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COMPARTMENTALISATION OF THE IMMUNE RESPONSE IN HUMAN SKIN: CROSS-TALK BETWEEN DENDRITIC CELLS AND T CELLS IN HEALTHY CONDITIONS AND IN PSORIASIS

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Cover picture: confocal microscopy image of dendritic cell-T cell aggregates in the skin. CD3 (magenta), CD74 (white), Foxp3 (blue).

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Compartmentalisation of the immune response in human skin: cross-talk between dendritic cells and T cells in healthy conditions and in psoriasis
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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to my dad

"You've got the future on your side
You're gonna be fine now
I know whatever you decide
You're gonna shine"

Dream Theater, "The answer lies within"

ABSTRACT

Human skin is highly compartmentalised and distinct subsets of dendritic cells (DCs) and T cells reside in epidermis and dermis. Alterations in the composition of DCs and T cells have been observed in psoriasis, a common cytokine-driven focal inflammatory skin disease. In this thesis we explore the functional profile of epidermal and dermal DCs and T cells in different phases of psoriasis, and how different cytokines present in homeostasis and inflammation affect the development of tissue resident memory T (Trm) cells.

PAPER I: In lesional psoriasis, langerin$^-$ DCs accumulated in the epidermis. Both Langerhans cells (LCs) and epidermal DCs (eDCs) displayed upregulated pro-inflammatory genes and produced IL-23 after stimulation with ligands binding to toll-like receptor (TLR) 4 or 7/8. Elevated gene expression of *IL23A* and *IL15* in LCs combined with their capacity to produce IL-23 upon TLR 7/8 stimulation suggested that LCs maintained their pro-inflammatory profile in resolved psoriasis.

PAPER II: Granzymes (Gzms) and perforin are produced by cytotoxic T cells. Here we show that epidermal T cells produced GzmA in absence of perforin within psoriatic lesions. IL-17-stimulated human keratinocytes, in presence of GzmA, upregulated the production of CXCL1, CXCL12 and CCL4, involved in neutrophil and lymphocyte recruitment, therefore highlighting a new pathway of T cell driven recruitment of immune cells.

PAPER III: In resolved psoriasis, epidermal resident T cells and LCs maintain a pronounced pro-inflammatory profile, whereas dermal T cells are less inflammatory. We observed an enrichment in the expression of the regulatory molecules *FOXP3*, *CTLA4* and *PD1* in T cells, and an increased IDO1 expression and IL-10 production by DCs. Clusters of Foxp3$^+$ T cells and DCs were identified in the skin of UVB treated patients, hinting at a possible interaction between regulatory T cells and tolerogenic DCs in the dermis of resolved psoriatic lesions.

PAPER IV: Healthy epidermis and dermis harbour phenotypically distinct subsets of Trm cells. Here we focused on the phenotypic and functional plasticity of skin CD8 Trm cells. Dermal Trm cells, mainly CD69$^+$CD103$^-$CD49a$^-$, upregulated CD103 or both CD103 and CD49a after stimulation with TGF-β, IL-2 and IL-15. TCR engagement increased the proportion of CD103$^+$CD49a$^+$ cells. Conversely, epidermal Trm cells exposed to the same conditions did not change their phenotype, but their functional profile was affected by IL-17 polarising cytokines.

Collectively, these studies highlight the high pro-inflammatory potential of epidermal DCs and T cells during different phases of psoriasis. In contrast, dermal cells acquire tolerogenic features in resolved disease. Moreover, we show that the functionality of Trm cells is affected by their surrounding milieu. These results encourage to take compartmental immune responses into consideration in the design of vaccines and targeted therapies.

LIST OF SCIENTIFIC PAPERS

I. **Dynamic changes in resident and infiltrating epidermal dendritic cells in active and resolved psoriasis**
Elisa Martini, Maria Wikèn, Stanley Cheuk, Irène Gallais Sérézal, Faezzah Baharom, Mona Ståhle, Anna Smed Sörensen, Liv Eidsmo
Journal of Investigative Dermatology, 2017 Apr;137(4):865-873.

II. **Granzyme A potentiates chemokine production in IL-17 stimulated keratinocytes**
Stanley Cheuk, Elisa Martini, Kerstin Bergh, David Chang, Bence Rethi, Mona Ståhle, Liv Eidsmo
Experimental Dermatology, 2017 Sep;26(9):824-827.

III. **Anti-inflammatory features of T cells and dendritic cells residing in the dermis of psoriasis-treated patients**
Elisa Martini, Stanley Cheuk, Elena Hoffer, Maria Wikèn, Irène Gallais Sérézal, Liv Eidsmo
Manuscript

IV. **Development and plasticity of human skin resident T cells**
Elisa Martini, Jaanika Kärner, Stanley Cheuk, Ebba Detlofsson, Yenan Bryceson, Liv Eidsmo
Manuscript

APPENDIX

I. **Human Langerhans cells with pro-inflammatory features relocate within psoriasis lesions**
Liv Eidsmo, Elisa Martini
Review - *Frontiers in Immunology*, 2018 Feb,22; 9:300.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Resident T cells in resolved psoriasis steer tissue responses that stratify clinical outcome
Irène Gallais Sérézal, Cajsa Classon, Stanley Cheuk, Mauricio Barrientos-Somarribas, Emma Wadman, Elisa Martini, David Chang, Ning Xu Landén, Marcus Ehrström, Susanne Nylén, Liv Eidsmo
Journal of Investigative Dermatology, 2018 Mar.

HIV-infected women have high numbers of CD103-CD8+ T cells residing close to the basal membrane of the ectocervical epithelium
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IL-22 binding protein regulates murine skin inflammation
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Experimental Dermatology, 2017 May;26(5);444-446.

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Immunity, 2017 Feb;46(2):287:300.

Fetal CD103+ IL-17–producing group 3 innate lymphoid cells represent the dominant lymphocyte subset in human amniotic fluid
Nicole Marquardt, Martin Ivarsson, Erik Sundström, Elisabet Åkesson, Elisa Martini, Liv Eidsmo, Jenny Mjösberg, Danielle Friberg, Marius Kublickas, Sverker Ek, Gunilla Tegerstedt, Åke Seiger, Magnus Westgren, Jakob Michaëlsson
The Journal of Immunology, 2016, Oct;197(8):3069-3075.

CONTENTS

LIST OF ABBREVIATIONS

αCD3	anti-CD3
AMP	Anti microbial peptide
APC	Antigen presenting cell
αTNF	anti-tumor necrosis factor therapy
C5aR	Complement component 5a receptor
CARD14	Caspase recruitment domain family member 14
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLA	Cutaneous lymphocyte-associated antigen
CTLA-4	Cytotoxic T-lympocyte antigen 4
CXCR	C-X-C chemokine receptor
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
EpCAM	Epithelial cell adhesion molecule
ERAP	Endoplasmic reticulum aminopeptidase 1
FACS	Fluorescence-activated cell-sorting
FBS	Fetal bovine serum
FLT3	Fms like tyrosine kinase 3
FMS	Feline McDonough Sarcoma
Foxp3	Forkhead box protein P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KLRG1	Killer cell lectin-like receptor subfamily G1
LC	Langerhans cell
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell-sorting
MHC	Major histocompatibility complex
NF-kB	Nuclear factor kappa-B
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PEST	Penicillin Streptomycin

PMA	Phorbol 12-myristate 13-acetate
PUVA	Psoralen and ultraviolet A
R848	Resiquimod
RPMI	Roswell Park Memorial Institute
slan-DC	6-sulfo LacNAc DC
STAT3	Signal transducer and activator of transcription 3
Tcm cell	Central memory T cell
TCR	T cell receptor
Tem cell	Effector memory T cell
TGF-β	Transforming growth factor beta
Tip-DC	TNF and iNOS producing DC
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
Trm	Tissue resident memory cells
UV	Ultraviolet
XCR1	X-C chemokine receptor 1

1 INTRODUCTION

Our skin is the most important interface with the external environment, as it is both part of the image we provide to the society and forms an important biological barrier. As such, suffering from skin diseases is associated with unique challenges that bring together the discomfort provoked by the disease itself and a considerable psychological burden due to issues in coping with the body image.

This thesis is an attempt to get a better understanding of the pathomechanisms of psoriasis and its key players within human skin. To achieve this, a high-resolution approach was employed by characterising dendritic cells and T cells from epidermis and dermis as separate entities, instead of analysing pooled cells in full thickness skin. Moreover, I decided not to distance myself from human immunology, despite the many limitations. Hopefully, a better understanding of the functional shades of dendritic cells and T cells from the two skin compartments would lead, one day, to the development of targeted therapeutical strategies for all the patients.

1.1 THE SKIN

The outermost barrier of the body, the skin, is a complex structure where multiple cell types cooperate to ensure the maintenance of the body temperature and hydric balance, the synthesis of vitamin D and the protection against external agents. The complexity of the skin is evident in its structural organisation containing three distinct anatomical compartments. Epidermis, dermis and hypodermis are connected by different types of skin-associated appendages, such as hair follicles, sebaceous glands and sweat glands. The epidermis is the outermost layer of the skin and is exposed to the external environment. The main cellular components of the epidermis are keratinocytes, actively proliferating in the stratum basale, the lowest part of the epidermis, and progressively differentiating until they complete their maturation into corneocytes in the stratum corneum, the apical part of the epidermis. Distinct subtypes of the keratinocyte-derived protein Keratin are expressed in the stratum basale, spinosum, granulosum and corneum, facilitating visualisation of altered keratinocyte differentiation in skin diseases. Once in the stratum corneum, the keratinocytes/corneocytes have lost the nucleus and the internal organelles and form a tight barrier that impedes the loss of water and forms a physical obstacle to the entrance of pathogens. The corneocytes persist in the stratum corneum for about 20 days (Wilhelm et al., 1990) before shedding off. The epidermis additionally contains a small population of melanocytes within the basement membrane, whose main function is to produce melanin. Melanin absorbs UV radiations and is delivered to keratinocytes to protect their nuclei from UV damage (Kanitakis, 2002).

The dermis is situated below the epidermis and is mainly composed by connective tissue made of collagen and elastin, both components produced by skin fibroblasts. The dermis harbours a variety of anatomical structures interacting with the rest of the body. Blood and lymph vessels in the dermis act as gateways for cell trafficking to and from the skin. Nerve endings reach the dermis and the epidermis and transmit a wide range of perception of stimuli coming from the external environment. Sweat and sebaceous glands, as well as the innermost part of the hair follicle, are also located in the dermis and serve the important function of regulating the body temperature (figure 1.1). The skin is one site of Vitamin D synthesis and thus also acts as an endocrine organ. As the first interface with the external environment, several immune cells inhabit the skin in healthy conditions: dendritic cells (DCs), Langerhans cells (LCs), macrophages, T cells and mast cells are the main components of the innate and adaptive immune system that reside in the skin. Moreover, additional cell types such as neutrophils and blood derived T cells and DCs infiltrate the skin during inflammation. Resident non-immune cells such as keratinocytes and fibroblasts participate in the immune defence mechanisms by secreting pro-inflammatory and immune cell recruiting factors in case of infection or inflammation.

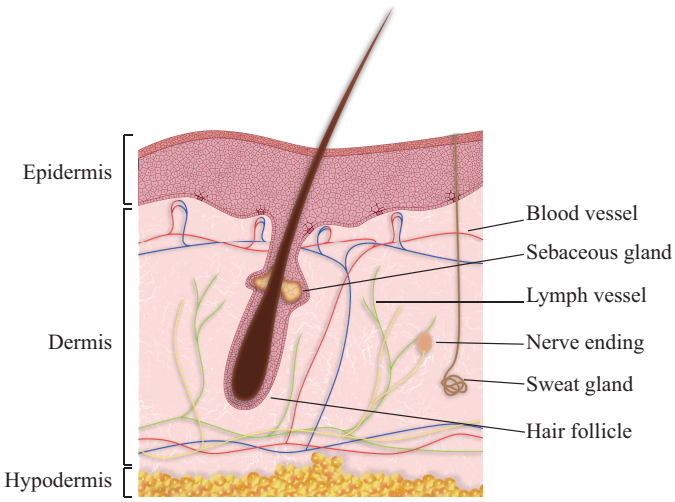

Figure 1.1. The skin structure and its components.

As functional studies on immune cells are mainly performed in mice, it is important to understand the structural and cellular differences existing between the two species. Mouse skin is densely covered in fur and the epidermis is composed of fewer layers compared to human skin. On the contrary, human skin has a larger interfollicular area and is thicker compared to mouse skin. LCs are the sole professional antigen presenting cells present in both mouse and human epidermis, whereas the dermis of mice and humans contains two distinct populations of dermal DCs (Segura, 2016). Although the markers used to describe DCs are not the same between the two species, their functional profile are comparable, as will be described in more detail further on in this thesis. The most strikingly difference in the immune cell composition of the skin of mice and humans can be found in the T cell compartment. The skin of mice bred in research facilities contains mainly γδ T cells and $\gamma\delta^{+}$ dendritic epidermal T cells (DETCs), whereas αβ T cells are prominent in human skin and

human epidermis does not contain DETCs (Pasparakis et al., 2014). These observations have important implications when establishing mouse models of skin diseases, as the basis of the pathological immune mechanisms in mouse skin inflammation does not necessarily reflect what happens in human skin, as will be discussed later for psoriasis.

As the very first interface towards the external environment, keratinocytes actively participate to the skin immune defence by sensing the presence of pathogen associated molecular patterns (PAMPs) through Toll-like receptors (TLRs). TLRs are invariant receptors sensing structurally conserved molecules present on pathogens (Ermertcan et al., 2011, Medzhitov, 2001). Keratinocytes express a wide range of surface and intracellular TLRs (Kollisch et al., 2005, Miller, 2008, Nestle et al., 2009), that make them capable of alerting the immune system and initiate the inflammatory process after bacterial or viral challenge. As a consequence of TLR activation, keratinocytes produce pro-inflammatory cytokines and chemokines whose type, magnitude and kinetics depend on the type of TLR stimulation received by keratinocytes (Lebre et al., 2007). Keratinocytes are the main producers of anti-microbial peptides (AMPs) in the skin, that fight bacterial infections by interfering with the bacterial cell wall (Zhang and Gallo, 2016). The production of AMPs by keratinocytes is an interesting example of the bilateral communication between epidermal immune and non-immune cells. Induction of several AMPs belonging to the S100 family is mediated by T cell derived IL-17 and IL-22 (Liang et al., 2006). On the other side, the AMPs beta defensins serve a chemoattractant for T cells and DCs (Yang et al., 1999) and monocytes (Rohrl et al., 2010). Moreover, beta defensins stimulate keratinocytes to produce chemoattractant for lymphocytes and monocytes (Niyonsaba et al., 2007), and induce the expression of maturation markers in blood DCs (Funderburg et al., 2007) and in LCs generated from peripheral blood (Ferris et al., 2013). Similarly to defensins, the AMP LL-37 modulates chemokine production in keratinocytes (Chen et al., 2013, Nijnik et al., 2012) and induces DC maturation (Davidson et al., 2004), exemplifying the ongoing cross-talk between keratinocytes and immune cells.

