Characterising the Plasticity of Cutaneous Myeloid Cells in Graft-Versus-Host Disease

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DOCTOR OF PHILOSOPHY

Declaration

I, Heather Carol West, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The skin is an important barrier to the external environment. Within the skin, immune cells interact to maintain a healthy protective environment in the presence of daily challenges. This requires the induction of tolerance to innocuous insults, but activation of adaptive immunity upon infection with pathogens. Within healthy skin, myeloid cells such as monocytes, macrophages, dendritic cells and Langerhans cells (LC) cooperate to maintain this balance. However, we know little about how disease may impact on these dynamic cell populations and their control of skin immunity.

Haematopoietic stem cell transplantation is a curative treatment for some blood cancers. However, the beneficial anti-tumour effect is often associated with the pathophysiology of graft-versus-host disease (GVHD). This thesis is focused on understanding how the skin myeloid compartment is altered after GVHD, and the consequences of these changes on skin immunity.

We have used a murine model of sub-lethal GVHD to investigate the developmental and functional impact of skin pathology on different myeloid cell populations. We have characterised the immune environment of the skin during and after GVHD and identified lasting changes to the myeloid compartment. In particular, pathology resulted in the recruitment of blood monocytes with the plasticity to differentiate into different cell types in the skin environment. Alterations to dermal myeloid cells led to defects in cutaneous immunity, including a breakdown in peripheral tolerance and protective barrier immunity. We demonstrated a profound loss of regulatory T cell function *in situ* and identified monocyte-derived IL-6 as a potential mediator of loss of function.

We have further utilised transcriptional analyses with a reductionist *in vitro* culture model to infer information about myeloid cell differentiation during GVHD. The approach has identified proliferation of myeloid precursors as a critical and unreported step in LC differentiation, and highlighted the importance of interleukin (IL)-34 in the generation of a persistent LC network following injury.

Together, this work has demonstrated the plasticity of myeloid cells within the skin after disease and the potential long-term consequences for skin immunity in patients who have recovered from GVHD.

Impact Statement

Acute GVHD remains the second leading cause of death following allogeneic HSCT. The skin, gut and liver are the principle target organs in acute GVHD, with cutaneous manifestations the most common and often the presenting sign of the disease. During the course of the disease, the immune environment of the skin is drastically altered resulting in a significantly disrupted myeloid compartment. The full effect of these changes on long-term patient immune function has not previously been characterised.

In work funded by the Medical Research Council, the Royal Free Charity and the Biotechnology and Biological Science Research Council, we used an *in vitro* and *in vivo* combinatorial experimental strategy to further understand how the skin myeloid compartment is altered after GVHD, and the consequences of these changes on skin immunity.

This project has identified a breakdown in local immune responses in the skin following GVHD, affecting both cellular and humoral responses. Crucially, we have demonstrated a long-term breakdown in cutaneous tolerance resulting from loss of regulatory T cell function in a murine model of GVHD. We have revealed IL-6 as one potential mediator of this breakdown, pointing to anti-IL-6 therapy as an attractive target for restoring tolerogenic responses in the skin. This work highlights the need to further investigate regulatory T cell function and tolerogenic responses in patients following GVHD.

Aside from the clear clinical implications of this project, we have generated data that furthers the understanding of the basic immunology field. We have identified lasting changes to the cutaneous immune environment after bone marrow transplantation and have utilised transcriptional analyses of cells to infer information about myeloid cell differentiation following injury. Our data strongly supports a monocytic origin for repopulating LC that can become long-term LC due to IL-34-dependent upregulation of Id2. We have further defined proliferation of LC precursors as a vital, and unreported, step in LC development. Finally, our data highlights the benefits of utilising *in vitro* culture models to study the differentiation and survival of LC in a reductionist environment.

Overall, the implications of this work are potentially far-reaching with regards to directing patient therapies and understanding core skin immunology.

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Table of Contents

Table of Figures

List of Tables

Abbreviations

AMP	Antimicrobial peptide
APC	Antigen presenting cell
BM	Bone marrow
BMP	Bone morphogenic protein
BMT	Bone marrow transplant
BSA	Bocine serum albumin
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDP	Common dendritic cell progenitor
CLR	C-type lectin receptor
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
CTL	Cytoxic T lympocyte
CXCL	(C-X-C motif) ligand
DAMP	Danger-associated molecular pattern
DETC	Dendritic epidermal T cell
DLI	Delayed lymphocyte infusion
DNA	Deoxyribonucleic acid
DNFB	2,4-dinitro-1-fluorobenzene
DSEARCH	Dendritic surveillance extension and retraction cycling habitude
DTR	Diphtheria toxin receptor
E7.0	Embryonic day 7.0
ECM	Extracellular matrix
EMP	Erythro-myeloid progenitors
EMT	Epithelial-to-mesenchymal transition
GVHD	Graft-versus-host disease

