Collection of human skin punch biopsies

The study subjects for **paper II** and **III** were recruited in Cape Town, South Africa or San Francisco and Cleveland, USA. Exclusion criteria included previously diagnosed tuberculosis and/or presentations of one of several symptoms e.g. cough, chest pain or fever. TST was performed according to international standards and considered positive at ≥10 mm. Punch biopsies were taken as described (212) from the antigen injection site and a saline injected site on the opposite arm at 48 h and snap frozen. The skin test for *C. albicans* and mumps were performed in the same way, but considered positive at ≥5 mm, and only the Cleveland cohort was tested with these antigens. The methods for sectioning, staining and analysis of the biopsies is described in section 7.2.4.

7.3.2 Stimulation of pDC with LL-37-DNA complexes

In **paper II**, in order to study the affects of LL37-DNA complexes on DCs, either human DNA (Biochain) or CpG ODN (class B 10103, Coley) were co-incubated with LL37 (Innovagen) for 30 min at RT. pDCs were exposed to the complexes for 16 h at 37°C. Cell free supernatants were then harvested and analyzed for IFNα production by ELISA (VeriKine TM) according to the manufacturers instructions, and used for culture experiments with mDCs. Stimulated pDCs were also set in co-cultures with T cells.

7.3.3 Functional assays of pDC effects on mDCs

In **paper II,** supernatants from pDCs (at 1:1) stimulated by LL37-DNA or LL37-CpG complexes, or recombinant IFNα as a control, were added to cultures of mDCs. The mDCs were incubated for 24 h, washed, exposed to PPD-Alexa 488 and analyzed for PPD uptake and CD80/CD86 expression, to illustrate whether activated pDCs could affect the antigen uptake capacity of the mDCs. To investigate the affects on T cell stimulatory capacity by stimulated pDCs or mDCs, the subsets were co-cultured with unlabeled or CFSE-labeled, purified allogeneic $CD4^+$ T cells. The T cells were then analyzed for detection of IL-2, TNFα and IFNγ expression by intracellular staining and FACS analysis after 16 h incubation or for assessment of proliferation by CFSE dilution after 5 days, as described (155, 213). Alternatively, proliferation was measured by thymidine incorporation as described (214). Luminex assays (Human Cytokine 10-Plex Panel, Invitrogen) were also used for analysis of cytokines in supernatants from DC and DC:T cell cultures, and performed according to the manufacturers instructions and analyzed by Luminex 200TM system (Invitrogen). As a control, PPD only was used in several of these experiments to ensure no stimulatory effects were exerted by that alone.

7.3.4 Evaluation of PPD uptake capacity

To allow detection of uptake in **paper II**, PPD (Statens Serum Institut, Denmark) was labelled by Alexa 488 protein labelling kit (Molecular Probes). Isolated pDCs and mDCs were then pulsed with PPD Alexa 488 and analyzed by flow cytometry. Alternatively, monocyte-enriched populations (Rosette Separation, StemCell technologies) were exposed to PPD Alexa 488 and cells expressing CD1c, CD123 or CD141 were analyzed. For confocal microscopy imaging, sorted pDCs and mDCs were transferred to adhesion slides.

8 RESULTS AND DISCUSSION

The studies in this thesis have all revolved around the functions of human DCs residing in, or infiltrating, the skin in health and HIV-1 infection. The specific aims were to first refine the isolation of skin DCs and to characterize the isolated subsets and their ability to internalize antigen. Further, in order to study skin DCs *in vivo* we took an approach to analyze the recruitment of DCs to antigen skin test sites, which represent examples of antigen administration in the skin. Finally, we examined whether this recruitment was affected in HIV-1 infected individuals. Below I will present and discuss highlights of our findings from **papers I-III**.

8.1 ISOLATION OF DENDRITIC CELLS FROM HUMAN SKIN

It is critically important to perform studies based on physiologically relevant primary human DCs, including skin DC subsets, and not solely *in vitro* derived DCs. However, studies using primary DCs are hampered by restrictions in existing isolation protocols. Hence, our first objective in **paper I** was to refine isolation procedures for skin DCs. With skin DCs being rare, large amounts of skin need to be processed to yield sufficient cells for experiments. At the onset of our studies, most available protocols were very time-consuming and laborious, much due to a step where the skin had to be cut manually by a scalpel into thin slices. This step is needed to prepare the skin for enzymatic disruption that enables separation of the epidermis and dermis so that higher yields can be obtained, and the DC populations from the various anatomical sites can be studied separately. The fact that the slicing step is time consuming and labour intensive is not only a practical but also a biological problem since prolonged *in vitro* handling of the DCs increases the risk of maturation of the cells. In clinical practice, an instrument called a skin graft mesher (Figure 5) is used to expand skin grafts for transplantation. This instrument contains built-in blades that cut the skin into a delicate net-like structure. This structure resembles the manually sliced pieces of skin. We therefore evaluated the use of the skin graft mesher in skin DC isolation protocols. We found that there were a number of advantages with the skin graft mesher. The slicing step was markedly faster, on average 10 times faster when processing small samples (10 g of skin) and even greater with larger samples. We also found that the net structure created by the skin graft mesher not only allowed for efficient enzymatic treatment of the skin, but also that the subsequent step where the epidermis is peeled off from the dermis was much faster. When using the skin graft mesher, the epidermis would often come off in large intact sheets instead of having to be peeled off in countless small pieces as with the manually cut pieces. After separation of the dermis and epidermis the respective sheets were cultured for 48 hours to allow migration of the DCs into the culture media (205, 215-219). The cells harvested by the skin graft mesher method did not differ in phenotype, number of cells per gram of donor-matched skin, or viability as compared to the manual slicing procedure.