1.2 LANGERHANS CELLS: THE OUTPOST OF THE SKIN IMMUNE DEFENCE

1.2.1 Langerhans cell ontogeny

Langerhans cells reside in the outermost layer of the skin and pierce through keratinocytes' tight junctions with their dendrites to scout the skin surface and sense the antigens that penetrated into the stratum corneum (Kubo et al., 2009). In homeostasis, LCs are the sole professional antigen presenting cells (APCs) within the epidermis and are second to keratinocytes in terms of cellular frequency (Kanitakis, 2002, Kashem et al., 2017, Merad et al., 2008). LCs have a peculiar ontogeny compared to the other populations of skin resident DCs. LCs are seeded in the epidermis during embryonic development (Collin and Milne, 2016, Merad et al., 2008) and their local development within the skin depends on IL-34,

ligand of CFS-1R produced by epidermal keratinocytes (Wang and Colonna, 2014, Wang et al., 2012). Extensive studies in multiple experimental settings also showed that LCs self-renew throughout life within the epidermis, contrary to the dermal DC population that is constantly replenished by circulating precursors. The clear demonstration of the self-renewal capacity of LCs came from research performed by Merad and colleagues: lethally irradiated mice, where all LCs and DCs are depleted, maintain host-derived LCs following reconstitution with congenital bone marrow cells. On the contrary, DCs in the spleen, liver and kidney are replaced by donor-derived cells (Merad et al., 2008, Merad et al., 2002). Further experiments based on parabiotic mice performed by the same authors reached the same conclusions (Merad et al., 2002). Interestingly, the same study also showed that $CCR2^{+}$ LCs of donor origin successfully colonised the epidermis after UV exposure and were present in the skin for at least 12 weeks (Merad et al., 2002). Further studies in mice confirmed that the strong damage produced by HSV infection or UV irradiation causes LC depletion and replenishment of the epidermal LC pool by monocytes (Eidsmo et al., 2009, Ginhoux et al., 2006, Sere et al., 2012), transiently infiltrating the skin, and subsequently by bone marrow precursors (Sere et al., 2012).

Clever observations on transplanted patients, based on HLA mismatch between donor and recipient, seemed to confirm the longevity of LCs in a human setting. By following one patient after hand transplantation over a period of time reaching 10 years from the transplant, it has been shown that epidermal LCs remained of donor origin and were not replaced by cells of recipient origin (Kanitakis et al., 2011, Kanitakis et al., 2004) but the evidence on the dynamics of LC replenishment after an inflammatory insult in humans is so far lacking.

1.2.2 TGF-β in LC physiology

TGF-β appears to be one of the master cytokines in the regulation of LC physiology reflected by the complete absence of LCs in the skin of the TGF-β1 null mice ($TGF\text{-}\beta1^{-/-}$) (Borkowski et al., 1996, Borkowski et al., 1997). Furthermore, the epidermis of mice lacking the receptors TGF-βRII (Bobr et al., 2012, Kaplan et al., 2007) and TGF-βR1 (Kel et al., 2010) presents a severe loss of LCs. Kel et al presented the interesting observation that LCs in the skin of the TGF-βR1 mouse seed and expand in the epidermis in the first days of life, although in reduced numbers compared to the wild-type control. However, after this initial expansion, LCs cannot keep their residency in the absence of the functional TGF-βR1, and gradually disappear from the epidermis (Kel et al., 2010). These results suggest that TGF-β signalling plays a crucial role in ensuring the residency of LCs in the epidermis after the cells seed and expand at the beginning of life (Chorro et al., 2009, Schuster et al., 2009). Indeed, LCs in the absence of TGF-β or TGF-β receptor-signalling display an increased migratory potential by expressing CCR7 (Bobr et al., 2012, Kel et al., 2010). Of relevance is the role of keratinocytes in ensuring the residency of LCs by converting the latent form of TGF-β to the active form (Mohammed et al., 2016). It is difficult to explore whether these basic biologic

processes discovered in mouse hold true for the human species, but it has been shown that TGF-β production in embryonic skin precedes the acquisition of langerin and CD1a markers by epidermal HLA-DR$^+$ cells (Schuster et al., 2009). Another hint comes from the observation that patients suffering from hypertension treated with losartan, known to diminish the levels of TGF-β (Bar-Klein et al., 2014, Campistol et al., 1999, Khalil et al., 2000), display reduced numbers of LCs in the epidermis (Mohammed et al., 2016). In *in vitro* experimental settings, TGF-β has been used to differentiate LC-like cells from CD34$^+$ circulating precursors from peripheral blood or cord blood, together with different combinations of GM-CSF, TNF-α and IL-4 (Geissmann et al., 1998, Jaksits et al., 1999, Strobl et al., 1996). The criteria used to discriminate LCs from DCs after stimulating CD34$^+$ precursors are the presence of structures resembling Birbeck granules and the expression of langerin, CD1a and E-cadherin. By adding TGF-β to the stimulation cocktail, the cell yield of LCs vs DCs increases compared to the original cocktail comprising only GM-CSF and TNF-α (Caux et al., 1992, Caux et al., 1996, Strunk et al., 1996). This observation also stresses the importance of TGF-β in relation to LCs.

1.2.3 LC phenotype and *in vitro* models

The expression of langerin (CD207) and the presence of Birbeck granules are the key factors that distinguish LCs from any other cell type in mice and humans (Birbeck et al., 1961, Kissenpfennig et al., 2005a, Mc Dermott et al., 2002, Reynolds and Haniffa, 2015, Valladeau et al., 2000). Human LCs also express high levels of the surface markers CD1a (Fithian et al., 1981, Klareskog et al., 1977, Rowden et al., 1977) and EpCAM (Eisenwort et al., 2011, Gaiser et al., 2012). Despite this ample choice of surface markers to identify LCs in healthy conditions, it is important to bear in mind that marker expression may vary on both LCs and other DCs in inflammatory conditions, as shown in atopic eczema (Wollenberg et al., 1996). Through the years, experimental models to study human LC functionality have been developed and used: LCs differentiated from circulating precursors, migrated LCs from skin explants and freshly isolated cells after enzymatic treatment of the epidermis. Choosing the appropriate *in vitro* system for functional studies can be challenging, as each of these models display advantages and disadvantages, in particular in cell yield and responsiveness to stimulation. Cell yield is rarely an issue when working with blood derived cells and CD34$^+$ circulating precursors can be differentiated into cells expressing high levels of CD1a and langerin, deceivingly identical to epidermal LCs (Geissmann et al., 1998, Jaksits et al., 1999, Strobl et al., 1996). However, these cells display a mature phenotype, with high expression of CD80 and CD86 (Caux et al., 1992, Klechevsky et al., 2008), high cross-presentation capacity (Klechevsky et al., 2008) and unresponsiveness to thymic stromal lymphoprotein (Nguyen et al., 2011), characteristics that functionally differentiate them from skin-derived LCs. Migrated cells obtained after short term culture of skin explants give acceptable cell-yield of skin-derived LCs, but with a mature phenotype like blood-differentiated LCs (Klechevsky et al., 2008). The immature features of LCs obtained after enzymatic treatment

of the skin (de Witte et al., 2007, Fujita et al., 2009, Peiser et al., 2003, Pena-Cruz et al., 2001) make them appropriate for studies on the changes that LCs undergo after stimulation or antigen encounter. However, enzymatic isolation of skin-LCs results in a low and even prohibiting number of cells recovered after the tissue digestion, especially when the intent is to examine the function of LCs obtained from patient material. LC-like cell lines also exist, mimicking both mouse and human LCs, although their usage is not as widespread as other LC *in vitro* models. The MUTZ-3 cell line is a cytokine-dependent cell line where cells acquire the expression of langerin and CD1a after culture with TGF-β. Transcriptomic analysis of MUTZ-3 LCs compared to LCs, blood-derived or skin-derived DCs and *in vitro* DCs (like monocyte-derived DCs) showed that they cluster together with skin DCs rather than with blood DCs, but their gene expression profile resembles more mature monocyte-derived DCs rather than skin LCs (Harman et al., 2013).

1.3 DERMAL DENDRITIC CELLS: SURVEILLANCE DEEP IN THE SKIN

Two populations of conventional dermal (dDCs), distinct from pDCs, inhabit the dermis of mice and humans in the steady state. The discovery of conventional DCs is attributed to Ralph Steinman, that first described DCs in lymphoid organs (Steinman and Cohn, 1973), but only in the nineties these cells have been detected in the dermis (Lenz et al., 1993). Dermal DCs are defined by their expression of surface markers. The most numerous population is composed by $CD1c^+$ DCs in human skin, whose mouse orthologue is the $CD11b^+$ population. In humans, $CD1c^+$ DCs also express CD1a, but at a lower level compared to LCs, and CD11c, although this marker does not uniquely define $CD1c^+$ DCs since it's also expressed by other monocytes (Collin et al., 2013) and $CD141^+$ DCs (Haniffa et al., 2012). Human $CD141^+$ DCs are a small subset of recently discovered DCs. They are better defined by their expression of XCR1, since CD141 is upregulated by other cell types in inflammatory conditions (Kashem et al., 2017). These DCs are the human orthologue of the murine $CD103^+$langerin$^+$ DCs. A small subset of langerin-expressing non-LCs has been identified in the dermis and across different organs and, interestingly, these cells are more related to $CD1c^+$ DCs than to $CD141^+$ DCs (Bennett, 2015, Bigley et al., 2015). A circulating counterpart of both $CD1c^+$ and $CD141^+$ DC subsets, expressing the same markers, can be identified in blood (Haniffa et al., 2015). Both human dDC populations develop from a common precursor present in bone marrow, blood and peripheral lymphoid organs and, as in mouse, their development requires the fms (feline McDonough sarcoma) like tyrosine kinase 3 ligand (FLT3L) signalling through FLT3 (Breton et al., 2015, Lee et al., 2015). Differently from LCs, dDCs express a wide variety of TLRs (van der Aar et al., 2007), that make dDCs perfectly equipped to sense different types of pathogens penetrating the deeper layer of the skin. Similarly to LCs, the surface expression of CD1c and CD1a makes dDCs capable to present lipid antigens.

1.4 OTHER MYELOID CELLS INHABITING THE SKIN: MACROPHAGES AND MONOCYTES

Macrophages are resident non-migratory cells present in mouse and human skin and can be distinguished from DCs by their autofluorescent properties at the flow cytometer, their adhesion properties when cultured in plastic wells and their surface expression of CD14 and FXIIIa (Haniffa et al., 2015). A seminal study on human dermal macrophages revealed that they are long lived cells with slow turnover rate, inferior to dDCs in activating naïve T cells but capable of activating memory T cells (Haniffa et al., 2009). Their non-migratory properties make skin macrophages distinguishable from $CD14^+$ monocytes, also resident in the skin, but capable of migrating from *ex vivo* dermal explants. Previously considered a part of DC family with high MHC class II expression, migratory $CD14^+$ cells have been shown to be phenotypically and transcriptionally closer to monocytes than to DCs (McGovern et al., 2014).

1.5 T CELLS AND T CELL MEMORY – AN OVERVIEW

T cells, as part of the adaptive branch of the immune system, provide a tailored immune response in the event of an infection by a foreign antigen. $CD4^+$ T cells, or T helper (Th) cells, contribute to the inflammatory process by secreting mediators that recall other immune cells to the site of infection, activate B and T cells or enhance the phagocytic activity of macrophages. $CD8^+$ T cells have cytotoxic functions and kill infected cells after recognition mediated by MHC class I interaction with the T cell receptor. The killing of the target cell is a complex process that involves production of both granzymes (Gzms) and perforin by the cytotoxic T cell, release of these components in the immunological synapse formed between the T cell and the target cell, formation of pores on the membrane of the target cell mediated by perforin and entrance of Gzms inside the target cell (Barry and Bleackley, 2002). Gzms subsequently activate caspase-mediated death programs inside the infected cell, resulting in its killing (Barry and Bleackley, 2002). T cells also exert important regulatory functions by maintaining self-tolerance and suppressing the pro-inflammatory functions of DCs and other T cells. Regulatory T (Treg) cells are $CD4^+$ T cells specialised in these regulatory functions (Josefowicz et al., 2012). The transcription factor Foxp3 is widely used to define Treg cells, although it is not possible to directly link Foxp3 to T cell suppression. Indeed, $Foxp3^+$ "Treg" cells lacking immunosuppressive functions can be identified in some settings (Allan et al., 2007, Tran et al., 2007). In addition, Treg cells can lose the expression of Foxp3 and become pro-inflammatory (Zhou et al., 2009). Treg cells mediate inhibition of the immune response by producing the anti-inflammatory cytokines TGF-β and IL-10 (Josefowicz et al., 2012).

One peculiar function of the members of the adaptive immune system is the ability to generate a memory of previously encountered threats. This allows the body to quickly respond to secondary infection, therefore making the immune response more efficient.

During the primary infection, naïve T cells receiving the activation signal differentiate to effector T cells. After the resolution of the inflammation, the vast majority of the effector cells dies, but a small fraction survives and becomes memory T cells. Central memory T (Tcm) cells and effector memory T (Tem) cells are found in the circulation. Tcm cells express the lymph node homing receptors CD62L and CCR7 and have a high proliferative capacity, whereas Tem do not express CD62L and CCR7, have a lower proliferative capacity but increased cytotoxic capacity (Mueller et al., 2013). More recently, the addition of CX3CR1 to the set of markers used to delineate memory T cell subsets revealed the existence of a population expressing intermediate levels of CX3CR1, as opposed to $CX3CR1^{hi}$ Tem cells and $CX3CR1^{neg}$ Tcm cells. This new population, called peripheral memory T (Tpm) cells, recirculates between blood and tissues and is responsible for the surveillance of peripheral organs (Gerlach et al., 2016).

1.6 TISSUE RESIDENT MEMORY T (TRM) CELLS IN THE SKIN

Tissue resident memory T (Trm) cells are additional members of the memory T cell family. They are non-migratory (Jiang et al., 2012), can survive for a long time in the tissue (Mackay et al., 2012) and confer superior protection in case of secondary infections (Gebhardt et al., 2009). $CD8^+$ Trm cells have been better characterised compared to $CD4^+$ Trm cells. The work included in this thesis focuses on $CD8^+$ Trm cells, thus the caption Trm cells will be used in this thesis to refer solely to the $CD8^+$ T cell subset. Phenotypically, Trm cells are CD62L negative, express CD69 or co-express CD69 and CD103 (Gebhardt et al., 2009, Masopust et al., 2006, Mueller and Mackay, 2016). CD69 is an inhibitor of the sphingosine-1-phosphate receptor, used by T cells to follow the sphingosine-1-phosphate gradient towards the blood stream (Mackay et al., 2015), whereas CD103 binds to E-cadherin, abundantly present in epithelial surfaces (Cepek et al., 1994, Pauls et al., 2001). Another Trm cell marker is CD49a, the ligand for collagen IV present in the basement membrane separating epidermis and dermis (Gebhardt et al., 2009). Although Trm cells are most commonly defined by the expression of CD69, $CD69^-$ cells can be sorted from different human tissues. The transcriptional profile of tissue resident $CD69^-$ cells shows that they are more similar to the blood $CD69^-$ counterpart, whereas $CD69^+$ Trm cells have a distinct transcriptional profile (Kumar et al., 2017).

Studies on congenically transferred $CD69^{-/-}$ or $CD103^{-/-}$ cells in mouse showed that T cells lacking CD69 or CD103 have a lower capacity to be retained in the skin, therefore CD69 and CD103 have an important role in retaining the cells into the tissue (Mackay et al., 2013). Similarly to what has been shown for LCs, TGF-β collaborates with the cell surface markers to maintain Trm cell residency both in the skin and in the gut (Mohammed et al., 2016).

1.6.1 Development of skin Trm cells

The development of skin Trm cells has been well-characterised in mice. Killer cell lectin-like receptor subfamily G1 (KLRG1) negative precursors entering the skin sequentially express CD69 and CD103 and give rise to Trm cells. The CXCR3-dependent infiltration in the epidermis exposes the cells to a cytokine-rich environment, in particular to TGF-β and IL-15, critical factors in the induction of CD69 and CD103 expression (Mackay et al., 2013). This mechanism is not skin specific, as the formation of Trm cells in other tissues such as gut (Sheridan et al., 2014, Zhang and Bevan, 2013) and lungs (Laidlaw et al., 2014) requires the same set of cytokines. One source of IL-15 in the skin has been identified in the keratinocytes forming the hair follicle, and the hair follicle itself is thought to be the entry portal for T cells in the epidermis in mouse skin (Adachi et al., 2015). Similar observations are yet to be made in human skin, but the morphological differences between mouse and human skin might lead to divergent conclusions than what has been observed in mice. Whereas Trm cells in the skin (Mackay et al., 2012) and gut (Casey et al., 2012) do not need the antigen stimulation for the upregulation of CD103, an antigen is required for the induction of CD103 expression in the brain (Wakim et al., 2010) and in the lungs (Lee et al., 2011, McMaster et al., 2018).