GVT	Graft-versus-tumour
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IMQ	Imiquimod
iNOS	Inducible nitric oxide synthase
LC	Langerhans cell
LN	Lymph node
MET	Mesenchymal-to-epithelial transition
MHC	Major histocompatibility complex
miHA	Minor histocompatibility antigen
MMP	Matrix metalloproteinase
moDC	Monocyte-derived dendritic cell
mRNA	Messenger ribonucleic acid
NK	Natural killer
NLR	Nod-like receptor
OVA	Ovalbumin
PAMP	Pattern-associated molecular pattern
PRR	Pattern recognition receptor
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TBI	Total body irradiation
Tconv	Conventional T cell
TCR	T cell receptor
Tfh	T follicular helper cell
TGF	Transforming growth factor

Th	T helper cell
TipDC	TNF-α/inducible nitric oxide synthase producing dendritic cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TRM	Resident memory T cell
UV	Ultraviolet
WT	Wild type

Chapter 1 Introduction

1.1.   Haematopoietic stem cell transplantation

1.1.1.   HSCT: the double-edged sword

Allogeneic haematopoietic stem cell transplantation (HSCT) is a curative treatment for various haematologic, neoplastic and congenital disorders, and involves the replacement of a patient's haematopoietic system with donor haematopoietic stem cells (HSC). First successfully carried out in 1959 (Thomas et al., 1959), this treatment was initially regarded as a method to rescue lymphohaematopoiesis in patients after marrow-ablative therapy; however, it is now exploited due to the beneficial graft-versus-tumour (GVT) effect of allogeneic T cells. Accordingly, the vast majority of allogeneic HSCT (70%) are carried out on patients with haematologic malignancies, including acute myeloid leukaemia (32%), acute lymphoblastic leukaemia (16%), myelodysplastic syndromes (16%) and non-Hodgkin lymphoma (9%) (Koreth et al., 2009, Wynn, 2011, Ljungman et al., 2006, Cornelissen et al., 2009, Vellenga et al., 2011, Bruno et al., 2007, Peggs et al., 2005, Passweg et al., 2012).

Graft-versus-host disease (GVHD) is one of the major complications of allogeneic HSCT, where allogeneic donor T cells recognise recipient tissues as foreign and mount an immune response. Observations of GVHD were made in some of the earliest mouse models of HSCT, leading to the proposal of three crucial requirements for GVHD: (1) the graft must contain immunologically active cells; (2) the recipient must express antigens not present in the donor, and; (3) the recipient must be unable to mount an immune response capable of eliminating the transplanted cells (Billingham, 1966). It is now established that the immunologically active cells are donor T cells, and GVHD occurs when donor T cells respond to genetically defined proteins on recipient cells, most importantly human leukocyte antigens (HLA), which are highly polymorphic and are encoded by the major histocompatibility complex (MHC) (Welniak et al., 2007, Petersdorf et al., 1995, Krensky et al., 1990, Ferrara et al., 2009). The incidence of acute GVHD is directly related to the degree of mismatch between HLA proteins (Loiseau et al., 2007, Ratanatharathorn et al., 1998), with more HLA-mismatches increasing the likelihood of developing GVHD (Kernan et al., 1993, Flomenberg et al., 2004). In addition, GVHD frequently occurs in HLA-matched transplants, due to genetic differences in minor histocompatibility antigens (miHA), immunogenic peptides derived from polymorphic proteins presented on the cell surface by MHC molecules (Goulmy et al., 1996). Some miHA, such as HY and HA-3, are expressed on all tissues and are targets for both GVHD

and GVT (Bleakley and Riddell, 2004), whereas others, such as HA-1 and HA-2, are expressed abundantly on haematopoietic cells and may induce a greater GVT effect with less GVHD (Goulmy et al., 1996, Bleakley and Riddell, 2004).

Other risk factors for GVHD include advanced age of the recipient, donor multiparity and the use of peripheral blood stem cells as the source of the graft (Jagasia et al., 2012, Storb et al., 1983, Cutler et al., 2001, James et al., 2003, Atkinson et al., 1986).

Marrow-ablative conditioning prior to HSCT prevents an immune response mounted on the graft by the recipient, in addition to eradicating residual tumour cells. Historically, the emphasis of the therapeutic effect of HSCT was on the conditioning regimen and therefore it was assumed that the more intense and ablative the conditioning, the more effective the treatment was (Shimoni and Nagler, 2011, Thomas et al., 1977). It was later recognised that the GVT effect of allogeneic T cells could be sufficient to eradicate malignant disease, and that more aggressive conditioning caused more GVHD, leading to the development of reduced intensity conditioning regimens and expanding the number of patients eligible to undergo HSCT (Storb et al., 1997, Giralt et al., 1997, Giralt et al., 2001, Slavin et al., 1998, McSweeney et al., 2001).