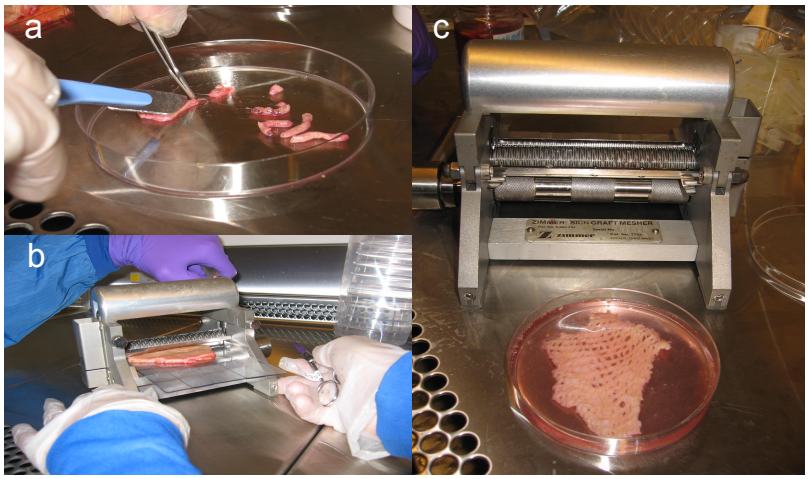

Figure 5: a) Manual slicing of skin in thin pieces by scalpel and forceps. b-c) The skin graft mesher, and the net shape of skin that has been run through the machine. (from figure 1, paper I)

8.1.1 Phenotypic characterization of isolated DC subsets

In **paper I,** we also performed an extensive analysis of the phenotype of the cells that we harvested from the skin. By flow cytometry, the cells with high expression of HLA-DR were further analyzed for a variety of DC markers. We found that the DCs isolated from epidermis consisted of a fairly homogenous population of Langerin^{+} CD1a^{+} CD11c^{+} DEC205^{+} DC-LAMP$^{+/-}$ CD123^{-} DC-SIGN^{-} CD14^{-} LCs. In contrast, the subsets isolated from dermis represented a heterogeneous population of DCs. They all expressed CD11c, DEC205 and some degree of DC-LAMP, and lacked DC-SIGN and CD123. However, they showed varying expression of CD1a, and could therefore be separated into populations based on their level of CD1a expression (Figure 6a). The subset with the highest CD1a expression was found to co-express Langerin (Figure 6b). This led us to speculate that they represent migratory LCs that are in transition from epidermis through the dermis towards afferent lymph as suggested earlier (220, 221). CD14^{+} cells were present among the CD1a^{dim} and CD1a^{-} dDC subsets which suggests that these cells might have a more monocyte/macrophage phenotype and function. DEC-205 expression was higher in the CD1a$^{high/dim}$ than in the CD1a^{-} subset (Figure 6b).

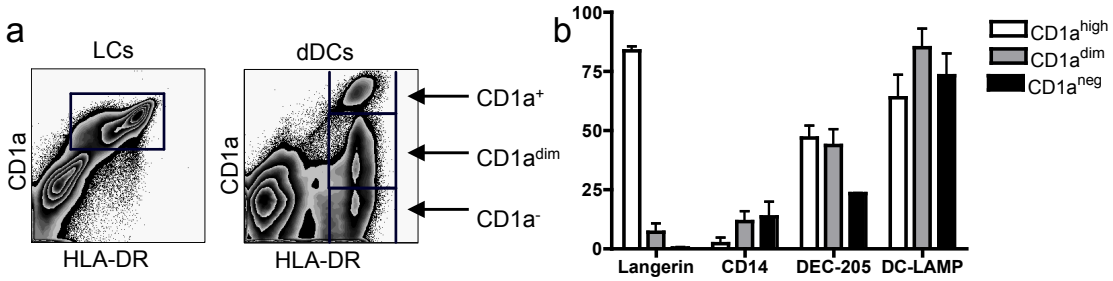

Figure 6. a) Flow cytometric plots show the varying CD1a expression found on DC subsets harvested from epidermis (left) versus dermis (right). b) Bar graphs depict the expression of Langerin, CD14, DEC-205 and DC-LAMP in dDCs based on their CD1a expression. (from figure 1, paper 1)

Both the epidermal and dermal DC subsets expressed intermediate to high levels of maturation markers CD25, CD86 and CD83.

There is limited knowledge about the dermal DC subsets. Several of the previously reported isolation procedures of DCs from dermis involve exclusion of Langerin or

CD14 expressing cells which leads to these cells being neglected in the analysis. Also, DC-SIGN has been shown to be downregulated after migration (222), while DC-LAMP expression can be upregulated during maturation in all skin DCs (221), which would affect the analysis of migrated subsets. As discussed in the introduction, a common classification of dermal DC subsets does not exist, although several have been suggested (122, 128, 220, 223-227). A strict classification is complicated due to the fact that most of the phenotypic markers are not specific for DCs, or that the expression of the markers can change with maturation. There is also controversy as to the ontogeny of the various skin DC subsets, including the LCs. To this end, some of the dDC subsets have been suggested as precursor cells rather than independent subsets. A classification of dDCs based on CD1a expression has previously been suggested (225). Our data are in favour of this since we consistently in our experiments could see a clear division of three dDC subsets based on CD1a expression. Further, separating the DCs based on CD1a expression correlated to significant functional differences in antigen uptake capacity between the subsets. While we hypothesize that the $CD1a^{high}$ subset represents migratory LCs, additional studies are needed to further characterize the DC subsets in terms of origin and function. The fact that CD14 expression was the highest in the $CD1a^{dim}$ subset, could support prior data showing that these cells represent precursors to other skin DC subsets (109).