Trm cells slowly scout the epidermis in search of foreign antigens (Gebhardt et al., 2011) and, in case of secondary infections, they decrease their speed to proliferate in an antigen-specific way (Beura et al., 2018, Park et al., 2018). In viral infections, recent data convincingly show that Trm cells proliferate in situ (Beura et al., 2018, Park et al., 2018). Additionally, newly formed Trm cells are established in the epidermis and share the epidermal niche with previously formed Trm cells (Park et al., 2018). In inflammation, other T cells with different TCR specificity can be recruited to the challenged site (Schenkel et al., 2013). Very recent reports show that some of these non-specific recruited cells also upregulate tissue resident markers and are detected in the tissue long after the resolution of the inflammation (Beura et al., 2018, Park et al., 2018).

1.6.2 Human skin Trm cells

A high number of T cells reside in the adult human skin in the steady state (Clark et al., 2006). The majority of T cells extracted from skin specimens express the cutaneous lymphocyte-associated antigen (CLA), that is also expressed on blood T cells with skin tropism (Fuhlbrigge et al., 1997). Skin resident T cells are most likely established in the skin as a result of repeated immunological challenges, since embryonic and foetal skin contain less T cells compared to adult skin (Schuster et al., 2012). As already mentioned, human Trm cells express CD69 and CD103 (Cheuk et al., 2017, Watanabe, 2015). Differences in the expression pattern of these markers exist in the two skin compartments: most of the epidermal $CD69^{+}$ cells co-express CD103, whereas dermal Trm cells are mainly CD69 single positive (Cheuk et al., 2017). Additionally, CD49a is unevenly distributed on skin Trm cells,

being almost exclusively expressed in a subpopulation of $CD69^+CD103^+$ Trm cells in the epidermis (Cheuk et al., 2017). Little evidence is available on the developmental pathway of human Trm cells, except that TGF-β is important for the upregulation of CD103 on T cells derived from peripheral blood (Watanabe, 2015).

1.6.3 Regulatory skin T cells

In mice, Treg cells are established in the skin within the first days of life (Scharschmidt et al., 2015), and both skin microbes and the development of the hair follicle promote the establishment of Treg cells in the skin (Scharschmidt et al., 2017). Treg cells reside in human skin, mainly in the dermis (Clark and Kupper, 2007, Sanchez Rodriguez et al., 2014), in close proximity to the hair follicle (Sanchez Rodriguez et al., 2014). They are present in the skin in a higher proportion compared to the circulating counterpart, and they accumulate in the skin over time as foetal skin has a lower frequency of Trm cells (Sanchez Rodriguez et al., 2014). Recently, the concept of "memory" was extended to the Treg cell family, and there are few indications obtained from murine models of inflammation that Treg cells possess memory-like properties (Ali and Rosenblum, 2017, van der Veeken et al., 2016). It was shown that human skin Treg cells express CD45RO, commonly present on memory T cells, but at the moment this is the only clue on the existence of tissue resident memory Treg cells in humans (Sanchez Rodriguez et al., 2014). Further studies in the field might elucidate if Treg cells persist at the site of inflammation and their reaction to multiple inflammatory challenges. This information will be extremely valuable to understand the dynamics of recurrent inflammatory diseases and to find strategies to dampen the inflammation or prolong the time in remission via Treg cell modulation.

1.7 FUNCTIONALITY OF SKIN RESIDENT IMMUNE CELLS

1.7.1 Immune regulation and non-classical T cell activation by LCs

It is a difficult task to summarise published data on LC-functionality in a few unifying concepts. As it is often the case, a wealth of reports on the function of LCs comes from murine models, while human immunology lags behind. Functional studies in mouse are possible thanks to the existence of models based on the use of the diphtheria toxin (DT) to ablate LCs. These models are knock-in models where the high affinity human DT Receptor (DTR) is inserted into the mouse langerin gene (Bennett et al., 2005, Kissenpfennig et al., 2005a, Kissenpfennig et al., 2005b). In this way, DT injection causes the ablation of all langerin-expressing cells. It is important to point out that LCs are not the only langerin-expressing dendritic cells in mouse, but a subpopulation of dDCs also expresses langerin (Bursch et al., 2007, Ginhoux et al., 2007, Nagao et al., 2009, Poulin et al., 2007). However, these populations can be differentiated by taking advantage of the fact that LCs and dDCs repopulate the skin with different kinetics after ablation of all skin langerin-expressing cells with DT. $Langerin^+$ DCs reappear in the dermis after few days following DT injection,

whereas it takes weeks for LCs to reappear in the epidermis. This discrepancy can be exploited to study the effect of a treatment/infection in presence or absence of these two distinct cell populations. Moreover, langerin$^+$ dDCs express CD103, whereas LCs lack this marker, and currently CD103 is preferentially used to describe this subset of dDCs, instead of langerin. A second murine model based on DT introduces human langerin regulated by DT subunit A (DTA) in mice, that results in the complete absence of epidermal LCs, whereas the dermal langerin$^+$ dDCs are still present (Bursch et al., 2007, Kaplan et al., 2005, Romani et al., 2010). These two murine models, together with the discovery of DCs residing in the dermis in human and mouse (Lenz et al., 1993), were fundamental to give new impulse to LC research, by challenging the view of LCs as the stereotype of the immunogenic DCs that sense the antigen and migrate to the lymph node to elicit the immune response (Allan et al., 2003, Romani et al., 2012, Romani et al., 2010). With the aid of the LC ablation models, it was possible to assign a specific function to epidermal LCs and dermal DCs and it was discovered that in some settings, and contrary to the common knowledge, dDCs have a prominent role in inflammation, whereas LCs were found to be dispensable (Kissenpfennig et al., 2005b) if not tolerogenic (Kaplan et al., 2005). Aside from these studies on lymph node migration and presentation to T cells mediated by the MHC class II machinery, LCs might have some sort of niche function strongly related to their unique phenotypic features. For example, the high expression of the lipid antigen presenting molecule CD1a (Van Rhijn et al., 2015) could confer LCs superior antigen presenting abilities compared to dDCs. A murine model in which LCs express the human CD1a molecule proved that the lipid antigen urushiol, that causes severe dermatitis after the contact with urshiol-producing plants like the poison ivy, is presented to CD1a-reactive T cells and leads to the production of IL-17, and in the imiquimod model of psoriasis, self-lipid antigens presented to T cells by CD1a elicit IL-17 production by T cells (Kim et al., 2016). In humans, a pioneer study showed that LCs present lipid antigens from *Mycobacterium leprae* to T cells (Hunger et al., 2004) and another report showed that CD1a-reactive T cells are present in human skin and can produce IL-22 upon activation (de Jong et al., 2010). Other than the high expression of CD1a, LCs are associated with the unique presence of langerin and the organelles Birbeck granules. The function of these organelles has been enigmatic for a long time, but recent insights show that Birbeck granules are subdomains of the endocytic recycling compartment (Mc Dermott et al., 2002). Interestingly, langerin can recognise microbial structures, such as the glycoprotein gp120 on HIV, internalise them and lead them to the Birbeck granules where they are degraded, therefore conferring LCs a protective role in case of HIV infection (de Witte et al., 2007, van der Vlist and Geijtenbeek, 2010).

TLR activation is one of the ways to study LC functionality. Skin LCs express a lower variety of TLRs (Renn et al., 2006, Takeuchi et al., 2003, van der Aar et al., 2007) (table 1.7.1). Allogenic co-cultures of LCs differentiated from blood CD34$^+$ precursors and T cells show that TLR-stimulated LCs can induce Th17 differentiation and IL-17 production, and that blocking TGF-β, IL-23 and IL-6 derived from LCs impairs the differentiation of Th17

cells (Aliahmadi et al., 2009). A similar study with the same setup showed that differentiated LCs induce Th17 polarisation specifically in the memory T cell subset (Duraisingham et al., 2009).

TLR	Ligand	Ligand species	Location	LCs	dDCs
TLR1	Triacyl lipopeptide	Bacteria	Cell surface	Yes	Yes
TLR2	Zymosan	Fungi	Cell surface	No	Yes
TLR3	Double stranded RNA	Viruses	Intracellular	Yes	Yes
TLR4	Lipopolysaccharide (LPS)	Bacteria	Cell surface	No	Yes
TLR5	Flagellin	Bacteria	Cell surface	No	Yes
TLR6	Mycoplasma lipopeptide	Mycoplasma	Cell surface	Yes	Yes
TLR7	Single stranded RNA	Viruses, endogenous	Intracellular	Yes	Yes
TLR8	Single stranded RNA	Viruses, endogenous	Intracellular	No	Yes
TLR9	Unmethylated CpG DNA	Bacteria, viruses, endogenous	Intracellular	No	No

Table 1.7.1. TLR ligands and expression on skin LCs and dDCs (Ermertcan et al., 2011, Medzhitov, 2001, van der Aar et al., 2007).

The efficacy of LCs in stimulating $CD4^+$ T cells to release IL-17 was shown also by co-culturing LCs migrated out of skin explants with allogenic T cells (Aliahmadi et al., 2009, Mathers et al., 2009). In the same manner, sorted LCs obtained after enzymatic digestion of the epidermis induced proliferation and IL-22 production in co-cultured allogenic T cells in both $CD8^+$ and $CD4^+$ T cells. The same effect was obtained after co-culturing dDCs and T cells (Fujita et al., 2009). A completely opposite outcome has been obtained by co-culturing skin LCs with autologous skin T cells: $Foxp3^+$ Treg cells are the T cell subset proliferating in this setup (Seneschal et al., 2012). A precedent study reported the capacity of vitamin D3-primed skin LCs in inducing $Foxp3^+$ Treg cells with a mechanism mediated by TGF-β (van der Aar et al., 2011). This evidence gathered by studying skin derived LCs, together with the lack of different TLRs sensing bacterial antigens on the LC cell surface (van der Aar et al., 2007), advocates for a physiologic role of LCs in maintaining tolerance within the skin. LCs derived from $CD34^+$ blood precursors seem to be capable to cross-present antigens

(Klechevsky et al., 2010, Ratzinger et al., 2004), but this is probably not the case for skin isolated LCs (Haniffa et al., 2012, Igyarto and Kaplan, 2013, van der Vlist et al., 2011). These results highlight that the model used to study LCs can greatly influence the results, especially in human studies, and phenotypical differences between LCs generated from $CD34^+$ precursors, migrated from skin explants or isolated after enzymatic treatment of the epidermis might translate to functional dissimilarities. When approaching the literature on this subject, these aspects are essential to be kept in mind.

1.7.2 Pro-inflammatory role of dermal DCs in inflammation and infection

In inflammation, additional populations of DCs are recruited into the skin and the distribution of the surface markers across the DC subsets changes, thus it is difficult to study the contribution of single populations of resident or infiltrating DCs in inflammation (Haniffa et al., 2015). The first observation on the massive infiltration of DCs in peripheral tissues by inflammatory DCs was obtained in a model of *Listeria monocytogenes* infection, where monocytes accumulated in infected spleens, developed into DCs that produced TNF and inducible nitric oxide synthase (iNOS), therefore were called TNF iNOS producing (Tip)-DCs (Serbina et al., 2003). In humans, infiltration of DCs in the skin during active inflammatory skin diseases has been noted both in atopic dermatitis and in psoriasis, but it is difficult to establish the origin and fate of these cells because of the inability to track human cells and to follow the disease evolution from the early phase to the resolution. Dermal DCs excel in the capacity to stimulate T cells: they engage the CD3 receptor by presenting the processed antigen on their MHC molecules (signal 1), they can upregulate CD80 and CD86 expression (signal 2) and produce cytokines upon activation (signal 3). Both $CD1c^+$ and $CD141^+$ DC subsets are migratory. The functional difference between the two DC populations, both in mice and in humans, stems from their ability to elicit a response from $CD4^+$ or $CD8^+$ T cells. $CD1c^+$ DCs, in mouse and in humans, are superior in eliciting a CD4 T cell response and produce a set of cytokines capable of inducing Th1, Th2 and Th17 subsets depending on the type of antigen encountered (Kashem et al., 2017). Conversely, murine $CD103^+$ DCs and their human orthologue $CD141^+$ DCs, of more recent discovery, are superior in cross-presentation to $CD8^+$ T cells (Bedoui et al., 2009, del Rio et al., 2010, Haniffa et al., 2012).

1.7.3 Dermal DCs and tolerance

Numerous evidence points towards the involvement of DCs in tolerogenesis. At a central level, DCs contribute to the establishment of immune tolerance (Bonasio et al., 2006, Brocker et al., 1997, Manicassamy and Pulendran, 2011, Proietto et al., 2008, Watanabe et al., 2005). At a peripheral level, mature IL-10 producing pulmonary DCs maintain tolerance in murine lungs (Akbari et al., 2001) and, in the gut, $CD103^+$ DCs promote Treg cell formation (Matteoli et al., 2010, Russler-Germain et al., 2017, Siddiqui and Powrie, 2008). In human

foetal development, foetal DCs seem to promote tolerogenesis by inhibiting T cell release of pro-inflammatory cytokines and induce differentiation of Treg cells (McGovern et al., 2017). In vitro, human blood-derived DCs, displaying an immature phenotype with low expression of CD80, CD83 and CD86, have a poor capacity to stimulate T cell proliferation and preferentially generate IL-10 producing T cells instead (Dhodapkar et al., 2001, Jonuleit et al., 2000). How dDCs contribute to the tolerance in the skin has not been clearly elucidated, especially regarding human dDCs. However, one report identifies $CD141^+$ dDCs as IL-10 producer cells capable of inducing T cells with some Treg cell phenotype features (Chu et al., 2012). Another report instead compares skin-derived vitamin D3-primed LCs and dDCs in their ability to induce a tolerogenic phenotype in co-cultured allogenic T cells (van der Aar et al., 2011). Vitamin D3 as a stimulant has been chosen because of its known immunosuppressive properties, especially concerning DCs (Anderson et al., 2009, Arnson et al., 2007, Berer et al., 2000, Griffin et al., 2001, Griffin et al., 2000, Penna and Adorini, 2000, Piemonti et al., 2000). The investigators show that vitamin D3-primed LCs induce the expression of CD25 and Foxp3 in co-cultured T cells, but these cells are not able to produce IL-10. On the contrary, T cells derived from vitamin D3-stimulated dDC co-cultures do not express Foxp3 but are functionally tolerogenic, being capable of producing IL-10. Moreover, an interesting piece of data in the study is that LCs and dDCs achieve the induction of tolerogenic T cells through the secretion of different mediators, namely TGF-β for LCs and IL-10 for dDCs (van der Aar et al., 2011).

In active inflammation, immunosuppressive mechanisms are activated together with the pro-inflammatory program to control extensive inflammation. One of these regulatory mechanisms is driven by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1). IDO1 is the first enzyme in the tryptophan degradation pathway and, as such, acts as a limiting factor in the degradation of the essential amino acid tryptophan to kynurenine. IDO1 expression is upregulated in DCs by pro-inflammatory molecules, especially IFN-γ, but it's also induced by the interaction of CD80/CD86 on DCs with the cytotoxic T lymphocyte antigen 4 (CTLA-4) (Cederbom et al., 2000, Fallarino et al., 2003, Grohmann et al., 2002, Mellor et al., 2003, Mellor and Munn, 2004). The depletion of tryptophan by the IDO1 pathway causes inhibition of T cell proliferation and cell cycle arrest because the cells are deprived of one amino acid essential for protein synthesis (Hwu et al., 2000, Munn et al., 1999). Moreover, toxic effects on T cells by the metabolites generated after tryptophan degradation have been reported (Fallarino et al., 2002, Hayashi et al., 2007, Moffett and Namboodiri, 2003). Conversely, these metabolites are also involved in the expansion of Treg cells (Baban et al., 2009, Hill et al., 2007). Overall, these mechanisms triggered by reduction in tryptophan result in immunosuppression (figure 1.7.1).