1.1.2. The graft-versus-tumour effect

Evidence for the GVT effect came from a number of studies. In 1979, the risk of leukaemia relapse was reported as 2.5 times lower in patients with GVHD than without GVHD (Weiden et al., 1979). Later studies reported a decreased relapse rate in patients with chronic GVHD (Weiden et al., 1981, Horowitz et al., 1990, Sullivan et al., 1989). Other studies identified an increased risk of relapse in recipients of syngeneic donor transplants compared with other HLA-matched transplants (Horowitz et al., 1990), or when *ex vivo* T cell depletion was used as prophylaxis for GVHD (Marmont et al., 1991). Development of delayed lymphocyte infusions (DLI) led to the successful treatment of relapsing patients after transplantation (Kolb et al., 1990).

1.1.3. Pathophysiology of acute GVHD

Acute GVHD was classically defined as a disease that developed within 100 days of HSCT. However, it is now accepted that the kinetics of acute GVHD and the chronic form of the disease are variable and that disease features frequently overlap (Filipovich et al., 2005). The pathophysiology of acute GVHD can be conceptualised in three sequential phases: (1) tissue damage to the recipient caused by conditioning regimens; (2) donor T cell activation, and; (3) efferent effector phase. The first phase involves the conditioning regimen administered to the patient that includes total body irradiation (TBI) and/or chemotherapy and is crucial to eradicate underlying disease and support engraftment of donor cells (Bacigalupo et al., 2009). Prior to HSCT, the patient's tissues

have been damaged by a multitude of factors, including conditioning, underlying disease, treatment and comorbidity (Kaitin, 1991, Hill et al., 1997). Consequently, damaged recipient tissues release danger-associated molecular patterns (DAMP), including the pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and interferon (IFN)-γ, that signal tissue injury and activate recipient antigen presenting cells (APC) (Hill and Ferrara, 2000, Ferrara et al., 2003, Zeiser et al., 2004, Xun et al., 1994, Teshima et al., 2002). The inflammatory environment enhances the ability of professional APC to prime T cells and also promotes antigen presentation by non-professional APC within tissue (Shlomchik et al., 1999, Koyama et al., 2011). The inflammatory cytokines may also provide complimentary costimulatory signals to T cells (Hill et al., 2000), and increase the access of donor T cells to GVHD target tissue (Chakraverty et al., 2006).

The priming and activation of donor T cells is the hallmark of the second phase of GVHD pathophysiology. Murine studies have demonstrated that recipient APC are necessary and sufficient to activate donor T cells and initiate GVHD (Shlomchik et al., 1999, Teshima et al., 2002, Koyama et al., 2011). Although recipient haematopoietic APC have been proposed to be central players in GVHD initiation, other studies have shown that there may be considerable redundancy between professional haematopoietic APC and non-haematopoietic APC in the activation of donor T cells (Koyama et al., 2011, Markey et al., 2014, Toubai et al., 2012).

$CD4^+$ and $CD8^+$ T cell responses are dependent on the disparity between recipient and host with regard to MHC II (HLA-DR, -DP, -DQ) and MHC I (HLA-A, -B, -C), respectively (Ferrara et al., 2009, Sprent et al., 1988), although HLA-identical transplants can still give rise to GVHD due to differences in minor histocompatibility antigens (Goulmy et al., 1996, den Haan et al., 1998, den Haan et al., 1995, Goulmy et al., 1983, Goulmy et al., 1977). The T cell receptor (TCR) of donor T cells can recognise alloantigens on either recipient APC (direct presentation) or donor APC (indirect presentation) (Shlomchik, 2003, Lechler et al., 2001, Koyama and Hill, 2016). In direct presentation, donor T cells recognise either the peptide bound to allogeneic MHC molecules or allogeneic MHC molecules without peptide (Lechler et al., 2001, Sayegh and Carpenter, 1996, Newton-Nash, 1994). During indirect presentation, donor T cells respond to peptide derived from allogeneic MHC molecules presented on self-MHC (Sayegh and Carpenter, 1996, Markey et al., 2009). This, together with costimulation (signal 2) and inflammatory cytokines (signal 3), leads to donor T cell activation, proliferation and differentiation, characterised by secretion of cytokines and chemokines (Briones et al., 2011). T helper (Th)1 cytokines, including IL-2 and IFN-γ, are preferentially secreted and are critical in the pathophysiology of GVHD (Hill and Ferrara, 2000, Goker et al., 2001). While Th1

responses are considered the paradigm for T cell differentiation during GVHD, studies have demonstrated that Th2 and Th17 polarised cells can also induce skin injury in mice (Yi et al., 2009, Tawara et al., 2008, Carlson et al., 2009, Hill et al., 2010).