8.2 THE ANTIGEN UPTAKE CAPACITY OF DC SUBSETS

8.2.1 Differential ability of antigen uptake by isolated skin DCs

While mouse studies have contributed with a significant volume of data on the antigen uptake capacity of skin DCs, many of these results remain to be confirmed in the human system. To functionally examine the distinct DC subsets isolated from epidermis and dermis, we focused on how efficiently they could internalize antigen in **paper I**. DCs were exposed to the well-characterized antigen OVA either during their migration process, or after harvesting. We found that LCs isolated from the epidermis were much more efficient at internalizing OVA than donor-matched dDCs when the cells were exposed during migration. Both subsets were more efficient if exposed to OVA during migration than after (Figure 7a). These data may not be surprising as it is well known that mature DCs have reduced antigen uptake capacity (64, 66, 68), and LCs have been described to have a more immature phenotype than dDCs (220, 221). This could explain part of the difference between the antigen uptake capacity of LCs and dDCs, and also the fact that DCs exposed post-migration were less prone to antigen uptake, since incubation and migration in itself matures the DCs. A mature phenotype may also explain why the $CD1a^{high}$ $Langerin^{high}$ dDCs showed the lowest uptake capacity of the dDC subsets; given our hypothesis that these cells are epidermal LCs in transition is correct. LCs that migrate through the dermis would likely be activated and carry antigen. In fact, the uptake capacity of $CD1a^{high}$ $Langerin^{high}$ dDCs often matched that of LCs that were exposed to antigen after emigrating out of the skin. The $CD1a^{-}$ dDC subset, which possibly represents non-DC-$HLA\text{-}DR^{+}$ cells, or a DC precursor, showed an intermediate uptake while the $CD1a^{dim}$ cells were the superior dDC subset for internalizing OVA (Figure 7b).

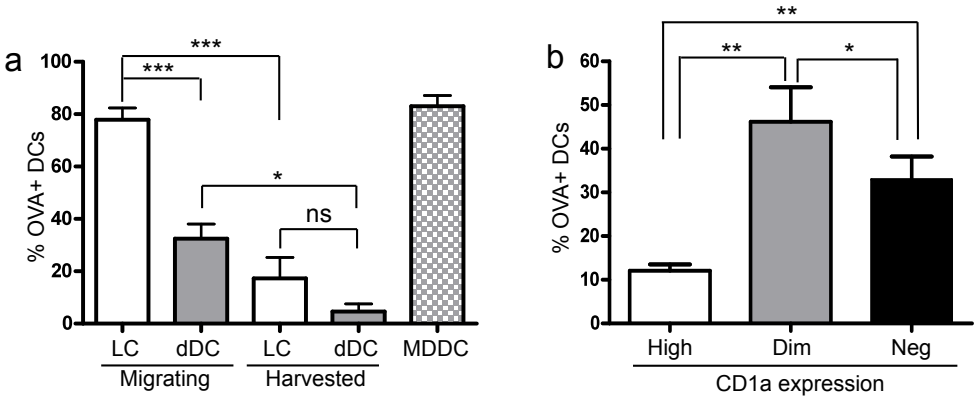

Figure 7. a) OVA uptake by skin DCs exposed to OVA before and after migration. Immature MDDCs were included for comparison. B) OVA uptake in dDCs exposed during migration, shown in relation to their CD1a expression. (from figure 3, paper I)

The functional differences in antigen uptake capacity could support a division of dDCs based on CD1a expression, but further studies of the cells phenotype and function are needed to elucidate whether there is biological relevance to such a division.

8.2.2 *In situ* injection of OVA in skin explants

Since *in vitro* handling induces maturation in DCs, we sought to develop a method to be able to study the DCs in an immature state. We therefore established a skin explant injection model in **paper I**, where we could track antigen uptake by these DC subsets *in situ* shortly after antigen administration. In human skin explants, we performed multiple shallow injections of OVA labelled with Alexa 488. The explants were placed in culture and the cells were allowed to migrate out of the explant for harvesting and analysis by flow cytometry. Since there is no separation of dermis and epidermis in this protocol, the cells emigrated from epidermis versus dermis could not be completely distinguished. However, based on the CD1a expression of the emigrated cells, we found that the cells harvested by this method closely resembled those of DCs harvested from dermis. The antigen uptake capacity of the DCs harvested from the explant model was higher than observed in the dDCs isolated by our mesher protocol. This suggests that the dDCs targeted in the explant model were in a more immature state when exposed to the antigen. This advantage makes the explant model a good complement to studies using the mesher isolation protocol, while the latter has the advantage of producing higher cell yields and definite separation of the epidermal and dermal DC subsets.

To further characterize the uptake of antigen by intact skin DCs prior to migration and maturation, we injected skin explants with OVA Alexa 488 and collected biopsies of the injection sites shortly after administration (90 min). OVA^+ cells were readily detected throughout the dermis, but most prominently in the superficial dermis close to the basement membrane. While a diffuse Alexa 488 signal was seen in the deep dermis (where the injection took place), it seemed like the OVA^+ cells were migrating towards the lymphatic capillary plexus (i.e. natural DC migration routes) in both the upper, papillary dermis, and in the deep dermis. No or very few OVA^+ cells were found in the epidermis, in line with the fact that the injection occurred in the deep dermis and that it is unlikely that antigen-bearing cells would enter the epidermis after the injection. The vast majority of cells showing OVA

uptake expressed HLA-DR, and several also expressed DC-SIGN (Figure 8). In these stainings, Langerin and CD1a expressing cells were most evident in the epidermis, consistent with the high expression of these markers in LCs. Some OVA^+ $CD1a^+$ cells, and rare OVA^+ $Langerin^+$ cells, were observed in the dermis, but the generally low CD1a expression observed on dDCs in this system could relate to difficulties in detecting dDCs with low CD1a expression above the autofluorescence in the tissues.