IDO1 is upregulated in inflammatory skin diseases, including psoriasis (Harden et al., 2016, Scheler et al., 2007). IDO1 has also been reported in different types of cancer, therefore finding ways to modulate the expression of IDO1 is particularly interesting in the field of

cancer immunology (Brochez et al., 2017) (figure 1.7.3). Tryptophan-mimetic compounds that bind to IDO1 and inhibit its activity, aiming at reverting the immunosuppression state that impedes the immune system to fight the tumour, are now in clinical trials (Brochez et al., 2017, Prendergast et al., 2017). However, in the context of skin diseases, the opposite tactic aiming at increasing IDO1 activity would be beneficial to shut down the inflammation or to prolong the time in remission.

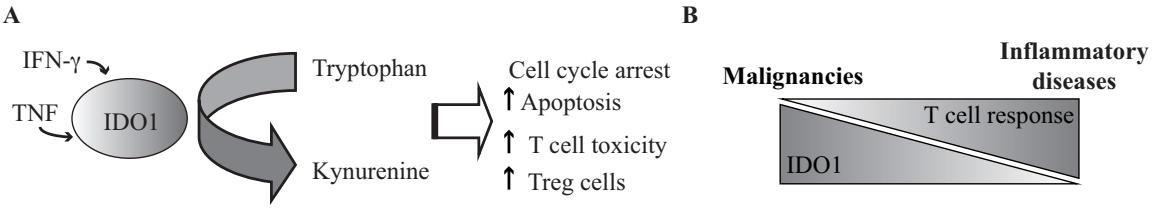

Figure 1.7.3. IDO1 in the tryptophan metabolism and its expression in diseased conditions. (A) IDO1 expression is increased by IFN-γ and TNF. IDO1 transforms the tryptophan to kynurenine, with implications for the T cell response. (B) The T cell response is inhibited in cancer, where IDO1 is highly expressed. Inflammatory diseases would benefit from an increased IDO1 expression to contrast the T cell activation.

1.7.4 Human Trm cell function in skin diseases

It is difficult to establish antigen specificity combined with functionality at single cell resolution in human T cells and the vast majority of the studies on Trm cell functionality focuses on cytokine production in the absence of defined antigen specificity, comparing healthy skin and diseased skin. Several skin conditions have been associated with pathogenic Trm cells, and these diseases all present with well-demarcated lesions in fixed spots on the skin. Fixed drug eruption, vitiligo and psoriasis are all examples of Trm cell associated skin diseases (Clark, 2015). Fixed drug eruption is a phenomenon that occurs on the skin after systemic administration of a drug, that spontaneously resolves after the interruption of the therapy but comes back in the same spot if the same drug is administered again (Shiohara, 2009). $CD8^+$ effector cells are implicated in the pathogenesis of fixed drug eruption, and $CD8^+$ cells producing IFN-γ have been identified in the epidermis of fixed drug eruption resting lesions (Teraki and Shiohara, 2003). Moreover, an enrichment of $CD69^+$ and $CD103^+$ T cells has been noted in the epidermis of patients with fixed drug eruptions, and these cells quickly upregulate *IFNG* expression (Mizukawa et al., 2002). Vitiligo is a skin disease characterised by local loss of melanocytes. T cell-mediated killing of melanocytes is one of the mechanisms responsible for the loss of pigment on fixed patches of the skin. Cytotoxic Trm cells are enriched in vitiligo lesions and are excellent producers of IFN-γ and TNF, as demonstrated by two independent reports on the phenotype and functional profile of Trm cells in vitiligo-affected skin (Boniface et al., 2018, Cheuk et al., 2017).

1.8 PSORIASIS

Psoriasis is a common inflammatory skin disease affecting 2-3% of the world population. The most common psoriasis phenotype is plaque psoriasis, or psoriasis vulgaris, characterised by well-demarcated, red and scaly plaques on the skin. Other clinical variants of psoriasis include guttate psoriasis, where small lesions appear all over the body in association with streptococcal throat infections, inverse psoriasis that affects skin folds and is characterised by red shiny lesions lacking scales, pustular psoriasis with a massive influx of neutrophils into the epidermis, and erythrodermic psoriasis where a large portion of the skin is covered by psoriasis (Griffiths, 2004). Any part of the body can be affected and, depending on the percentage of the body surface involved, the disease severity is classified as mild (less than 3% of the body surface involved), moderate (between 3 and 10% of the body surface involved) or severe (more than 10% of the body surface involved). The treatment options vary depending on the severity of the disease and on the body location. Psoriasis per se is not a life-threatening condition, but the disease affects the quality of life in several ways, from social isolation to employment problems (Kimball et al., 2005). Patients report problems in coping with the body image, the symptoms of the disease (itch on the site of the lesions) or the treatments' side effects. The patients' condition can worsen by the onset of common co-morbidities, such as psoriatic arthritis, metabolic syndromes and depressive disorders (de Oliveira et al., 2015). Around 30% of the patients with psoriasis develop joint diseases, as reported in a study on more than 5000 psoriatic patients in the Nordic countries (Zachariae, 2003). Metabolic syndrome, obesity, diabetes and heart failure are noted primarily in patients with severe psoriasis (Henseler and Christophers, 1995).

1.8.1 Genetics

Genetic predisposition plays a paramount role in the risk of developing psoriasis, as firstly demonstrated in studies involving monozygotic and dizygotic twins (Bowcock, 2005, Duffy et al., 1993, Farber et al., 1974). Many psoriasis-associated genes are related to immunological pathways. Some types of HLA genes, in particular *HLA-Cw6*, show a strong association with psoriasis. HLA-C is an MHC class I gene, it is therefore implicated in presentation of intracellular peptides to $CD8^+$ T cells and NK cells. Other genes of more recent discovery, thanks to genome-wide association studies, establish a link between psoriasis and polymorphism of genes related to the MHC class I pathway (*ERAP1*), IL-23 (*IL12B*, *IL23A* and *IL23R*), Th17 polarisation (*STAT3*) or NF-kB pathway (*CARD14*) (Harden et al., 2015b). The correlation of the psoriasis-associated genes with the immune system clearly enforces the view of psoriasis as a skin disease with a strong immune-related component.

1.8.2 Psoriasis pathogenesis and hallmarks of the disease

One proposed pathogenic mechanism for the development of early lesions is based on the activation of pDCs by LL-37 and self DNA complexes, which leads to IFN-α production that consequently initiates the inflammatory cascade leading to profound epithelial alterations (Nestle, 2009, Nestle et al., 2005).

Other proposed adaptive immune mechanisms are based on the presence of CD1a- or melanocyte- autoreactive T cells in the skin of psoriatic patients (Arakawa et al., 2015, Cheung et al., 2016). The inflammation in the skin causes acanthosis, parakeratosis, vessels alterations and infiltration of immune cells in the skin (Lin et al., 2011, Teunissen et al., 2014, Villanova et al., 2014) (figure 1.8.2).

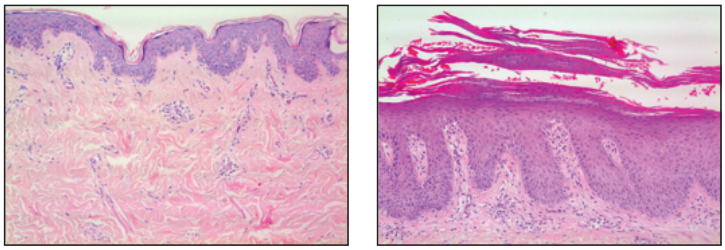

Figure 1.8.2. Histological features of plaque psoriasis. Healthy skin (left) and lesional psoriatic skin (right). Courtesy of Dr. Hedblad.

Recent drug development has highlighted the role of the IL-23/Th17 axis in the maintenance of psoriasis. Activation of Th17/Tc17 cells leads to the production of IL-17 which stimulates the release of pro-inflammatory cytokines and neutrophil-attracting chemokines from a variety of immune and epithelial cells. IL-17 itself acts as a potent recruiter and activator of neutrophils (Di Cesare et al., 2009), and neutrophils themselves are also proposed as a source of IL-17 in psoriasis (Lin et al., 2011). Th17/Tc17 cells can also produce IL-22. In contrast to IL-17, IL-22 does not act on immune cells but on epithelial cells, promoting proliferation and release of antimicrobial mediators (Wolk and Sabat, 2006), therefore it is evident how IL-17 and IL-22 play a fundamental role in the generation of the hallmarks of the disease. One of the peculiarity of psoriasis is that the disease comes back in previously affected sites of the skin after successful treatment. This feature led investigators to suggest that a memory of the disease is maintained in cells residing in psoriatic-treated patches on the skin (Clark, 2011), indeed treated skin sites present residual alterations in immune and non-immune genes, even if the morphology of the skin is normalised, that has been named “molecular scar” (Cheuk et al., 2014, Matos et al., 2017, Suarez-Farinas et al., 2011).

1.8.3 Current treatments

The therapeutic decision in psoriasis depends on the severity of the disease and the location of the affected skin. Currently, the major clinical challenges are to induce long-term disease remission and to treat psoriasis in body areas that are particularly difficult to treat, such as the nails, the scalp and the genital area. Mild psoriasis is treated with topic application of corticosteroids alone or in combination with Vitamin D. For moderate to severe psoriasis,

systemic treatment with immunosuppressive agents, such as ultraviolet (UV) therapy or methotrexate, is widely used. Narrow-band UVB therapy reduces the inflammation by several mechanisms, including T cell apoptosis (Ozawa et al., 1999) and inhibition of cytokine production including IL-12 and IL-23 (Johnson-Huang et al., 2010, Piskin et al., 2004). Despite UV rays causing skin cancer, studies on narrow-band UVB therapy used according to clinical guidelines show no increase in cancer risk (Lee et al., 2005). In contrast to UVB therapy, the exact mechanism of action of methotrexate is not fully understood, but this remains a safe and cheap treatment for moderate-to-severe psoriasis. Currently still a second or third line of treatment for severe disease, biologic therapy targeting TNF, IL-12/23 or IL-17 has been introduced with astounding efficacy in relatively recent times. The term "biologics" refers to a class of protein (usually monoclonal antibodies) targeting pro-inflammatory cytokines or cytokine receptors. Regarding the safety profile of biologics, it seems that these agents are well tolerated, even after long term therapy (Rustin, 2012).

1.8.4 LCs in psoriasis

LCs in active psoriasis are exposed to multiple environmental pro-inflammatory signals from keratinocytes and immune cells that can alter their function and their phenotype. TLRs on keratinocytes have an altered expression pattern in psoriasis (Miller, 2008), so it would not be surprising if the expression or activating threshold of TLRs would differ in LCs between healthy and psoriatic skin. Another potential environmental factor is the microbiome. Several investigations on the psoriasis microbiome have been conducted, but the results do not show drastic differences between healthy and psoriatic microbiome, as is the case with atopic dermatitis (Fahlen et al., 2012, Gao et al., 2008, Grice et al., 2009, Paulino et al., 2006, Tett et al., 2017).

The challenge in enumerating LCs in healthy and diseased skin has been documented since the end of the eighties, where a methodological article describing six different ways to count LCs showed profound differences in LCs in psoriasis compared to healthy skin depending on the method employed for counting (Bieber et al., 1988). Several publications report an increase (Baker et al., 1985, Fujita et al., 2011, Komine et al., 2007), a decrease (Bos et al., 1983, Glitzner et al., 2014, Lisi, 1973) or a stable (Czernielewski et al., 1985, Gommans et al., 1987, Gunther et al., 2012, Guttman-Yassky et al., 2007) number of LCs in lesional psoriasis, compared to healthy skin, using different enumeration methods. The reasons for such contradicting results could lie in the cellular markers used for the enumeration and the location where the biopsies were collected within the plaque. Many authors used CD1a for the identification of LCs and report an increase in the number of cells within the lesional plaques. However, CD1a expression in active lesions is not a prerogative of LCs but is shared with a subpopulation of langerin$^-$ DCs infiltrating the epidermis. The presence of this additional population may contribute to increase the number of counted cells, unless an additional marker such as langerin is used for co-staining. Less, but more consistent data are

available on the number of LCs in perilesional skin during acute psoriasis, with several articles agreeing in reporting an increased number of LCs close to the border of the lesions (Alshenawy and Hasby, 2011, Gordon et al., 2005, Komine et al., 2007). In resolved skin, LC numbers have been reported to be reduced after psoralen and ultraviolet A (PUVA) treatment (Czernielewski et al., 1985, Erkin et al., 2007, Ree, 1982), and increased after biologics administration in resolved skin after treatment (Gordon et al., 2005, Hendriks et al., 2014) or uninvolved skin (Piaserico et al., 2013). Of relevance is the disappearance of the pro-inflammatory infiltrating langerin$^-$ cells from the epidermis after treatment with biologics or narrow band UVB (Johnson-Huang et al., 2010, Lowes et al., 2005).

Functional studies on human LCs in psoriasis are scarce but suggest an active role in sustaining the inflammation. Early reports show migrational impairment of LCs from non-lesional skin of psoriasis patients in response to the administration of subcutaneous TNF and IL-1β correlating with the time of onset of the disease (Cumberbatch et al., 2006, Eaton et al., 2014, Eaton et al., 2017, Shaw et al., 2010). Interestingly, a recent follow-up identified IL-17 stimulated keratinocytes as the source of unidentified factors causing the migrational impairment of LCs (Eaton et al., 2017). LCs sorted from atopic dermatitis or psoriasis skin showed comparable abilities to stimulate T cells in an allogenic setup, but displayed some differences in the chemokine gene expression profile, as LCs from psoriasis skin expressed more *CXCL1* and *CXCL10*, immune cell chemoattractants, and *CCL18* and *CCL20*, important pro-inflammatory mediators (Fujita et al., 2011). Conversely, LCs from atopic dermatitis showed upregulation of *CCL17*, and collectively, these result show that LC profile is disease-specific, despite the similar ability to stimulate T cells in the artificial context of the mixed leucocyte reaction. More recently, LCs were found to produce IL-23 after stimulation with the TLR2 ligand zymosan (Sweeney et al., 2016). This finding is important for two reasons: one reason is that it attributes LCs the capacity to skew T cells towards a Th17/Tc17 phenotype, the second reason is that it indirectly shows an altered TLR expression pattern on psoriatic LCs. The presence of a subset of LCs expressing CD5 has been recently reported in healthy skin, and increased frequency of this population is found in lesional psoriasis (Korenfeld et al., 2017). As already mentioned, LCs express high levels of CD1a, important for the presentation of lipid antigens. In psoriasis, CD1a-restricted T cells capable of producing IL-17 and IL-22 have been detected (Cheung et al., 2016) but, despite the existing premises for local antigen presentation and elicitation of the T cell response within the skin, the direct engagement of LCs and CD1a-restricted T cells in skin lesions has not been reported yet. Even more interesting is the understanding of the contribution of resident cells in disease flares. Resident T cells with high IL-23R expression persist in the skin of treated patients (Cheuk et al., 2014), and gene expression profile alterations persist in morphologically healed skin, thus raising the possibility that LCs could have a role in psoriasis relapses (Eidsmo and Martini, 2018) (figure 1.8.4).

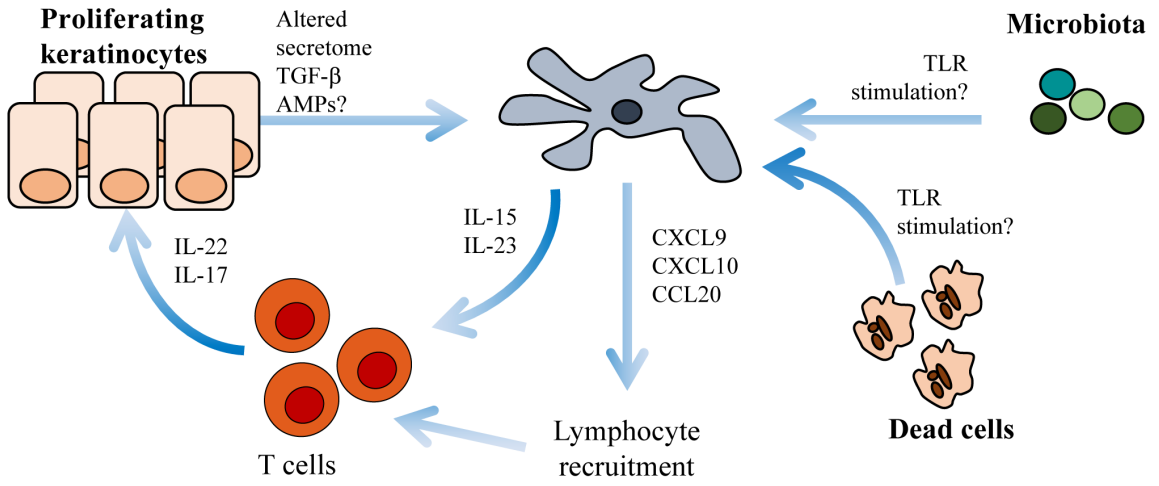

Figure 1.8.4. Interactions between Langerhans cells, T cells and keratinocytes in lesional psoriasis. Schematic representing the mediators involved in the cross-talk between cells in the epidermis of psoriatic lesions (Eidsmo and Martini, 2018).