Following activation in lymphoid tissues, donor T cells traffic to target organs and cause tissue damage (Wysocki Blood 2005; Morris STM 2013). Migration is facilitated by the expression of numerous chemokines, such as MIP-1α, CCL2-5, CXCL2, CXCL9-11, CCL17 and CCL27 (Wysocki et al., 2005, Mapara et al., 2006, Serody et al., 2000), and the expression of selectins and integrins and their ligands, such as α4β7/MadCAM-1, αLβ2/ICAM1, and α4β1/VCAM-2 (Cyster, 2005, Murai et al., 2003, Waldman et al., 2006, Wysocki et al., 2005, Pribila et al., 2004).

The final phase of GVHD pathophysiology involves the generation of a complex cascade of multiple cytotoxic effectors of target tissue injury, which can be grouped into cellular effectors (e.g. cytotoxic T lymphocytes (CTL) and natural killer (NK) cells) and inflammatory effectors (e.g. TNF-α and IL-1) (Hill and Ferrara, 2000).

CTL and NK cells lyse target cells using the Fas/Fas ligand (FasL) pathway and the perforin/granzyme pathway (Kagi et al., 1994, Lowin et al., 1994, Schmaltz et al., 2001, Wasem et al., 2001). The secretion of inflammatory cytokines synergises with cellular effectors, resulting in amplification of local tissue injury and target organ dysfunction (Ferrara et al., 2009). TNF-α has a central role in the pathophysiology and can activate APC and enhance alloantigen presentation, recruit effector cells into target organs through the induction of chemokines and cause direct tissue damage by inducing apoptosis and necrosis (Hill and Ferrara, 2000, Teshima et al., 2002, Laster et al., 1988, Couriel et al., 2004, Zeiser et al., 2004, Antin and Ferrara, 1992, Piguet et al., 1987). IL-1 is also an important mediator of pathology (Antin and Ferrara, 1992) and its secretion occurs predominantly in the skin and spleen in experimental models of GVHD (Abhyankar et al., 1993).
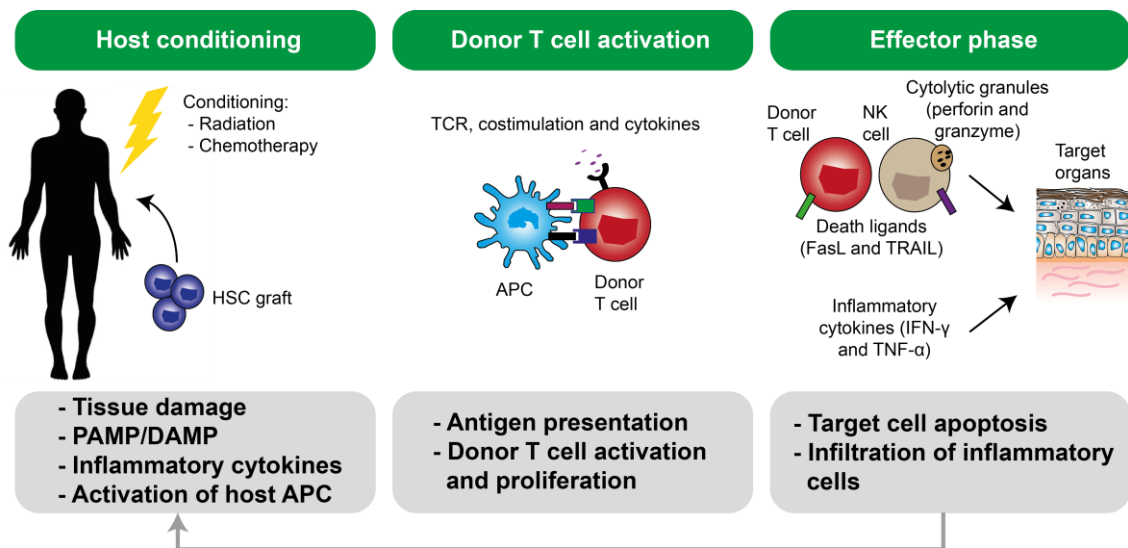
Figure 1.1 Pathophysiology of acute graft-versus-host disease

The three stages of acute GVHD. In the host conditioning phase, microbial products are released and tissue damage and inflammation occur, leading to host APC activation. Antigen presentation leads to donor T cell activation and proliferation. In the effector phase, donor effector immune cells (CTL and NK cells) attack host tissues by various mechanisms including target cell apoptosis by cytolytic granules or death ligands. A strong inflammatory cytokine "storm" results in target organ damage and further promotes antigen presentation and the recruitment of effector T cells and innate immune cells. Adapted from (Jenq and van den Brink, 2010).

1.1.4. Chronic GVHD

Chronic GVHD is the most common complication and the major cause of late non-relapse death after HSCT (Lee et al., 2002, Socie et al., 1999, MacDonald et al., 2017, Flowers and Martin, 2015). Disease may evolve directly from acute GVHD (progressive), or following a period of resolution (quiescent), or develop with no prior history of acute GVHD (*de novo*) (Shimabukuro-Vornhagen et al., 2009). The greatest risk factors for chronic GVHD are older recipient age and prior history of acute GVHD (Carlens et al., 1998). The clinical manifestations of chronic GVHD are many and varied, affect multiple organs and often resemble autoimmune syndromes. This complex disease is characterised by immune dysregulation, immunodeficiency, impaired organ function and decreased survival (Pavletic et al., 2005).