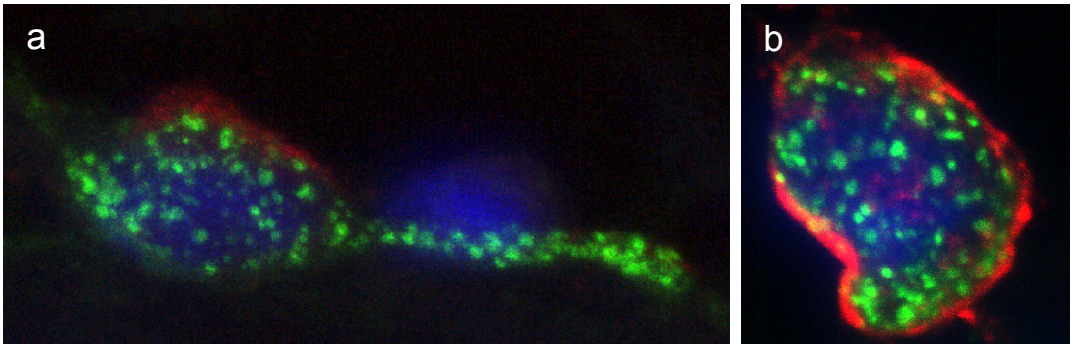

Figure 8. Immunofluorescent stainings of skin biopsies taken from skin explants injected with OVA Alexa488. OVA uptake (green) is detected in a) DC-SIGN (red) and b) HLA-DR (red) expressing dDC. (from figure 5, paper I)

We concluded that the *in situ* model could be used to study the first events in the skin after antigen exposure. However, since no recruitment of new cells into the tissue can occur, this model can not fully appreciate all the components of innate immune responses. Nonetheless, it has the potential to provide important clues as to what the primary target cells are for antigens or adjuvants administrated intradermally.

8.2.3 Potential OVA uptake mechanisms by DCs

To investigate the pathway(s) by which OVA is taken up by the DCs, we performed a series of experiments in **paper I** using mannan to block CLR-mediated endocytosis (68) and rottlerin to block macropinocytosis (228). Pre-incubation with mannan and rottlerin is necessary for these studies, and since there was no way to ensure the inhibitors would efficiently penetrate the skin, we had to perform the experiment using MDDCs. We found that both blocking strategies led to a significant reduction of OVA uptake. Mannan, known to block CLRs like the mannose receptor (MR/CD206), DC-SIGN and Langerin, was the most potent inhibitor. MR is known to traffic antigen to stable early endosomes, while DC-SIGN also can deliver cargo to lysosomes. Macropinocytosed antigen, which uptake can be blocked by rottlerin, would also end up in lysosomes (35). Early endosomes express the marker EEA-1, while lysosomes express Lamp-1 (68, 71, 229). Since we were unable to perform the blocking experiments in skin DCs, we instead analyzed the co-localization of OVA and EEA-1 versus Lamp-1. Skin explant biopsies were collected 90 min after OVA injection and stained to visualize potential co-localization. We observed co-localization with both EEA-1 and Lamp-1, indicating possible uptake by both endocytosis and macropinocytosis, although it was not as frequent as found in the control experiments using MDDCs. However, our data indicate that skin DCs might utilize several receptors and pathways to internalize OVA.

While our data confirmed co-localization of OVA and EEA-1, and OVA and LAMP-1, in the injection model, implying plural uptake mechanisms, we could not quantify the contributions of macropinocytosis versus endocytosis, due to the constraints on blocking studies in skin DCs.

8.3 DC SUBSETS INFILTRATE THE TST REACTION

As mentioned above, a significant disadvantage with our *in situ* explant model is that studies of recruitment of immune cells, including DCs, to the skin after antigen administration cannot be performed. Determining the infiltration patterns of DC subsets to the skin after antigen exposure and/or during inflammation is important for increasing the understanding of the innate immune mechanisms involved in the response to various pathogens, allergens, topical treatments or vaccine components targeting the skin. In this regard, samples of human skin after antigen exposure offer a unique opportunity to study recruitment of cells. In the work presented in **paper II** and **III**, we had the opportunity to perform studies using skin punch biopsies obtained from individuals who had received standard DTH-inducing skin tests. In **paper II**, healthy volunteers living in Khayelitsha Township, Cape Town, South Africa, an area highly endemic for tuberculosis, were recruited (230). Due to the high TB exposure in the area, all donors expressed a positive, ≥10 mm, TST. As described in section 4.5, TST is the most commonly used skin test in humans and monitors prior exposure to MTB or its vaccine. TST gives a measure of the cellular immunity and DCs have previously been postulated to play a role in TST reactions (231, 232). In **paper II**, we therefore started by analyzing the presence of various DC subsets in biopsies taken from the positive TST sites and compared that with the level of DCs in biopsies taken from saline injected control sites in the opposite arm of the same individuals. As expected, there was a significant increase in the cellularity of the skin in the TST reaction. This is a hallmark of inflammation. The presence of DCs was first evaluated by HLA-DR staining which showed that the positively stained area was significantly higher in the TST reaction than in saline-injected control sites. In line with that, several markers for myeloid DCs, including Langerin, DC-SIGN, CD11c, CD68 and CD141, were also significantly upregulated at the TST site compared to controls (Figure 9). This suggests that there is a rather robust infiltration of several DC subsets into the positive TST indurations. Interestingly, while the $CD141^+$ cross-presenting DC subset was rarely detected in skin from the control site, it was consistently detected at the TST site. Infiltration of $CD141^+$ DC in response to antigen delivery and inflammation in the skin had not earlier been described, and since they are potent stimulators of $CD8^+$ T cell responses (84), they may play a particular role in presenting PPD to infiltrating $CD8^+$ T cells. Their recruitment to skin might mean that there is a possibility of targeting these cells in transdermal vaccination strategies. Since our paper was published, a study has reported a skin resident $CD141^+$ dDC with tolerance-inducing capacity, thus potentially important for maintaining skin homeostasis (121). Hence, as for many DC subsets, the tolerance-inducing versus immune-activating roles of this subset needs to be further elucidated.

pDCs are rare in the skin at steady state (223). In accordance with this, $CD123^+$ pDCs were rare or absent in controls, but we detected the presence of pDCs in all positive TST biopsies.