1.8.5 Dermal DCs in psoriasis

The majority of studies on psoriatic DCs investigates $CD11c^+$ cells in the dermal compartment. It is important to underline that, in humans, CD11c has been shown not to be an absolute marker for DCs but can be expressed by monocytes and neutrophils (Loike et al., 1991, Maecker et al., 2012). Nevertheless, many studies, including ours, on active psoriasis use CD11c in combination with HLA-DR as a primary marker to define DCs, although markers such as CD14 and CD16 would help to distinguish monocytes and macrophages from DCs. Shortly after the discovery of IL-23 and the first studies on Th17 T cells, Lee and co-workers analysed *IL23A* mRNA expression of whole skin cell suspensions and found it upregulated in active psoriatic lesions. At that time, a good antibody for IL-23p19 staining was not available, so the group analysed the staining pattern of the common IL-12/IL-23 subunit, p40, and found that this protein was mainly present in the dermis of psoriatic patients and a dendritic cell shape of the stained cells was observed (Lee et al., 2004). This discovery led to a deeper effort to delineate the pro-inflammatory profile of dDCs. An increase in the number of $CD11c^+$ cells is apparent in the dermis in active psoriasis (Guttman-Yassky et al., 2007, Lowes et al., 2005, Zaba et al., 2009) but their clear phenotypic description is still lacking. These cells have been described in different ways (Tip-DCs or slan-DCs) but when it comes to their pro-inflammatory properties, they seem to be overlapping. Human TNF and iNOS producing (Tip-) DCs, as the related mouse population, produce TNF and iNOS and are considered non-resident because of the lack of CD1c and CD141 surface markers. It is a very heterogeneous population, with overlapping expression of CD163 (macrophage marker), DC-SIGN (immature DCs and macrophages) and CD14 (monocyte, DCs and macrophages) (Zaba et al., 2009). 6-sulfo LacNAc (Slan)-DCs have been originally described in blood (Schakel et al., 2002) and subsequently identified in inflamed skin (Hansel et al., 2011) as TNF and iNOS producer population. The blood slan-DCs express CXCR4 and the

complement component 5a receptor (C5aR): their ligands, CXCL12 and C5a, are abundant in psoriasis skin and taken the presence of skin slan-DCs in proximity of the dermal blood vessels, the authors speculate that slan-DCs in skin directly come from the blood (Hansel et al., 2011). Regarding the functionality of Tip-DCs, a population of dermal $CD11c^+$ non-resident DCs, that includes Tip-DCs, emigrated from cultured biopsies showed the capacity to stimulate T cell proliferation and IL-17 and IFN-γ production in an alloreactive experiment (Zaba et al., 2009). Similar results have been achieved when culturing blood slan-DCs with allogenic T cells (Hansel et al., 2011). Taken together, the bulk of descriptive data and the few mechanistic studies in an alloreactive context suggest a prominent role for DCs, both resident and infiltrating, in sustaining the inflammation in active psoriasis.

Psoriasis treatments seem to normalise the number of dDCs and reduce the infiltration of inflammatory DCs in the skin. UVB treatment, one of the first treatment options in the treatment of moderate-to-severe psoriasis, reduces the number of inflammatory DCs in the dermis in responding subjects, whereas inflammatory dDCs persist in non-responsive lesions. Anti-TNF (αTNF) treatment (Infliximab, an αTNF-antibody) reduced the expression of *IL1B*, *IL12B* and *IFNG* in skin after treatment and reduced the production of IL-12 and IL-23 in blood derived slan-DCs from treated patients (Brunner et al., 2013). Another αTNF agent (Etanercept, soluble TNF receptor) gave analogue results on blood slan-DCs cytokine production but, interestingly, slan-DCs that remain in the skin retained the production of TNF-α in biopsies taken after 24 weeks of treatment (Gunther et al., 2013). Specific therapies against IL-23/IL-17, the major players in psoriasis pathogenesis, have been developed. Early studies on Ixekizumab (anti-IL-17 monoclonal antibody) showed a decrease of immune cell infiltration (both T cells and $CD11c^+$ cells) and decreased *IL17A*, *IL22*, *IL12B* and *IL23A* gene expression after 6 weeks of treatment (Krueger et al., 2012). Two anti-IL-23 agents, Aprilimod (small molecule inhibiting the synthesis of IL-12 and IL-23) and Guselkumab (specific anti-IL-23 monoclonal antibody), ameliorated the clinical phenotype of the disease and inhibited the infiltration of immune cells in psoriatic skin (Sofen et al., 2014, Wada et al., 2012). Therefore, the common denominator of successful therapies is the capacity to reduce the immune infiltration into the skin and to decrease the production of pro-inflammatory cytokines.

1.8.6 T cells in psoriasis

Evidence points towards a pathogenic role of T cells and Trm cells in psoriasis. Early studies showed that depleting T cells temporarily heals human psoriasis, proving the involvement of T cells in the psoriasis pathology (Prinz et al., 1991). In landmark experiments, non-lesional psoriatic skin was transplanted on immunodeficient mice and spontaneously gave rise to a psoriasis-like dermatitis. The administration of an anti-CD3 antibody prevented the exacerbation of the pathology, showing the central role of T cells in the development of psoriasis (Boyman et al., 2004). Overall, the number of T cells increases in the skin during

the disease flare (Boyman et al., 2004, Cheuk et al., 2014). The impressive response to biologic drugs targeting the IL-23/Th17 axis clearly shows the strong link between psoriasis and IL-17 (Di Cesare et al., 2009). T cells within psoriatic lesions are one of the sources of IL-17 and are also capable of producing IFN-γ, IL-13 and IL-22 (Cheuk et al., 2017, Cheuk et al., 2014, Hijnen et al., 2013, Lowes et al., 2008). Interestingly, double-producing IFN-γ and IL-17 cells are detected among Trm cells in lesional psoriasis, highlighting the strong inflammatory potential of Trm cells in active disease. These cells are absent in healthy skin or in vitiligo (Cheuk et al., 2017). A recent paper analysed the variety in the TCR repertoire and the clonality of skin T cells in full thickness skin from lesional and resolved psoriatic skin (Matos et al., 2017). Although the evidence that T cells in psoriasis are not monoclonal is not new (Harden et al., 2015a), the interesting finding from Matos and colleagues is the presence of psoriasis-specific T cell clones possessing similar TCR nucleotide sequences or giving rise to overlapping amino acid sequences, within the same individual or across different patients (Matos et al., 2017). With the fast development of current technologies (Dash et al., 2017, Glanville et al., 2017), the hope is that one day it will be possible to know what these "pathogenic" T cells recognise, with the scope of identifying one or multiple antigens responsible for triggering the aberrant immune response. CD1a-reactive T cells producing IL-17 and IL-22 after activation have been identified in both non-lesional and lesional skin of patients with psoriasis (Cheung et al., 2016), and T cell clones specific for a melanocyte-derived antigen were isolated from the skin of a psoriatic patient (Arakawa et al., 2015). Despite the challenges to verify to which extent these autoreactive T cells are found in a broader population of patients with psoriasis, these two papers open up to the existence of alternative models of psoriasis pathogenesis and disease maintenance. In resolved lesions, the number of epidermal and dermal T cells is dramatically reduced (Cheuk et al., 2014, Krueger et al., 2012, Lowes et al., 2005), but molecular alterations still persist despite the normalised skin morphology. T cells in resolved epidermis display a high expression of IL-23R and a higher proportion of cells produce or co-produce IL-17 and IL-22 after stimulation, compared to healthy epidermal T cells and regardless of the treatment method (UVB or biologics) (Cheuk et al., 2014). An interesting feature of T cells in resolved skin is that this high pro-inflammatory potential concerns only T cells in the epidermis, but not T cells in the dermis whose pro-inflammatory profile is much milder compared to the epidermal counterpart, thus highlighting how different the cell behaviour is in the two compartments of the skin. Previous studies therefore highlight the need to examine dermal T cells in depth.

1.8.7 Mouse models of psoriasis

Despite the fact that psoriasis is a human disease and not naturally occurring in mice, murine models have been developed to unravel specific pathogenic roles for immune cell subsets and to test active compounds (Eidsmo and Martini, 2018). Early studies employed genetically modified mouse strains to recapitulate the skin phenotype of psoriasis, although some of the models fail to reproduce the infiltration of immune cells seen in human psoriasis (Gudjonsson et al., 2007, Schon et al., 2001, Sundberg et al., 1993). Currently, the most commonly used

mouse model of psoriasis is based on the application of the TLR7 agonist Imiquimod (IMQ) to induce a psoriasiform dermatitis. In the IMQ mouse model of psoriasis, the phenotypical features of the disease are reproduced, as well as the immune cell infiltration typical of human psoriasis (van der Fits et al., 2009). The number of T cells in the skin, of prominent γδ phenotype, increases few days after the application of IMQ, and T cells are the source of IL-17 driving the inflammation (Wohn et al., 2013, Yoshiki et al., 2014). LCs might be the source of IL-23 necessary to trigger IL-17 release from $\gamma\delta^+$ T cells (Sweeney et al., 2016, Xiao et al., 2017, Yoshiki et al., 2014), but another study ascribes this function to dDCs (Wohn et al., 2013). The number of DCs in the dermis is increased in the IMQ mouse model of psoriasis (Terhorst et al., 2015, van der Fits et al., 2009) as well as in the model based on IL-23 injection in the skin (Singh et al., 2016) or in the *Jun*$^{f/f}$ *JunB*$^{f/f}$ *K5cre*^{ER} (DKO*) mouse (Glitzner et al., 2014), and all these studies agree on their marked pro-inflammatory function.

LCs have been found to have an immunomodulatory function in the DKO* mouse, where LCs display high *IL10* expression and upregulate PD-L1 (Glitzner et al., 2014) , and when the application of IMQ is prolonged up to two weeks to mimic the chronicity of psoriasis (Terhorst et al., 2015). However, the study does not present data on which cytokines produced by LCs are responsible for this immunomodulatory function, nor what is the consequence for the skin T cell population.

2 AIMS

This thesis aims at dissecting the phenotype and function of LCs, DCs and T cells in different anatomical skin compartments in different phases of psoriasis.

Aims:

- Characterise the functional profiles of LCs and eDCs in the epidermis of active and resolved psoriatic skin (PAPER I);

- Explore the role of the pro-inflammatory T cell derived granzyme A in active psoriasis (PAPER II);

- Examine the anti-inflammatory features of dermal DCs and T cells residing in the skin of successfully treated patients (PAPER III) and

- Investigate the phenotypic and functional plasticity of epidermal and dermal Trm cells in healthy skin (PAPER IV).

3 MATERIALS AND METHODS

3.1 TISSUE SAMPLES (PAPER I, II, III AND IV)

Healthy skin from patients undergoing reconstructive surgery was obtained from Karolinska University hospital or from AdVita clinic (Stockholm, Sweden). Samples from lesional, non-lesional or resolved psoriatic skin were collected from patients at Karolinska University hospital (Stockholm, Sweden) or at the Psoriasis association clinic (Sundbyberg, Sweden). The studies were approved by the Stockholm Regional Committee of Ethics and signed consent was collected from all the patients.

3.2 BIOPSY COLLECTION AND CELL SUSPENSIONS (PAPER I, II, III AND IV)

Six millimetre skin biopsies were collected from healthy skin, four millimetre biopsies were collected from patients with psoriasis. The biopsies were placed in 5 U/ml dispase (Life technologies) in PBS (Gibco) overnight at 4°C. After incubation, epidermis and dermis were separated and placed in a solution containing Collagenase III 570 U/ml (Worthington) DNase 5 μg/ml (Roche) in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco) supplemented with 10% Fetal bovine serum (FBS, HyClone) and 100 U/ml Penicillin and Streptomycin (PEST, Gibco), for 90 minutes at 37°C. After incubation, the epidermal cell suspension was obtained by mechanical disruption of the tissue by repeated pipetting. The dermal cell suspension was obtained by repeated pipetting or after mechanical disruption with the Medicon/Medimachine system (BD Biosciences). The whole process is outlined in PAPER I, figure S1.

3.3 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PAPER II AND IV)

Peripheral blood mononuclear cells (PBMCs) were prepared from 10-20 ml of peripheral blood using Ficoll Paque Plus (GE Healthcare) according to the manufacturer's protocol. PBMCs were then cultured in RPMI 1640 supplemented with 10% FBS and 100 U/ml PEST until further use. In Paper IV, $CD8^+$ T cells were negatively sorted from PBMCs using magnetic-activated cell-sorting MACS CD8 T cell isolation kit (Miltenyi Biotech) according to the manufacturer's protocol.

3.4 CULTURE AND STIMULATION OF DCS (PAPER I AND III)

Epidermal and dermal cell suspensions were cultured in 1 ml RPMI 1640 supplemented with 10% FBS and 100 U/ml PEST and left unstimulated or stimulated with 1 μg/ml lipopolysaccharide (LPS, Sigma Aldrich) or 1 μg/ml resiquimod (R848, GLSynthesis) for 6 hours. Brefeldin A (GolgiPlug, BD Biosciences) was added to the culture to impede the release of intracellular cytokines into the culture media (PAPER I and III). For the induction

of IDO1 expression in PAPER III, the dermal cell suspension was cultured for 18 hours in presence or absence of IFN-γ (10 ng/ml) and brefeldin A. After the incubation, cells were stained with antibodies directed against surface epitopes and, subsequently, against intracellular cytokines.

3.5 CULTURE AND STIMULATION OF T CELLS (PAPER II AND IV)

Epidermal and dermal cell suspensions and PBMCs were cultured in 1 ml RPMI 1640 supplemented with 10% FBS and 100 U/ml PEST. Different combinations of TGF-β (2 ng/ml), IL-2 (20 ng/ml), IL-15 (20 ng/ml), IL-1β (20 ng/ml), IL-23 (20 ng/ml) (all R&D Systems) and anti-CD3 (clone OKT3, Biolegend) were used as indicated in each figure to stimulate the cells for 1, 3 or 7 days. In some experiments (PAPER IV), epidermal and dermal cell suspensions and $CD8^+$ MACS sorted PBMCs were stained prior culturing with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) according to the manufacturer's protocol. In brief, cells were resuspended in 1 ml PBS and stained with CFSE for 20 minutes at 37°C, then the residual dye was quenched by adding 5 ml RPMI 1640 medium for 5 minutes at 37°C.

For the assessment of the production of intracellular cytokines in PAPER II and IV, cell suspensions were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 1 μg/ml ionomycin (Sigma Aldrich) in presence of brefeldin A for 4.5 (PAPER II) or 5 (PAPER IV) hours.

3.6 CULTURE AND STIMULATION OF HUMAN PRIMARY KERATINOCYTES (PAPER II)

Six millimetre punch biopsies from healthy skin were placed in 5 U/ml Dispase in PBS and incubated overnight at 4°C. The following day, the epidermis was separated from the dermis and treated with 0.05% Trypsin/EDTA (Gibco) for 30 minutes at 37°C. A solution containing Dulbecco's Modified Eagle Medium (DMEM, Gibco) and 10% FBS was used to interrupt the reaction. The obtained epidermal cell suspension was cultured in a cell culture flask pre-coated with collagen I (Gibco) in EpiLife medium (Gibco) supplemented with human keratinocyte growth supplement (Gibco), 100 U/ml PEST and Fungizone (Gibco). The expanded keratinocytes were frozen for later use.