1.1.5. Cutaneous GVHD

The skin, gut and liver are the principle target organs in acute GVHD, with cutaneous manifestations the most common and often the presenting sign of the disease (Martin et al., 1990, Santos et al., 2018a, Strong Rodrigues et al., 2018). The characteristic erythematous maculopapular rash can begin anywhere on the body but often starts with palm and sole involvement. Skin lesions often spread to the trunk and can affect extensive body surface area (BSA), with severe cases involving skin blistering and ulceration (Vogelsang et al., 2003). Characteristic histopathologic findings include

apoptosis at the base of epidermal rete ridges, dyskeratosis, exocytosis of lymphocytes, satellite lymphocytes adjacent to dyskeratotic epidermal keratinocytes and a perivascular lymphocytic infiltration into the dermis (Ferrara and Deeg, 1991, Goker et al., 2001).

Table 1.1 Acute GVHD grading

Histopathology described by Lerner et al. for diagnosing GVHD, 1994 Consensus Conference on Acute GVHD Grading, focusing on skin GVHD. (Villarreal et al., 2016). BSA = Body surface area.

Grade	Histopathology described by Lerner et al. for diagnosing GVHD (Lerner et al., Transplant Proc. 1974)	1994 Consensus Conference on Acute GVHD Grading (Przepiorka et al., Bone Marrow Transplant 1995)
0	Normal skin	No GVHD rash
1	Mild vacuolization of epidermal cells	Maculopapular rash < 25% BSA
2	Diffuse vacuolization of basal cells with scattered dyskeratotic bodies	Maculopapular rash 25 – 50% BSA
3	Sub-epidermal cleft formation	Maculopapular rash > 50% BSA
4	Complete epidermal separation	Generalised erythroderma plus bullous formation

The skin is affected in over 90% of cases of chronic GVHD, and cutaneous involvement can be classified into two stages: lichenoid chronic GVHD, which tends to have an earlier onset, and sclerodermatous chronic GVHD, tending to manifest later (Penas et al., 2004, Villarreal et al., 2016). Lichenoid manifestation may be clinically indistinguishable from classic lichen planus: hyperkeratosis, hypergranulosis, acanthosis and dyskeratotic keratinocytes with basal cell vacuolisation (Vargas-Diez et al., 2005, Filipovich et al., 2005). Sclerodermatous manifestation involves epidermal atrophy, edema and homogenisation of the collagen in the upper dermis (Penas et al., 2002) and may clinically resemble systemic sclerosis.

1.2.   The skin as an immunocompetent organ

1.2.1.   Anatomy of the skin in the steady state

The skin is the largest organ in the human body and is the primary interface between the body and the environment. It performs many vital homeostatic functions, including restricting water loss, maintaining body temperature, acting as a sensory organ and providing a physical barrier against external insults (Matejuk, 2018). However, the skin is far more than just a passive barrier and is an immunologically active organ, constituting a well-coordinated network of immune cells that ensure adequate responses against pathogens, toxins and injury, while preventing unnecessary reactions to harmless foreign

antigens that would otherwise cause allergies, atopic dermatitis (contact hypersensitivity) and autoimmunity. In addition, the skin is home to as many as 10^{12} resident microorganisms including bacteria, viruses and fungi known collectively as skin microbiota (Fyhrquist et al., 2016). These diverse microbial communities live in tight symbiosis and homeostasis with the host and can directly influence local and systemic immunity.

The structure of the skin reflects the complexity of its functions as a protective barrier. The epidermis is the outermost layer of the skin and is a stratified squamous keratinised epithelium. Keratinocytes are organised into four strata that correspond to progressive stages of differentiation, known as keratinisation (Sotiropoulou and Blanpain, 2012). The stratum basale is the bottom layer of the epidermis and contains a single row of columnar germinating basal cells that divide frequently and are responsible for replenishing epidermal cells (Sen, 2011, Eckert, 1989, Watt, 1989, Solanas and Benitah, 2013). Basal keratinocytes differentiate and move into to stratum spinosum, where they divide to replenish the basal layer but also begin a maturation process. Within this layer, keratinocytes begin to produce cytokeratin, used to generate tonofilaments and tonofibrils that are subsequently aligned to transition from the columnar keratinocyte form of the stratum basale to the more flattened cells of the upper strata. Maturing cells enter the stratum granulosum where they actively secrete lamellar bodies containing lipid granules that comprise the major permeability barrier of the epidermis (Elias, 2012). The ultimate product of maturing keratinocytes is the stratum corneum, the outermost of the four strata in the epidermis comprised of terminally differentiated, enucleated skin cells known as corneocytes (Sotiropoulou and Blanpain, 2012). Corneocytes are connected by corneodesmosomes and are embedded in an extracellular lipid matrix, synthesised by the stratum below (Williams and Elias, 1993). The stratum corneum serves as the principle barrier against external insult (Madison, 2003, Jackson et al., 1993). Keratinocytes in the deeper layers also contribute to initiation of local immunity by pathogen recognition through pattern recognition receptors (PRR) and secreting cytokines and chemokines (Heath and Carbone, 2013).