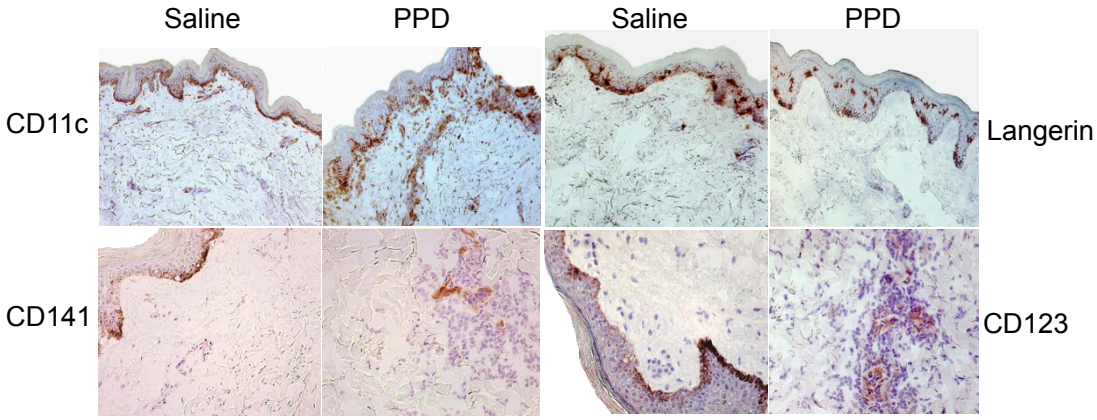

Figure 9. Expression of selected DC-markers (brown) in skin punch biopsies from saline-injected sites as well as positive TST sites. The cell nuclei are visualized in blue by haematoxylin counterstaining.

Since CD123 is not entirely specific for pDCs, we also stained the tissues for another pDC marker, CD303 (BDCA-2). Again, we found very few if any CD303+ pDCs in the control sites and significantly more at the TST sites. Most CD303+ cells co-expressed CD123. BDCA-2 is known to be downregulated in activated pDCs (224), which probably explains some of the lack of co-staining of $CD123^{high}$ and BDCA-2^{+}. The recruitment of pDCs to the TST sites had not been previously shown either, and prompted our investigation of their role in this immune reaction.

8.4 THE ROLE OF PDCS AT THE TST SITE

As discussed earlier, pDCs are potent producers of IFNα/β, hence their role in antiviral defences has been particularly explored (98). Indeed, pDCs have been shown to be recruited to the skin in viral infections such as herpes simplex (233, 234) and varicella (235, 236). They presumably have as important a role in anti-viral defences in the skin as elsewhere in the body. However, pDCs have also been shown to exert other immune-regulatory properties not involving pathogen responses (237). They have been described to be recruited to the skin not only in infection, but also to allergic contact hypersensitivity reactions (131) and to autoimmune skin manifestations in lupus erythematosus (132, 133) and psoriasis (102, 106). In the latter, infiltrating pDCs were shown to be activated and produce IFNα. Since we observed that pDCs were recruited to the TST site, we set up experiments aiming to reveal some of their roles in the DTH reaction. First, we stained for IFNα in the TST tissue, but were unable to detect any. However, we did find that the GTPase MxA, specifically induced by IFNα/β exposure (238, 239), was significantly expressed in the TST site, while it was undetectable in the saline controls. This strongly implied that there was production of IFNα/β in the positive TST reaction. Insufficient sensitivity of the IFNα/β staining or the time point (48 hrs) of the biopsy collection are probable explanations for the lack of IFNα detection. Assuming that the pDCs at the TST site were activated to produce IFNα, what activated them? The dogma has long been that pDCs respond with IFNα production in response to TLR stimulation by microbial invasion. However, the PPD mixture itself is designed to *not* evoke immune responses in naïve individuals. IFNα production in psoriatic lesions, a non-pathogen-containing yet inflammatory milieu, has been suggested to be induced by self-DNA or RNA from dying cells in complex with

the antimicrobial peptide LL37 (29, 30). We could detect strong upregulation of LL37 and also high levels of Caspase-3, high-mobility group protein B 1 (HMGB-1) and lactate dehydrogenase (LDH) in the TST tissue – all indications of cell death with leakage of cell matrix proteins, most likely also including self nucleic acids, at the site. Therefore, this mode of activation could be present and lead to pDC activation at the TST site as well. In line with previous studies (29, 30) we found that phenotypic maturation and IFNα secretion could be induced in pDCs by exposure to complexes of LL37 and human DNA *in vitro*. mDCs did not respond to stimulation by LL37-DNA, but if exposed to supernatants from pDCs exposed to LL37-DNA the mDCs matured. Also, mDCs exposed to pDC supernatants showed significantly reduced capacity to stimulate allogeneic $CD4^+$ T cell proliferation (Figure 10a-b). Neither of these effects could be reproduced by exposure of the mDCs to recombinant IFNα alone. Further, LL37/DNA-exposed pDCs directly suppressed T cell proliferation in pDC:T cell co-cultures. In addition to reducing proliferation of T cells, mDCs exposed to pDC supernatants altered the profile of cytokine production in the T cells, reducing the production of IL-2 (Figure 10c), while production of TNFα and IFNγ remained unchanged, and IL-6 and IL-10 were elevated. Thus, pDCs activated by LL37/DNA complexes in the TST site may regulate immune activation by directly or indirectly skewing cytokine production and controlling proliferation of T cells.