Thawed keratinocytes were seeded in EpiLife medium supplemented with human keratinocyte growth supplement, 100 U/ml PEST and Fungizone in a 24 or 48 well plate at 400000 cells/ml density. The following day, 35 nM Granzyme A (GzmA, Enzo Life Sciences), 10 ng/ml IL-17 or a combination of GzmA and IL-17 were added to the culture media and cells were incubated for 48 hours. In some experiments, 50 μM 3,4-dichloroisocumarin (Enzo Life Sciences) was incubated for 1 hour at 37°C together with GzmA before adding it to the culture media.

3.7 LUMINEX-BASED MULTIPLEX IMMUNOASSAY AND ELISA ASSAY (PAPER II)

The analytes released in cell culture supernatants were quantified using ProcartaPlex Human Cytokine & Chemokine Panel Immunoassay (eBioscience) and the plate was read by a Bio-Rad Bio-Plex 200 System (Bio-Rad Laboratories). The amount of CXCL-1 released in cell culture supernatants was quantified using R&D Quantikine ELISA human CXCL1 (R&D Systems) and the plate was read by a Spectramax plus 384 reader (Molecular Devices).

3.8 CONFOCAL MICROSCOPY (PAPER I, II AND III)

Eight μm frozen skin sections were fixed by immerging them in 100% acetone for 5 minutes at 4°C (PAPER I and II) or by using the Foxp3/Transcription factor staining buffer set (Thermo Fisher) for 20 minutes at room temperature. After fixation, skin sections were incubated with Protein block (Dako) and incubated overnight with primary antibodies diluted in PBS containing 2.5% horse serum (Immunokemi) (PAPER II and II) and Foxp3/Transcription factor permeabilisation buffer (PAPER III). The following day, the skin sections were washed and stained with Alexa-based secondary antibodies of the appropriate species specificity (Life Technologies) and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Images were acquired using Zeiss LSM780 (PAPER I and II) or LSM700 (PAPER III) confocal microscope (Zeiss) and processed using Fiji-ImageJ.

3.9 TRANSMISSION ELECTRON MICROSCOPY (PAPER I)

Skin biopsies were fixed in 3% paraformaldehyde (Agar Scientific), moved to 2.5% glutaraldehyde (Ladd Research) and osmium tetroxide (TAAB Laboratories). Subsequently, skin sections were dehydrated and embedded in LX-112 (Ladd Research). Ultrathin sections were obtained by cutting the skin sections with a Leica EM UC 6 (Leica Microsystems) and contrasted using uranyl acetate and lead citrate. Images were acquired with a Tecnai 10 transmission electron microscope (FEI) at 100 kV.

3.10 FLUORESCENCE ACTIVATED CELL SORTING, RNA EXTRACTION AND RT-PCR

Epidermal and dermal cell suspensions were stained with conjugated antibodies against cell surface epitopes and cells were sorted using MoFlo XDP (Beckman Coulter) (PAPER I and III) or BD FACSJazz (BD Biosciences) (PAPER IV). In PAPER I and III, sorted cells were collected in QIAzol lysis reagent (Qiagen) and mRNA was extracted using RNA Mini kit (Qiagen). The high-capacity RNA-to-cDNA kit (Applied Biosystems) was used to obtain cDNA from mRNA and TaqMan probes or TaqMan Low Density Array (Applied Biosystems) were used to analyse the gene expression by quantitative PCR. Gene expression was normalised against the house-keeping genes Ribosomal Protein Lateral Stalk Subunit P0

(DCs, PAPER I and III) or beta-2-microglobulin (T cells, PAPER III) and calculated as $2^{(CT(Target\ Gene)-CT(House\text{-}keeping\ gene)}$.

In PAPER IV, sorted $CD69^{-}CD103^{-}CD49a^{-}$ dermal T cells were collected in RPMI 1640 supplemented with 50% FBS and seeded in 96 well U bottom plate in presence of the cytokines previously enlisted.

3.11 FLOW CYTOMETRY (PAPER I, II, III AND IV)

Cells were collected and stained with Live/Dead Fixable Yellow Dead Cells kit (Thermo Fisher) in presence of human Fc receptor binding inhibitor (eBioscience, only when staining LCs and DCs). After washing, cells were stained with antibody cocktails directed against surface markers, then fixed and permeabilised using Cytofix/Cytoperm (BD Biosciences) and stained with antibodies directed against intracellular molecules. Samples were filtered through a 70 μm filter prior acquisition with CyAn ADP Analyzer (Beckman Coulter, PAPER I), LSRFortessa or LSRII (BD Biosciences, PAPER II, III and IV). Flow cytometric data and plots were analysed and generated using Flowjo version 9 (Tree Star).

3.12 STATISTICAL ANALYSIS

Mann-Whitney U test was used when comparing two groups with non-paired data, whereas the Wilcoxon matched-pair test was used when comparing two groups of paired data. The Kruskal-Wallis with Dunn's multiple comparisons test was used when comparing more than one group. All the statistical calculations were performed using Graphpad Prism version 6 and R (only PAPER I). All the plots were created with Graphpad prism except heat maps and spice charts in PAPER I, generated by MeV (TM4) and SPICE (NIH) respectively. P values < 0.05 were considered as significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4 RESULTS AND DISCUSSION

4.1 LANGERIN$^-$ DCS INFILTRATE THE SKIN IN LESIONAL PSORIASIS

In healthy skin, LCs are the only antigen presenting cells that reside in epidermis. In active psoriasis, an additional population of HLA-DR$^+$Langerin$^-$ cells infiltrates the epidermis, as shown by several reports (Alshenawy and Hasby, 2011, Fujita et al., 2011, Komine et al., 2007, Lowes et al., 2005, Zaba et al., 2009). This is not an exclusive feature of psoriasis, since a prominent epidermal infiltration of DCs is also seen in atopic dermatitis (Baadsgaard et al., 1989). When these cells were visualised by confocal and electron microscopy, we confirmed that the infiltrated epidermal DCs (eDCs) did not express langerin nor contain Birbeck granules (PAPER I, figure 1). In figure 4.1 we show the fluorescence intensity profile graphs obtained by analysing images of healthy or psoriatic epidermis. The graphs depict the quantification of the fluorescence intensity of a given region, in this case along a 250 μm line drawn across the fluorescent cells. The graphs clearly showed an overlap of the langerin and the CD74 (invariant chain of the MHC class II complex, thus identifying APCs) signals in healthy skin, whereas in psoriasis single-positive CD74 signals were detected. (figure 4.1).

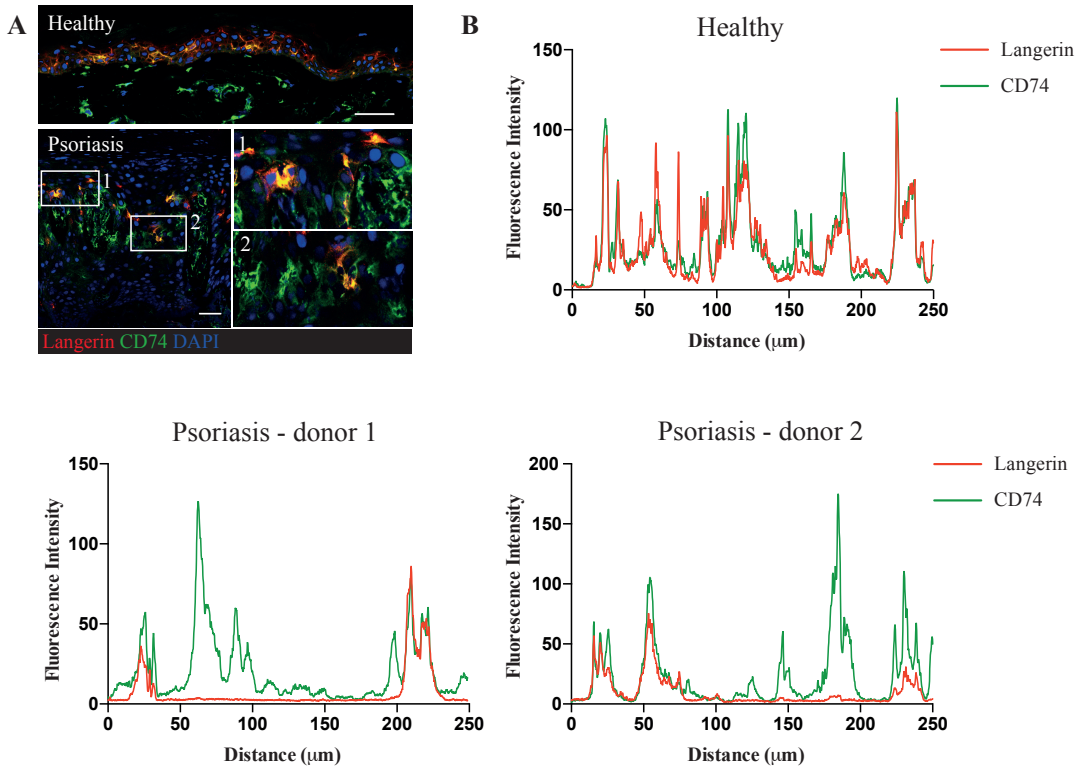

Figure 4.1. Infiltration of langerin$^-$ cells in lesional psoriatic epidermis. (A) Confocal microscopy image of healthy and lesional psoriatic skin. Langerin (red), CD74 (green), DAPI (blue). Scale bar: 50 μm. (B) Plots depicting the fluorescence intensity of langerin (red) and CD74 (green) in healthy or psoriatic epidermis.

Next, the eDC population was phenotyped by flow cytometry in enzymatically-digested cell suspension. Two populations of $CD11c^{bright}$ and $CD11c^{dim}CD141^{+}$ were detected (PAPER I, figure 1 and S1). The composition of the DC subsets based on CD11c and CD141 were similar in eDCs and dDCs. CD11c and CD141 expression is not confined to resident DCs but is upregulated by a variety of other cell types in inflammation (Kashem et al., 2017, Loike et al., 1991, Maecker et al., 2012). Therefore, whether eDCs originated from translocated dDCs or blood derived DC precursors could not be assessed. However, the expression of CCR2, both at the mRNA and protein level, might indicate a monocytic origin (PAPER I, figure 2). By analysing the expression of CD1a and CD1c on eDCs and dDCs, we observed that a high proportion of dDCs did not express these markers, typically expressed on resident dDCs in healthy skin (PAPER I, figure 2), which might be an indication of the non-dermal origin of these cells. A previous study on TNF and iNOS producing dDCs in psoriatic lesions proposed that these cells infiltrated the skin from the circulation, based on their vicinity to blood vessels (Hansel et al., 2011). Our phenotypic profile could be a further indication that the increase in the number of dDCs in lesional psoriasis might be due to non-resident cells infiltrating the skin. LCs in psoriatic epidermis maintained similar levels of EpCAM compared to healthy LCs and, as expected, expressed high CD1a (PAPER I, figure 1-2). The enumeration of LCs in psoriasis is still an open debate. In our experimental setup, we identified and sorted LCs from healthy, non lesional psoriasis and lesional psoriasis by using EpCAM expression, and we did not find significant differences in the number of LCs/mm^2 of skin (PAPER I, figure 2). Since we showed that the expression of EpCAM is comparable between healthy and lesional LCs and that sorted LCs expressed classical LC markers, such as *LANGERIN*, *ECADHERIN* and high *CD1A* (PAPER I, figure 2), we are confident that the sorted population is indeed composed by LCs.

Gene expression analysis of sorted LCs from healthy skin and LCs and eDCs from lesional psoriasis showed differential gene expression in cells obtained from lesional psoriatic skin compared to healthy skin (PAPER I, figure 3). LCs upregulated *IL15*, important for T cell proliferation, and *IL23A*, involved in the generation of Th17/Tc17 T cells. Interestingly, comparable levels of *IL15* mRNA were detected in LCs from non-lesional psoriasis compared to healthy LC, whereas *IL23A* mRNA was upregulated in both LCs from lesional psoriasis and non-lesional psoriasis (data not shown). This is an indication that, although LCs in non-lesional psoriasis display pro-inflammatory features, the gene expression of pro-inflammatory cytokines is not generally upregulated in patients with psoriasis. Epidermal DCs expressed high levels of of pro-inflammatory cytokines, such as *IL1B* and *IL6*, and chemokines involved in macrophage, neutrophil (*CXCL1*), and lymphocyte (*CCL22*, *CCL17*) recruitment. In parallel, LCs and eDCs from lesional psoriasis upregulated numerous genes associated to tolerogenesis, such as *PDL1*, *PDL2* (LCs and eDCs), *IL10*, *ILT3* and *IDO1* (eDCs). This double pro-inflammatory/regulatory nature of cells in inflammation in human tissues is not surprising. In a previous work dissecting functional properties of T cells in psoriasis (Cheuk et al., 2014), sorted T cells from the epidermis of lesional psoriasis displayed high gene expression of both pro-inflammatory (*IL17*, *IL22*, *IFNG*, Granzymes and

perforin) and inhibitory genes (*CTLA4*, *FOXP3* and *IL10*). Taken together, the gene expression profile of cells belonging to the DC compartment (PAPER I, figure 3) or to the T cell compartment (Cheuk et al., 2014) shows the complexity of the immune dysregulation in active psoriasis.

4.2 LCS AND EPIDERMAL DCS FROM LESIONAL EPIDERMIS PRODUCE IL-1β AND IL-23

As shown by van der Aar and colleagues, LCs do not express TLR2, TLR4 and TLR5 (van der Aar et al., 2007), (table 1.7.1). Therefore, when we tested the capacity of LCs to produce cytokines in response to TLR stimulation, lipopolysaccharide (LPS)-stimulated LCs from healthy skin did not produce IL-1β, IL23 or IL-10 (PAPER I, figure 4), as expected. In addition, LCs from healthy skin did not respond to the stimulation with resiquimod (R848) (PAPER I, figure 4), ligand of both TLR7 and TLR8 present in the intracellular compartment of LCs (van der Aar et al., 2007) (table 1.7.1). Conversely, LCs from lesional psoriasis responded to both LPS and R848 stimulation with IL-23 production. In an independent study, IL-23 production by LCs from lesional epidermis was shown as a consequence of TLR2 stimulation with zymosan (Sweeney et al., 2016). Collectively, the results shown in PAPER I and by Sweeney et al indicate a differential expression pattern (TLR2, TLR4) or activation threshold (TLR7/8) of TLRs on LCs from psoriatic skin. In accordance to the gene expression analysis (PAPER I, figure 3), eDCs displayed a high pro-inflammatory profile and produced both IL-23 and IL-1β after stimulation. It is important to highlight that eDCs produced IL-23 at similar or higher levels compared to dDCs, well known to produce IL-23 (Cai et al., 2011, Lee et al., 2004, Teunissen et al., 2012, Tonel et al., 2010, Yawalkar et al., 2009). In PAPER I, figure S1 we stained cryopreserved skin sections and analysed the localisation of CD74, langerin and CD3 expressing cells. As expected, CD74 and langerin co-localised and exclusively identified LCs, whereas the single-stained CD74 cells identified eDCs. The images showed that LCs and eDCs in psoriatic epidermis resided in close proximity to epidermal T cells. Considered the DC-T cell vicinity and the capacity of LCs and eDCs to produce cytokines important for T cell proliferation and activation, it is possible that both LCs and eDCs contribute to trigger the resident T cells to maintain focal pathology. In the attempt to provide definitive proof that LCs stimulate T cells in psoriasis lesions, skin-derived LCs, eDCs and dDCs were co-cultured with autologous blood-derived $CD45RA^-$ T cells in presence of the superantigen Staphylococcus Enterotoxin B. Proliferation and concentration of cytokines and chemokines released in the medium was assessed with flow cytometry and a multiplex immunoassay. The experimental design had several problems: 1) the number of cells recovered after sorting was very variable and did not allow reproducible co-culture conditions in terms of cell number and cell density in the wells; 2) LCs quickly died after sorting; 3) circulating T cells from patients with active psoriasis or during systemic treatment were more prone to secretion of cytokines compared to healthy T cells, thus the background levels were variable (figure 4.2.1); and 4) the choice of using autologous T cells,

made to closely resemble the *in vivo* situation, led to poor T cell stimulation and, as a consequence, low concentration of analytes released in the supernatants. In conclusion, we did not manage to setup a sound system and the obtained results were not of such quality to be fit for publication.