In addition, other important cell types can be found in the epidermis, including melanocytes and merkel cells. Melanocytes are found predominantly in the stratum basale and function in the production of the pigment melanin and melanosome transfer to keratinocytes (Cichorek et al., 2013). Melanin presence in the skin defines its pigmentation and is involved in photoprotection against ultraviolet (UV) radiation (Yamaguchi et al., 2007, Lin and Fisher, 2007). Merkel cells are neuroendocrine cells located in the stratum basale that interact with nerve cells to constitute the sensory nerve

component of the skin, acting as mechanoreceptors (Ogawa, 1996, Moll et al., 2005, Halata et al., 2003, Boulais and Misery, 2007).

The epidermis is separated from the dermis by the basement membrane that allows exchange of cells and fluid and holds the two layers together (Behrens et al., 2012). The underlying dermis is rich in extracellular matrix and is highly vascularised, and contains stromal cells such as fibroblasts, fibrocytes and structural cells of the blood and lymph vessels. The dermis is composed of an upper papillary (stratum papillare) and lower reticular (stratum reticulare) containing thick and thin collagen fibres, respectively. These offer a mechanical barrier as well as a structural framework in which to host many specialised immune cells.
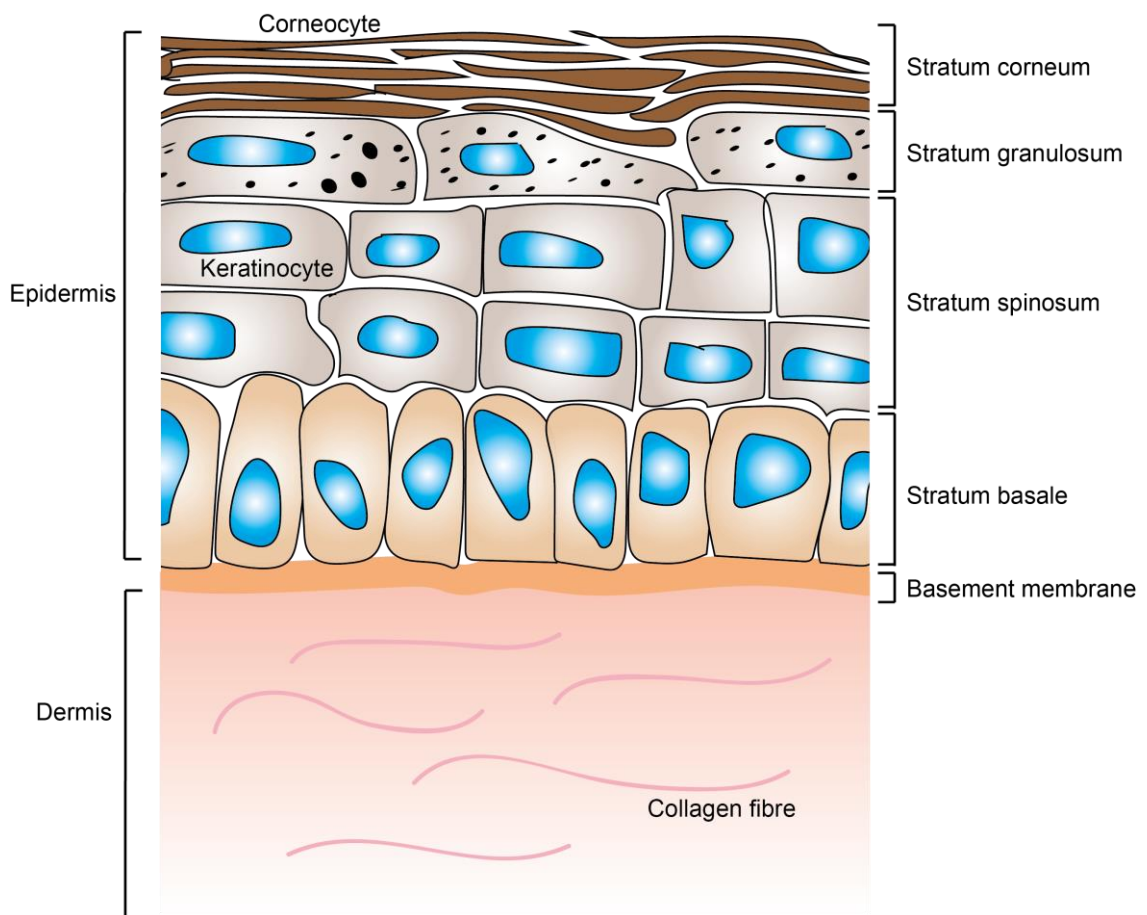
Figure 1.2 Anatomy of the skin

The epidermis is a stratified squamous keratinised epithelium, comprising four strata: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The epidermis is separated from the dermis by the basement membrane. The dermis is rich in extracellular matrix.