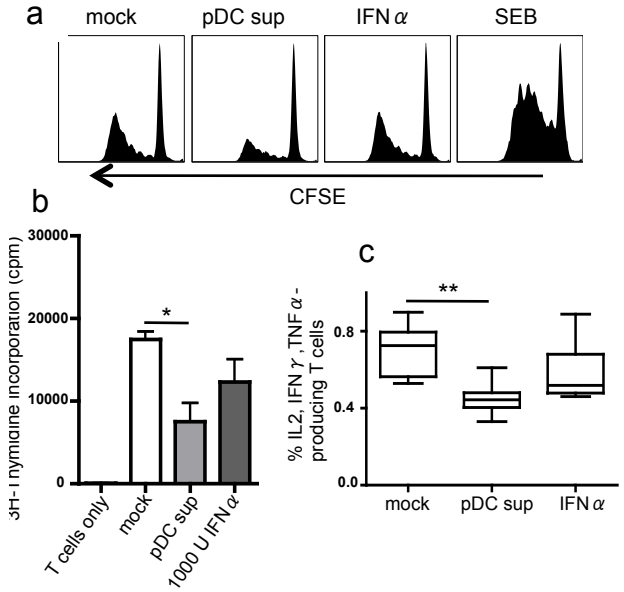

Figure 10. Reduced T cell stimulatory capacity of mDCs exposed to supernatants from pDC stimulated by LL-37/DNA complexes, seen as reduced proliferation of activated T cells, measured by CFSE dilution (a) and thymidine incorporation (b), as well as reduced cytokine production, measured as percentage of T cells staining positive for indicated cytokines by intracellular FACS staining (from figure 3 and 5, paper II).

In previous studies, pDCs have been shown to regulate the magnitude of the immune response, for example by limiting T cell proliferation (240) or inducing T regulatory cells (241, 242). pDCs have also been shown to have different immune-stimulatory capacities at different differentiation/activation stages (243-245). It is likely that the nature of the antigen as well as the cytokine milieu at the site of antigen exposure play a role in shaping the pDC function. Our results indicate that pDCs stimulated by LL37-DNA complexes, that are very likely present at the TST site, affect mDCs to reduce their antigen uptake and become less potent in activating T cells, actually limiting T cell proliferation and skewing the cytokine profile of the T cells away from a strong effector profile. The stimulated pDCs also had a direct effect on T cell, limiting their proliferation. We therefore believe that the role of pDCs at the TST is, at least in part, to control and limit the inflammation. It is not clear how the immune-stimulatory and

immune-regulatory capacities of the pDCs in this setting would come together. Potentially a response initiated by the pDCs could, as it becomes chronic, be propagated by other mechanism while the role of the pDCs instead would change towards a more tolerogenic role. Interestingly, pDCs have been shown to exert tolerogenic functions in other DTH reactions, like graft versus host disease and tolerance to liver grafts (136, 244, 246), and in the maintenance of tolerance to food antigens (247), but in conflicting studies have also been shown to mediate graft-versus-host disease (104). As for the specific roles and modes of recruitments of pDCs in the TST, further studies, preferably with varying kinetics, are required to decipher these mechanisms.

8.5 DIFFERENTIAL PPD UPTAKE BY DC SUBSETS *IN VITRO*

In **paper II**, we also characterized the interactions of PPD and different DC subsets. PPD consists of proteins from seven strains of MTB and is sterilized and tested to not induce immune responses in non-sensitized individuals (248). However, there have been some data showing that PPD can induce maturation of MDDCs (249). In our study, we performed a series of experiments exposing primary human DCs isolated from blood to PPD. Neither mDCs nor pDCs responded with maturation or cytokine production after exposure to even high concentrations of PPD *in vitro*. We also analyzed PPD uptake capacity by the DC subsets. We found that pDCs showed significantly lower uptake of PPD compared to both the conventional $CD1c^+$ mDCs and the cross-presenting $CD141^+$ DCs (Figure 11). The PPD uptake data are in line with earlier observations where pDCs have been shown to possess poorer uptake capacity of other antigens compared to the mDC subsets (250).

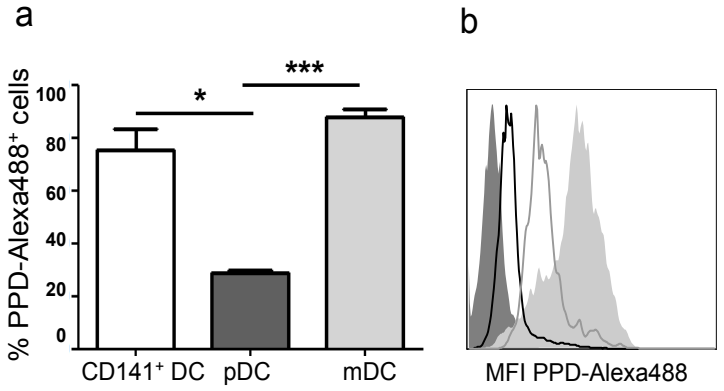

Figure 11. PPD uptake in DC subsets isolated from blood, a) percentage PPD-Alexa 488$^+$ DC in the various subsets and b) Alexa 488 MFI of mock (filled dark grey), pDC (black line), CD141$^+$ DC (grey line) and mDC (filled light gray). (from figure 4, paper I)

In conclusion, since pDCs showed much lower uptake of PPD their primary contribution in the TST reaction may not pertain to antigen processing and presentation to T cells. Instead, they may have an important bystander effect of conditioning surrounding mDC subsets. As discussed above, this bystander effect could consist of a suppressor function to restrain otherwise potentially harmful, excessive immune activation.