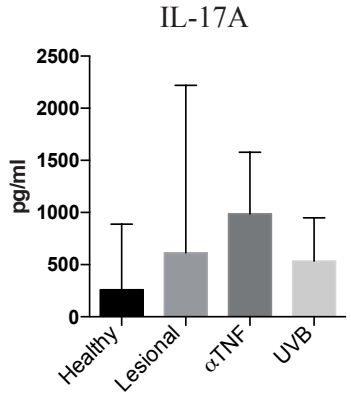

Figure 4.2.1. Peripheral blood T cells from healthy, lesional or treated individuals display differential IL-17 production after stimulation with SEB. Sorted $CD8^{+}CD45RA^{-}$ T cells from peripheral blood were cultured for 5 days in presence of the staphylococcus enterotoxin B (SEB). The levels of IL-17A released in the culture media were assessed with a multiplex immunoassay.

Despite the lack of this formal proof, the gene expression profile and cytokine production by LCs and eDCs, their vicinity to T cells and published data from independent studies (Sweeney et al., 2016) point towards an active pro-inflammatory role for LCs and eDCs in psoriasis plaques (figure 4.2.2).

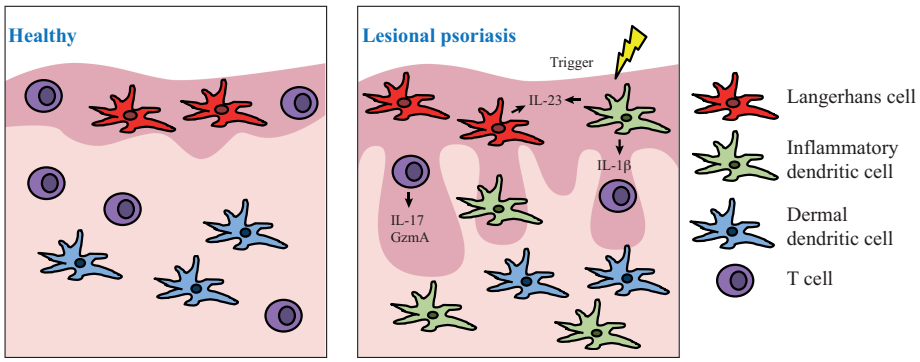

Figure 4.2.2. Schematic of the events occurring in the skin in lesional psoriasis. Adapted from Eidsmo, Martini "Tillsammans skapar stationära och cirkulerande immunceller kaos i psoriasisplack", BestPractice dermatologi, sep 2017.

4.3 GRANZYME A PRODUCED BY T CELLS CONTRIBUTE TO CHEMOKINE RELEASE FROM KERATINOCYTES

Psoriasis is a T cell-driven disease and T cells from lesional skin produce the cytokines IFN-γ, IL-17 and IL-22 (Cheuk et al., 2014, Hijnen et al., 2013, Lowes et al., 2008). Another essential function of CD8 T cells is cytotoxicity, mediated by granzymes (Gzms) and perforin, both upregulated at the RNA level in psoriasis (Cheuk et al., 2014, Suarez-Farinas et al., 2011). In addition to being upregulated during the active phase of psoriasis, *GZMA* is one of the genes that is retained at high levels, with less than 75% reduction after successful treatment (Suarez-Farinas et al., 2011).

In accordance to previously published gene expression data (Cheuk et al., 2014), CD8 T cells from lesional psoriasis displayed high levels of GzmA protein, but not of GzmB or perforin (PAPER II, figure 1 and figure 4.3).

The presence of GzmA in CD8 T cells in the absence of perforin has previously been observed in lymph node tissue sections obtained from HIV infected patients (Andersson et al., 1999), but not in skin, nor in the context of psoriasis.

To investigate if GzmA could affect skin homeostasis, we stimulated human primary keratinocytes with GzmA, alone or in combination with IL-17, and analysed the cytokines and chemokines released in the culture supernatant with a luminex-based multiplex panel. GzmA did not act alone, but in combination with IL-17, and caused an increase in the production of the chemokines CXCL1, CXCL12 and CCL4, known chemoattractants for neutrophils, lymphocytes and monocytes (PAPER II, figure 2 and S2). These results show an alternative function for GzmA outside its cytotoxic activity, which requires expression of perforin. Moreover, CD8 T cells may contribute to the pro-inflammatory environment in active psoriasis not only by producing cytokines, but also by enhancing keratinocyte-driven recruitment of other immune cells at the disease site (figure 4.2.2).

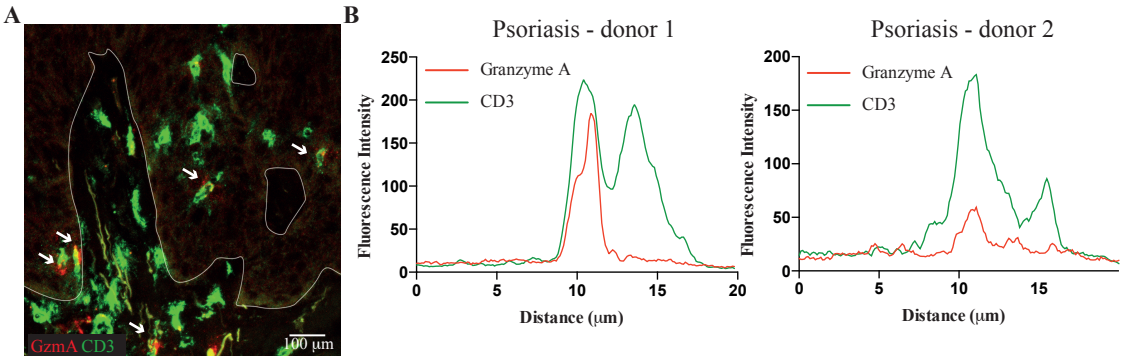

Figure 4.3. GzmA$^+$ T cells reside in the skin in psoriasis. (A) Confocal microscopy image of lesional psoriatic skin. White line outlines the profile of the epidermis, arrows indicate T cells containing GzmA granules. GzmA (red), CD3 (green). (B) Plots depicting the fluorescence intensity of Granzyme A (red) and CD3 (green).

4.4 LCS FROM THE EPIDERMIS OF SUCCESSFULLY TREATED PATIENTS MAINTAIN A PRO-INFLAMMATORY PROFILE

As previously mentioned, several strategies for the management of psoriasis are available, but these therapies do not cure psoriasis, and the disease comes back after therapy withdrawal. If the treatment is successful, the skin appearance ameliorates, and every sign of the previous inflammation disappears. Despite these fantastic macroscopic results, microscopic alterations persist in the skin (Suarez-Farinas et al., 2011). Epidermal T cells in resolved skin maintain a more active pro-inflammatory profile compared to dermal T cells, as displayed by their superior capacity to produce or co-produce IL-17 and IL-22 (Cheuk et al., 2014, figure 5). Taken the pro-inflammatory nature of resident T cells in resolved psoriasis, we next wanted to explore if neighbouring LCs and DCs accelerated or dampened this activity. We analysed

the DC compartment in the epidermis of patients with psoriasis after successful treatment with αTNF or UVB light therapies. By comparing the composition of the DC populations in non-resolved and resolved psoriasis plaques, we found that eDCs were restricted to the areas with ongoing disease (PAPER I, figure 5), therefore eDCs were absent from the epidermis after successful treatment with αTNF and UVB therapies. In contrast, LCs did not display variations in the number of cells/mm^2 compared to healthy skin (PAPER I, figure 5). The gene expression data obtained from sorted LCs from healthy or resolved skin revealed a residual upregulation of *IL23A* and *IL15* in LCs obtained from αTNF and UVB treated skin, respectively (PAPER I, figure 5), compared to healthy skin. We could not confirm the increased basal levels of IL-23 at a protein level, but when we stimulated LCs from αTNF treated skin with the TLR 7/8 agonist R848 we found increased IL-23 production, whereas LCs from healthy skin failed to do so, as previously shown (PAPER I, figure 5). Taken the vicinity of LCs and T cells in resolved skin (PAPER I, figure 5), into account, the results show that LCs can have the potential to stimulate resident T cells during both active and resolved disease (figure 4.2.2 and 4.4). In parallel, epidermal LCs in resolved psoriatic skin also retain their pro-inflammatory potential by quickly upregulating IL-23 after R848 stimulation.

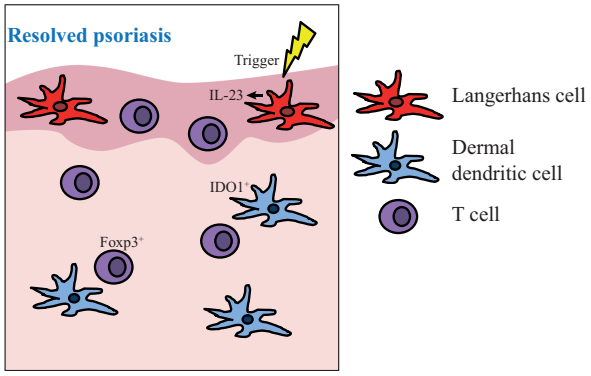

Figure 4.4. Schematic of the events occurring in the skin after successful treatment. Adapted from Eidsmo, Martini "Tillsammans skapar stationära och cirkulerande immunceller kaos i psoriasisplack" BestPractice dermatologi, sep 2017.

4.5 T CELLS AND DCS FROM THE DERMIS OF SUCCESSFULLY TREATED PATIENTS DISPLAY TOLEROGENIC FEATURES

Since dermal T cells are less active compared to their epidermal counterpart, we sought to investigate if T cells and DCs from resolved psoriatic skin presented a phenotype and functionality skewed towards tolerogenesis. Gene expression profiling of 45 genes in CD4 and CD8 T cells sorted from αTNF and UVB treated skin highlighted expression of several markers associated to T cell inhibition or tolerogenesis. *CTLA4* and the programmed cell death protein 1 (*PD1*) were upregulated in CD8 T cells sorted from UVB treated skin (PAPER III, figure 1). CTLA-4 is constitutively expressed on the surface of Treg cells and upregulated on activated T cells (Buchbinder and Desai, 2016, Takahashi et al., 2000). CTLA-4 is a T cell restricted marker that competes with CD28 on T cells for the binding to CD80 and CD86 present on the surface of APCs and binds these receptors with higher affinity (Collins et al., 2002). The binding of CTLA-4 does not result in an activation signal, as CD28 does, and affects T cell motility to impede T cell-APC engagement (Schneider et al., 2006). PD-1 is another important mediator of T cell tolerance. The binding of PD-1 to one of

its ligands result in inhibition of T cell proliferation, inhibition of the production of pro-inflammatory cytokines and of cytokines important for T cell survival (Keir et al., 2008). PD-1 has a broader expression compared to CTLA-4, and is present on B cells, NK cells, monocytes and DCs. PD-1 ligands, PD-L1, PD-L2 and CD80, are present on tissue cells and monocytes (PD-L1) or DCs (PD-L1, PD-L2 and CD80) (Keir et al., 2008). We did not find differences in the mRNA expression levels of *CD80*, *CD86* (PAPER III, supplementary figure 2) or *PDL2*, while the expression of *PDL1* was slightly upregulated in dDCs from αTNF treated dermis (figure 4.5.1). In the CD4 T cell compartment, we noticed the upregulation of *FOXP3* regardless of the treatment strategy (PAPER III, figure 1). Foxp3$^+$ T cells were visualised at the confocal microscope in UVB treated skin and were found in close contact with CD74$^+$ APCs (PAPER III, figure 1).

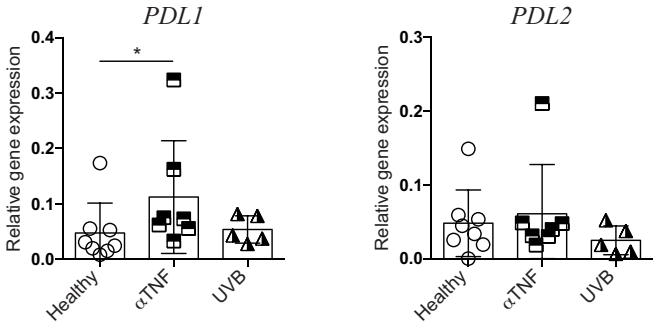

Figure 4.5.1. Expression levels of *PDL1* and *PDL2*. Relative gene expression of *PDL1* and *PDL2* in sorted dermal CD11c$^+$ cells from healthy, αTNF- or UVB- treated skin. Kruskal-Wallis with Dunn's multiple comparisons test.

Thereafter, we sorted CD11c$^+$ DCs from the dermis of αTNF and UVB treated patients and correlated their gene expression profile with healthy CD11c$^+$ cells (PAPER III, figure 2). The gene expression of the AMP *S100A8* remained upregulated after treatment, indicating residual low-grade inflammation, in accordance to previously published data (Gudjonsson et al., 2009, Suarez-Farinas et al., 2011). The interpretation of the upregulation of *IL27* in DCs from treated dermis is more complicated, as IL-27 has both pro- and anti- inflammatory functions depending on the surrounding conditions (Yoshida and Hunter, 2015). On the other hand, *ALOX15B* was found to be upregulated in healthy skin compared to treated skin. This result confirmed previously published data on gene expression analysis of healthy, lesional and non-lesional skin, where *ALOX15B* was shown to be upregulated in healthy skin (Gudjonsson et al., 2009). Interestingly, no major differences were noticed when correlating the gene expression profile of DCs between the two treatment strategies, but some differences were evident when comparing dDCs from lesional psoriasis with dDCs from treated skin (figure 4.5.2). Overall, by comparing the gene expression correlation plots of epidermal LCs (PAPER I, figure 5) with the correlation plots of dDCs (PAPER III, figure 2 and figure 4.5.2) in healthy and resolved skin, we could conclude that residual alterations were mainly found in the epidermis.

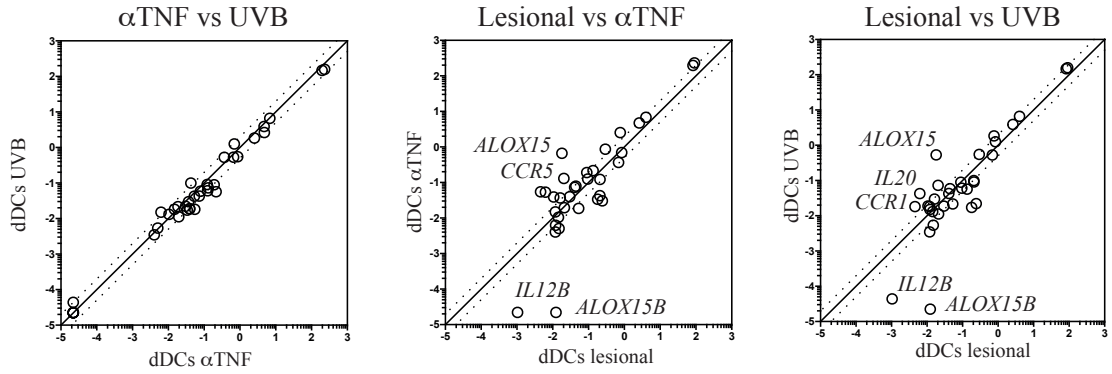

Figure 4.5.2. Differences in the gene expression profile between dDCs from lesional psoriasis or after treatment with αTNF or UVB. Gene expression correlation plots comparing sorted CD11c$^+$ dDCs from lesional psoriasis, αTNF treated dermis or UVB treated dermis. Dotted line indicates 2-fold difference.