1.2.2.   Immune cells in the skin in the steady state

Under steady state conditions, the epidermis contains Langerhans cells (LC) as the only APC population, and both conventional and non-conventional T cells. LC are in intimate association with keratinocytes and are found in the stratum spinosum and stratum

granulosum, where they extend their dendrites through tight junctions to the stratum corneum to sample antigens and sense danger (Nagao et al., 2009).

The human adult epidermis contains approximately 20 billion resident memory T cells (TRM), consisting of resident and recirculating populations (Watanabe et al., 2015, Carbone et al., 2013, Mackay et al., 2012, Gebhardt et al., 2011, Gebhardt et al., 2009, Mueller et al., 2013). These cells display a unique dendritic morphology and are long-lived after initial recruitment to the epidermis owing to infection or inflammation (Zaid et al., 2014, Gebhardt et al., 2011, Mackay et al., 2012, Ariotti et al., 2012, Davies et al., 2017, Zhu et al., 2013).

Mice, but not humans, possess Vγ5Vδ1 T cells, named dendritic epidermal T cells (DETC) that form an interdigitating network within the layers of the epidermis (Nielsen et al., 2017, Havran and Jameson, 2010). DETC are the predominant T cell subset in murine skin and are involving in promoting wound healing and immune surveillance in the skin (Sharp et al., 2005, MacLeod et al., 2013, Lai et al., 2012). In humans, Vδ1 T cells are the major γδ T cell subset homing to the skin and have been attributed similar functions to DETC (Toulon et al., 2009, Pang et al., 2012).

In comparison to the epidermis, the steady state dermis is host to a much broader array of immune cells, including dendritic cells (DC), macrophages, mast cells, NK T cells, CD4$^+$ T helper (Th) cells, γδ T cells and innate lymphoid cells (ILC). These immune cells are tightly coordinated to mediate functions of immunity and tolerance.

Dermal DC can be divided into two conventional (c)DC populations which are transcriptionally distinct. Dermal CD103$^+$ cDC (also known as cDC1) depend on the transcription factor IRF8 and may also be characterised by surface expression of CD24, Langerin (CD207) and XCR1 in mice, and CD141 and XCR1 in humans (Edelson et al., 2010, Henri et al., 2010, Haniffa et al., 2013). Dermal CD11b$^+$ cDC (cDC2) are IRF4-dependent and also express CD172α and CD301b in mice, and CD1c and CD172α in humans (Schlitzer et al., 2013, Kumamoto et al., 2013, Haniffa et al., 2013). Unlike mice, Langerin appears to be restricted to the CD11b$^+$ cDC2 population in humans (Bigley et al., 2015).

CD103$^+$ cDC are not dermis-specific but found in all tissues. This cell type was first identified with Langerin-eGFP mice (Kissenpfennig et al., 2005) and has been shown to dominate the priming of CTL and Th1 immunity after skin infection (Brewig et al., 2009, Hildner et al., 2008, Stoecklinger et al., 2011, Bedoui et al., 2009, Igyarto et al., 2011a, Seneschal et al., 2014). By comparison, CD11b$^+$ cDC may be more important for the induction of Th2 immunity (Kumamoto et al., 2013).