8.6 DC RECRUITMENT TO SKIN TEST SITES IN HIV-1+ INDIVIDUALS AND AIDS PATIENTS

As mentioned, the function (or dysfunction) of DCs in HIV-1 infection is not clear. However, there are multiple reports showing there is depletion of blood DC subsets in

HIV-1 infected individuals (discussed in more detail in section 6.3.). Antigen skin tests including TST are used to monitor the integrity of the cellular immune system during HIV-1 disease. The main objective of **paper III** was therefore to study whether the robust infiltration of DCs to TST sites as found in **paper II** could also be observed in HIV-1+ individuals. In addition, we extended our analyses to include alternative skin tests using antigens other than PPD.

For the studies of **paper III**, biopsies from both HIV-1+ and HIV- individuals were collected. One of our study cohorts comparing TST responses was again recruited in Khayelitsha Township, Cape Town, South Africa. In addition, we included another cohort from the University Hospital of Cleveland, which received mumps and *Candida albicans* skin tests. *C. albicans* and mumps virus are examples of antigens that most people have pre-existing immunity to, and skin tests for these antigens are therefore often used in the assessment of cellular immune response capacity. Amongst the HIV-1 infected individuals in all our cohorts, we had separate groups of subjects with asymptomatic HIV-1 infection and those with AIDS. All study subjects had a biopsy taken from the antigen test site as well as a control site.

The stainings of the control skin biopsies revealed that the steady state levels of DC marker expression in HIV-1+ individuals did not differ from that of the healthy individuals. This data complement previous studies demonstrating unaffected Langerhans cell counts in HIV-1+ patients (251). Hence the reduction in blood DCs during HIV-1 infection does not seem to be reflected in the skin. As expected, there was a significant increase of mDC subsets and pDCs at the TST sites as opposed to the control sites. The recruitment of $CD11c^+$ DCs including the $CD141^+$ cross-presenting subset was significantly lower in HIV^+ subjects than in healthy controls. In addition, there was also a significant decrease of the recruitment of $CD303^+$ pDCs in the HIV-1+ cohort (Figure 12). The difference was most pronounced in AIDS patients.

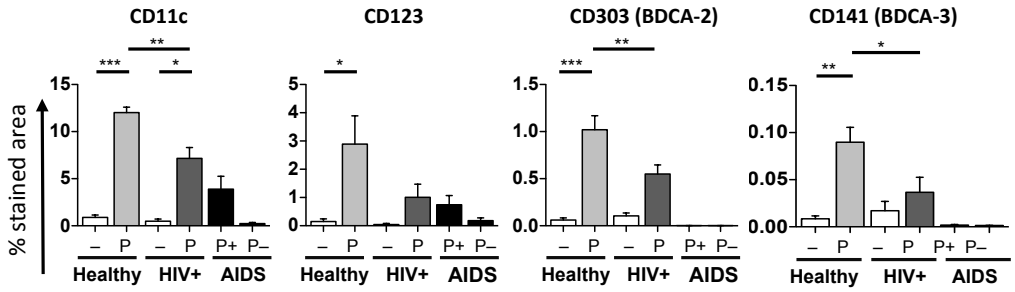

Figure 12. Expression of selected DC subset markers in skin punch biopsies from TST sites of healthy as well as HIV-1+ individuals, including subjects with AIDS. - = control site, P = PPD injected site, P+ = AIDS patient with positive TST, P- = AIDS patient with negative TST. (from figure 1, paper III)

As mentioned above, in **paper III**, we also performed a similar analysis of biopsies from healthy and HIV-1+ individuals that had received antigen skin tests based on antigens from *C. albicans* and the mumps virus. We found that the mumps virus skin test showed very similar results as the PPD test did, namely that there was a substantial recruitment of DC subsets in both healthy and HIV-1+ individuals in response to antigen injection, but that there was a trend toward lower DC levels in the HIV-1+

group. In contrast, the results from the *C. albicans* skin tests implied the reverse pattern i.e. the DC recruitment seemed to be increased in the HIV-1+ individuals. As discussed below, we hypothesize that these differences can be explained by the magnitude of the T cell responses to the various antigens.

8.7 DC INFILTRATION CORRELATES WITH THE LEVEL OF T CELL RECRUITMENT

There was significant recruitment of $CD3^+$ T cells in response to the TST (Figure 13a) in healthy individuals. Importantly, the number of recruited pDCs and mDCs at the TST sites correlated to the number of infiltrating $CD3^+$ T cells (Figure 13b). By immunofluorescent double-stainings, DCs and T cells were found closely adjacent in the tissue.