One of the genes that was upregulated in dDCs obtained from both αTNF and UVB treated skin was IDO1. IDO1 is the first enzyme of the tryptophan metabolism pathway and is a limiting factor in the conversion of tryptophan to kynurenine. The depletion of tryptophan interferes with T cell proliferation and produces toxic metabolites, with the overall result of inhibiting T cell proliferation and activation (Mellor and Munn, 2004). IDO1 expression was highly upregulated in DCs from UVB treated skin, therefore we recruited a new cohort of UVB treated patients, generated the dermal cell suspension and analysed the protein expression of IDO1 by flow cytometry. In these patients, IDO1 displayed a trend towards increased expression in UVB patients and a high variation in healthy skin, but the difference between healthy controls and UVB patients was not statistically significant (PAPER III, figure 2). Further analysis of the phenotype of IDO1-expressing cells showed an enrichment in the CD1c$^+$CD1a$^-$ subpopulation, indicating variations in the composition of the DC populations in UVB treated skin (PAPER III, figure 2). Interestingly, and in contrast to healthy skin, IDO1 expression was not upregulated in dDCs from UVB treated skin after IFN-γ stimulation of the cell suspension, which might indicate saturated expression or incapacity of IFN-γ alone to upregulate the production of IDO1 in dDCs from UVB treated skin (PAPER III, figure 2). In summary, the overall picture of the skin after psoriasis treatment shows that cells residing in the epidermis retain features of the disease (PAPER I, figure 5 and (Cheuk et al., 2014)), whereas both T cells and DCs in the dermis display anti-inflammatory features (figure 4.4). This compartmental difference is important to keep in mind because it could lead to the design of therapeutic strategies aiming at taking advantage of the enrichment of anti-inflammatory cells in the dermis, with the aim of prolonging the time in remission after therapy withdrawal.

4.6 ENVIRONMENTAL EFFECTS ON THE DEVELOPMENT AND FUNCTIONALITY OF TRM CELLS

The composition of the Trm cell populations residing in the skin in the steady state differs between epidermis and dermis. The epidermis contains a high proportion of CD69$^+$ Trm cells expressing CD103 or co-expressing CD103 and CD49a, whereas the majority of the Trm

cells in the dermis only expresses CD69, with few cells co-expressing CD103 (Cheuk et al., 2017, Cheuk et al., 2014, Watanabe, 2015, Wong et al., 2016). Previously published data revealed that T cells with shared clonality reside in both epidermis and dermis, therefore a common origin for epidermal and dermal Trm cells might be possible (Cheuk et al., 2017). Moreover, CD49a expression on $CD103^+$ epidermal Trm cells is linked to the enhanced capacity to produce IFN-γ, whereas the absence of CD49a favours the production of IL-17 (Cheuk et al., 2017).

In light of 1) the differential expression pattern of the Trm cell markers in the two skin compartments, 2) the differential environment established by the cytokines produced by LCs and DCs in epidermis and dermis and 3) differences in the skin milieu surrounding Trm cells in the steady state or in inflammation, we next set out to study the development and plasticity of skin Trm cells.

To this end, we stimulated epidermal and dermal cell suspensions with a cytokine cocktail composed by TGF-β, IL-2 and IL-15 in presence or absence of anti-CD3 (αCD3). The components of the cytokine cocktail were chosen based on their relevance for T cell survival or formation of memory cells. IL-2 is important for the long-term survival of T cells *in vitro* (Hedfors and Brinchmann, 2003), although it has never been implicated in the formation of tissue resident T cells. TGF-β and IL-15 are instead important for the upregulation of CD103 in mouse across different tissues (Casey et al., 2012, Mackay et al., 2013, Masopust et al., 2006, Schenkel et al., 2016, Zhang and Bevan, 2013) and in human T cells from peripheral blood (Watanabe, 2015). Moreover, IL-15 can efficiently activate $CD103^+CD49a^+$ cells to exert their cytotoxic capacity (Cheuk et al., 2017). The requirement of antigen stimulation for the upregulation of resident markers is less clear. Since our experimental setup consists on the stimulation of a pool of T cells with unknown antigen specificity, a broad CD3 activation by an αCD3 antibody was used to mimic TCR engagement.

In parallel to the skin cell suspensions, we stimulated magnetic-activated cell sorted (MACS) $CD8^+$ T cells from peripheral blood mononuclear cells. As expected, the kinetics of the upregulation of resident markers in CD8 T cells from peripheral blood showed that the early activation marker CD69 was quickly upregulated already at day 1 and declined after 7 days of stimulation, whereas both CD103 and CD49a were slowly induced and reached high levels of expression at day 7 when cells were stimulated with TGF-β, IL-2 and IL-15 in presence of αCD3 (PAPER IV, figure 2). At day 1, blood-derived CD8 T cells did not express CD69, CD103 nor CD49a, whereas nearly all dermal and epidermal T cells expressed CD69 (PAPER IV, figure 2, 3 and 4). After 7 days of stimulation, we observed an increase in the proportion of blood and dermal CD8 T cells co-expressing CD103 and CD49a. The combination of TGF-β, IL-2, IL-15 and αCD3 was most efficient in inducing CD103 in combination with CD49a (PAPER IV, figure 2 and 3). Interestingly, and contrary to the blood and dermal samples, epidermal T cells did not change the expression of the Trm cell markers when exposed to cytokines in combination with αCD3 (PAPER IV, figure 4).

The upregulation of CD103 and CD49a by IL-2 stimulation is interesting in itself. The IL-2 receptor (IL-2R) is composed by the subunits alpha (IL-2RA, or CD25), beta (CD122) and gamma (CD132) (Minami et al., 1993). The beta and gamma subunits are also part of the IL-15 receptor (IL-15R) (Budagian et al., 2006), but the alpha subunit is specific for the IL-2R. It has been shown that CD69$^+$ cells collected from different human tissues (Kumar et al., 2017) including skin (Wong et al., 2016) express low levels of CD25, therefore it could be surprising that dermal cells responded to IL-2 stimulation, although the response of T cells to IL-2 signalling in absence of CD25 has been reported before (Hedfors and Brinchmann, 2003). It is important to point out that the stimulation with TGF-β, IL-2, IL-15 and αCD3 did not only affect the expression of CD103 and CD49a, but also caused the downregulation of CD69. However, this downregulation was less pronounced in epidermal T cells compared to blood and dermal T cells: only 20% of the epidermal cells lost the expression of CD69 (PAPER IV, figure 4), compared to 80% of downregulation in dermal cells (PAPER IV, figure 3). Taken together, these results indicate that dermal T cells have a high degree of phenotypic plasticity, whereas epidermal cells have a more rigid phenotype.

We also analysed the proliferative capacity of epidermal, dermal and blood CD8 T cells by marking cells with carboxyfluorescein succinimidyl ester (CFSE) prior to stimulation. CFSE penetrates the cell-membrane, covalently binds to intracellular proteins and is equally divided between dividing cells, thereby the expression is decreased two-fold when the cells divide. By analysing the CFSE expression pattern, we observed that dermal CD103$^+$CD49a$^-$ cells formed after stimulation with TGF-β, IL-2, IL-15 and αCD3 underwent multiple proliferation cycles, whereas the pattern of proliferation of the CD103$^+$CD49a$^+$ cells was donor-dependent (PAPER IV, figure 3). CD103$^+$CD49a$^+$ and CD103$^+$CD49a$^-$ cells differentiated from blood-derived CD8 T cells showed a comparable proliferation pattern (figure 4.6). The proliferative capacity of blood-derived cells was not consistent with that of dermal cells within the same donor (data not shown). Conversely, epidermal T cells belonging to both CD103$^+$CD49a$^+$ and CD103$^+$CD49a$^-$ cell populations from 3 out of 4 donors did not proliferate (PAPER IV, figure 4).

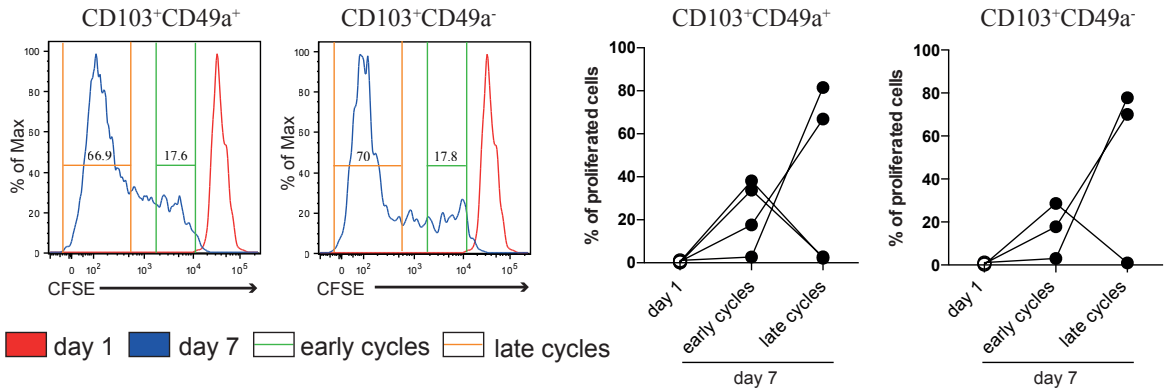

Figure 4.6. Proliferative capacity of CD103$^+$CD49a$^+$ and CD103$^+$CD49a$^-$ cell populations differentiated from CD8$^+$ peripheral blood cells. Cells were stained with 1μM CFSE and cultured for 7 days in presence of TGF-β, IL-2, IL-15 and αCD3. Representative histograms and graph depicting the proportion of cells undergone multiple proliferation cycles in the CD103$^+$CD49a$^+$ and CD103$^+$CD49a$^-$ cell populations.

Next, the cytokine producing properties of *in vitro* derived $CD103^+CD49a^+$ and $CD103^+CD49a^-$ cells were assessed with flow cytometry (PAPER IV, figure 5). We found that the magnitude of cytokine production was largely dependent on the type of stimulation received by the cells during the 7 days in culture. Few $CD103^+CD49a^+$ epidermal cells produced IFN-γ after 7-day stimulation with TGF-β, IL-2, IL-15 and αCD3, whereas a high proportion of dermal $CD103^+CD49a^+$ cells produced IFN-γ regardless of the stimulatory conditions. Conversely, IL-17 production was ascribed to the $CD103^+CD49a^-$ population in the epidermis independently of the stimulation received by the cells, whereas it was confined to the $CD49a^-$ cells stimulated with IL-2 alone in the dermis (PAPER IV, figure 5).

We have previously shown that LCs and eDCs contributed to the pro-inflammatory environment in lesional psoriasis by producing IL-1β and IL-23 (PAPER I) and we also showed that the type of stimulation received by epidermal and dermal cells for 7 days had a high impact on their cytokine production profile (PAPER IV, figure 5). To determine if pro-inflammatory cytokines would affect the phenotype or functionality of the epidermal and dermal cells, we stimulated the cell suspensions with a cytokine cocktail containing IL-1β and IL-23 besides TGF-β, IL-2 and αCD3 for 7 days. The expression of CD103 and CD49a in dermal cultured cells increased and reached the same levels achieved after stimulation with TGF-β, IL-2, IL-15 and αCD3. Epidermal cells, as previously shown with other stimulating conditions, did not alter their expression of CD103 and CD49a. Strikingly, the epidermal cells responded with increased IL-17 production compared to the levels achieved after stimulation with TGF-β, IL-2, IL-15 and αCD3. This increase was noticed in both $CD103^+CD49a^+$ and $CD103^+$ $CD49a^-$ cell populations, although $CD103^+CD49a^-$ cells were still better at producing IL-17 compared to the counterpart. The results obtained in PAPER IV clearly highlight the influence of the environment on the expression of resident markers, and how T cells residing in different compartments of the skin (or in peripheral blood) respond in different ways to the inputs that might be present in their surroundings by changing the surface marker expression pattern or their functionality. Understanding these dynamics is important because it could lead to the possibility to influence the cell phenotype and function, and to the possibility to skew them towards a non-pathogenic profile. We have shown here that the magnitude of IFN-γ and IL-17 production by the cells cultured for 7 days with different stimuli depends on the type of stimulation itself. Therefore, it is relevant to ponder over the consequences of cytokine-targeted strategies and how they might affect the composition and function of resident cells.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Skin resident immune cells are instrumental for ensuring protection against foreign threats, but their role in maintaining localised inflammatory diseases is increasingly appreciated. In this thesis, individual immune cell subsets, in particular LCs, DCs and T cells, were characterised in different phases of psoriasis. Moreover, the development and function of resident T cells in healthy skin was investigated. Here, epidermal and dermal DCs and T cells were studied separately, instead of in full-thickness skin.

In lesional psoriatic plaques, both resident and infiltrated immune cells contributed to the maintenance of a pro-inflammatory environment by secreting Th17/Tc17 skewing cytokines (PAPER I). The T cell derived granzyme A, in concert with IL-17, induced the release of neutrophil- and lymphocyte- attracting chemokines by keratinocytes, that might lead to the recruitment of additional pro-inflammatory cells perpetuating the inflammatory loop (PAPER II). The compartmentalised features of the skin immune system were evident when analysing DCs and T cells from resolved psoriatic skin after successful treatment. In the epidermis, LCs obtained from skin following anti-TNF inhibition therapy maintained the pro-inflammatory characteristics by producing IL-23 after TLR stimulation (PAPER I), whereas hints on the presence of anti-inflammatory DCs and T cells in the dermis from resolved psoriasis could be detected (PAPER III).

Epidermal immune cells in resolved psoriasis, directly exposed to the external environment, responded more vigorously to stimulation compared to the cells situated in the deeper strata of the skin. One conclusion from my thesis work is that this pro-inflammatory immune cell reaction combined with the enhanced reactivity, or lower activation threshold, in keratinocytes in resolved lesions could cause a new disease flare. In parallel, inhibitory dermal cells seem to be anti-inflammatory.

In healthy skin, different populations of Trm cells reside in epidermis and dermis. $CD103^{+}CD49a^{+}$ Trm cells are enriched in the epidermis, together with single positive CD103 expressing Trm cells, whereas the dermis is almost completely devoid of these two populations. Here we showed that dermal Trm cells could acquire the expression of both CD103 and CD49a when stimulated with a cocktail comprising TGF-β, IL-2 and IL-15, important for their T cell stimulatory capacity and involved in the formation of Trm cells in mouse. Contrary to dermal Trm cells, epidermal Trm cells did not possess this phenotypic plasticity, but their IL-17 production levels were increased when stimulated with IL-23 and IL-1β (PAPER IV). These results further highlight the different nature of Trm cells residing in epidermis and dermis and show that epidermal cells still have the capacity to adapt to the input received from the outside.

Future studies will aim at expanding the findings shown in PAPER IV, by analysing the phenotypic and functional characteristics of epidermal and dermal Trm cells from diseased skin. These studies would unravel potential differences in terms of phenotypic and functional

plasticity due to the different genetic background or differential capacity to respond to environmental clues. Moreover, it would be of great interest to sort $CD103^+CD49a^+$ and $CD103^+CD49a^-$ cells from the epidermis or from the cultured blood or dermal T cells and compare their transcriptional and epigenetic profile. These analyses will reveal if the cells obtained after 7 days of culture are similar to *bona fide* Trm cells or if they retain clues of their origin.

The experiments presented in this thesis show that it is possible to upregulate residency markers on dermal cells, but if these markers remain upregulated for a prolonged period of time or if they really confer residency to the cells is not known. One way to test this possibility is to transplant human skin on an immunodeficient mouse, deliver the cytokine cocktail to the skin and verify if the cells upregulate the residency markers *in vivo*, how long these markers remain upregulated and if the cells are retained into the skin. To verify if blood derived cells have the potential to generate a stable Trm cell population in the skin, it would be possible to sort peripheral memory T cells with skin tropism (CLA^+), label these cells and transfer them to the mice, then deliver the cytokine cocktail to the skin and follow the fate of the labelled cells. If the establishment of these two systems is possible, the following step would involve the induction of skin infection to test if the "induced" Trm cells would confer better microbial protection or would establish an exaggerated immune response.

A deep understanding of the phenotype, function and plasticity potential of resident DCs and T cells from different skin compartments would lead to targeted strategies aiming at eradicating or reprogramming resident immune cells, strategies that can be applied not only in the context of psoriasis but also in other inflammatory skin diseases or in skin malignancies.

6 ACKNOWLEDGEMENTS

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