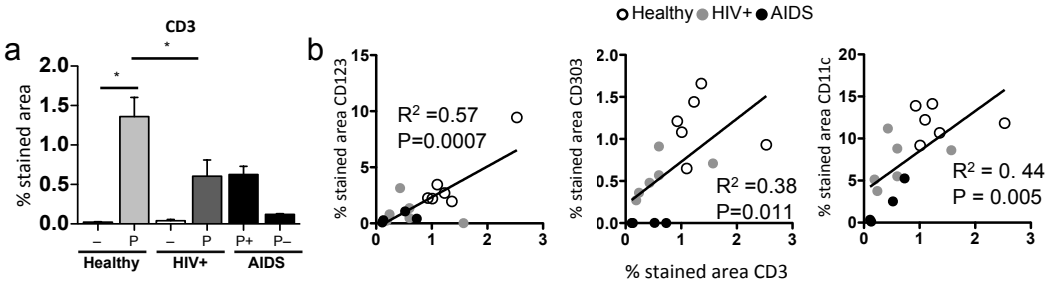

Figure 13. a) The recruitment of $CD3^+$ T cells was significantly impaired in HIV+ individuals. P indicates PPD injected site, as opposed to saline injected controls. P+ = AIDS patient with positive TST, P- = AIDS patient with negative TST. B) The levels of infiltrating pDC, illustrated by both CD123 and CD303 expression, and $CD11c^+$ mDCs, significantly correlated to the $CD3^+$ T cell infiltration, as shown by a correlation of the % of stained area for both markers.

We hypothesized that the level of T cells that infiltrated the site of the skin antigen test determined the DC recruitment. This would mean that when the frequency of antigen-specific T cell response is high, more DCs would be recruited to the site of antigen injection. As studied more in detail in **paper II**, administration of the skin antigen tests per se does not induce any immune activity, but if there are antigen-specific T cells available they will accumulate at the site of injection which in turn induces substantial inflammation, with subsequent recruitment of additional immune cells, including DCs. Maintained *C. albicans*-specific T cell responses in asymptomatic HIV infection (252, 253), as well as more preserved reactions to antigen skin tests compared to PPD and mumps (254) have been reported in HIV-1+ people. The comparably well-preserved *C. albicans*-specific T cell responses in the HIV-1+ individuals could relate to there being a higher level of exposure to *C. albicans* antigens in these people. It is well documented that there is increased permeability of the gut mucosa during HIV-1 infection, which results in translocation of endotoxins from the gut (255). This likely means that there is also translocation of *C. albicans* antigens, which could explain the elevated levels of Abs to *C. albicans* in HIV-1+ individuals (256). Thus, it is probable that there is increased exposure of antigens from *C. albicans* to the gut draining lymphatics, which could lead to persistent stimulation of memory T cell responses. In studies of blood T cell responses to various antigens in HIV-1+ individuals, the response to *C. albicans* has been shown to be of higher magnitude than that to several viral antigens (257). In

contrast, PPD-specific memory T cells have been shown to be preferentially depleted in HIV-1+ infection (258). Our findings that the T cell infiltration at the PPD injected test sites seem to be reduced compared to the *C. albicans* test sites are in line with these studies. As mentioned, the level of recruited DCs correlated with the T cell recruitment to the TST site, suggesting that the DC recruitment might be influenced by the T cell recruitment. The high recruitment of DCs in response to the *C. albicans* test supports this theory, and also indicates that the functional capacity of the DCs, regarding migration to the skin, is not impaired in HIV-1 infection. Instead the lower recruitment detected in the PPD test in the HIV-1 infected individuals compared to the healthy controls probably reflects the fact that the PPD-specific T cells are reduced and/or defective during HIV-1 disease. Unfortunately, data on the levels of T cells specific to the different antigens in the study subjects were not available. However, CD45RA, CD45 RO and Ki-67 staining showed that most of the infiltrating cells expressed CD45 RO and several of them expressed Ki-67 suggesting that the majority of infiltrating T cells were cycling memory T cells. Further studies addressing the kinetics of the recruitment of the various cell types are required to confirm whether the reduced DC infiltration reflects the depletion of blood DCs, indicates functional impairment affecting the migratory capacity of the DCs, or is secondary to reduced T cell recruitment.

9 CONCLUSIONS

In the studies in **paper I** we developed protocols to facilitate isolation of skin DCs, and methods to target immature DCs in a skin explant model. We concluded that these experimental systems allowed us to perform studies on a larger scale than previously possible, and that antigen uptake by skin DCs could be visualized *in situ*. Our data indicated that in this system, most antigens are likely to be taken up by a heterogeneous group of dermal DC subsets. Taken together, **paper I** provided improved tools for studies of antigen exposure of skin DCs.

In **paper II**, we showed that there was a robust and rapid infiltration of multiple DC subsets, including the cross-presenting $CD141^+$ myeloid subset and pDCs, to the indurations induced by positive tuberculin skin tests. This is particularly interesting since several of these DCs do not normally reside in the skin. We found that components in the positive indurations, such as self-DNA leaking out from dying cells and the antimicrobial peptide LL-37, induced activation, including production of IFNα, of pDCs *in vitro*. We further showed that stimulation of mDCs by supernatants of LL37-DNA-activated pDCs, reduced both their antigen uptake capacity and ability to activate T cells. This led us to speculate that the role of the pDCs at the skin antigen test site is mainly regulatory.

In **paper III**, we found that antigen skin test sites of HIV-1 infected individuals also had a substantial recruitment of DCs. In contrast to what has been reported in the blood, there was no reduction of resident skin DCs in the HIV-1 cohort at steady state. The level of DC recruitment to the antigen skin tests site correlated with the level of T cell infiltration. The data therefore suggested that mobilization of DCs to the skin is dependent on memory T cell activation and recruitment, rather than the DC count in blood, and that the blood DCs in HIV-1 infected individuals retain their capacity to migrate to a site of inflammation.

In summary, we hope our studies have contributed some clues to the innate immune functions of various DC subsets in human skin. While there is still much to learn about the role of DCs in health and disease, every piece of the puzzle brings us closer to a better understanding, and immense knowledge has been gained since the fairly recent realization that there is significant complexity among the DC subsets and their functional specialization in the human immune system.

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