This thesis focuses on the myeloid cell populations of the skin, which are discussed in more detail in section 1.3.
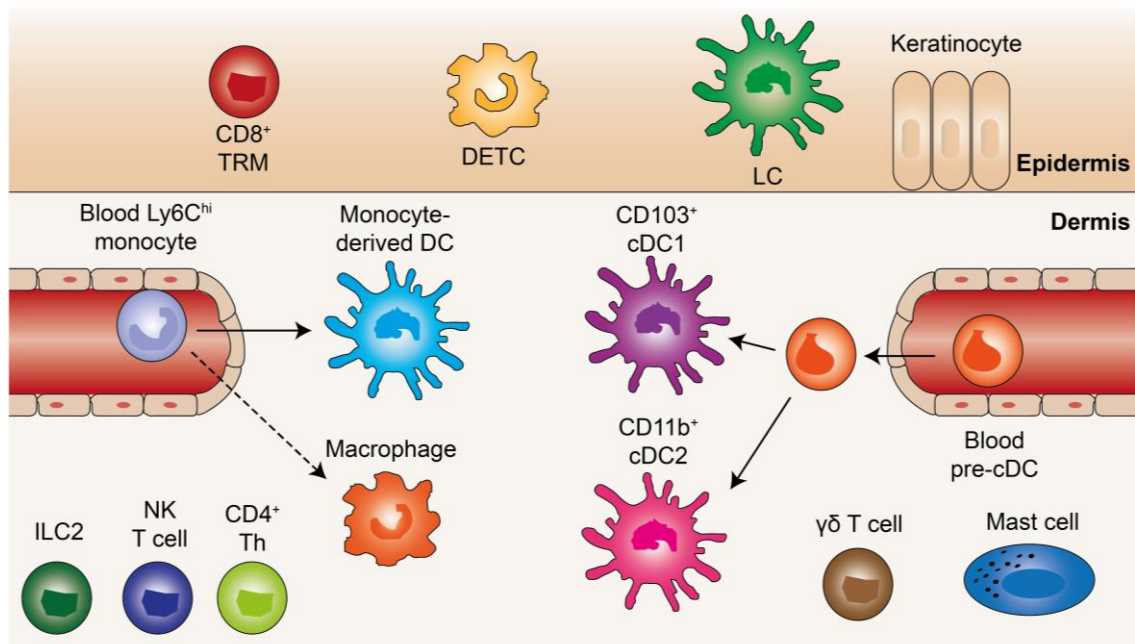
Figure 1.3 Cutaneous immune cells in the steady state

The epidermis contains Langerhans cells (LC), keratinocytes and CD8+ T resident memory cells (TRM). Murine skin additionally contains dendritic epidermal T cells (DETC). The dermis contains numerous immune cells, including conventional dendritic cells (cDC) (cDC1 and cDC2), monocyte-derived DC (moDC), macrophages, innate lymphoid cells (ILC)2, natural killer (NK) T cells, CD4+ T helper (Th) cells, γδ T cells and mast cells. Adapted from (Malissen et al., 2014).

1.2.3. Immune functions of the skin

In addition to its properties as a physical barrier, the skin has a host of active anti-microbial defence mechanisms. These include both innate and adaptive immune surveillance and encompass cells in both the epidermis and dermis.

<u>Sensing danger</u>

Detection of foreign or dangerous material in the skin depends on the expression of evolutionarily conserved PRR, which include Toll-like receptors (TLR), C-type lectin receptors (CLR), RIG-I-like receptors (RLR) and NOD-like receptors (NLR), and sense evolutionarily conserved microbial products termed pattern-associated molecular patterns (PAMP). PAMP include molecules such as lipopolysaccharide (LPS), flagellin, and nucleic acids (Mogensen, 2009). The binding of PAMP to their cognate PRR triggers intracellular signalling cascades that results in activation of the immune cell, increased pathogen killing, upregulation of pro-inflammatory cytokines, leukocyte recruitment and increased antigen presentation (Takeda et al., 2003).

Epidermal LC have a crucial role in immune surveillance. LC have been shown to continually extend and retract their dendrites between keratinocytes in a behaviour known as dendritic surveillance extension and retraction cycling habitude (DSEARCH; Nishibu et al., 2006). The *de novo* formation of tight junctions between LC and keratinocytes maintains skin barrier integrity, facilitating the sampling of the extra-tight junction environment without loss of integrity (Kubo et al., 2009).

Mouse LC express TLR2, TLR4 and TLR9, but do not express TLR7 (Mitsui et al., 2004), while human LC express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR10 (Flacher et al., 2006). It has been suggested that LC are more sensitive to viral rather than bacterial infection (van der Aar et al., 2013), however this is still unclear. Consistent with their macrophage origin, LC express the CLR Dectin-1 and Dectin-2, which are important for response to fungal infection (Brown et al., 2002, Ariizumi et al., 2000, Kashem et al., 2015).

In the dermis, a range of PRR are expressed by DC, macrophages, mast cells and stromal cells (Miller and Modlin, 2007). The differential expression of PRR between APC subsets probably accounts for the diversity of cutaneous immune responses (Segura et al., 2012).

Innate responses to infection or damage

Antimicrobial peptides (AMP) are released in response to invading pathogens by many skin cell subsets, including epidermal keratinocytes and LC, dermal mast cells, DC and macrophages and infiltrating neutrophils and NK cells. Cathelicidins and β-defensins are the best characterised of cutaneous AMP, functioning as direct anti-microbial chemicals but also as alarmins, in which they act as further triggers of the innate and adaptive immune systems (Braff et al., 2005, Schauber and Gallo, 2007, Oppenheim et al., 2007). For example, LL-37 has anti-fungal, -bacterial and -viral activity (Braff and Gallo, 2006, Lopez-Garcia et al., 2005), and can influence host responses, such as inducing keratinocyte migration and IL-18 release, inducing histamine release from mast cells, inducing ROS generation by neutrophils and influencing TLR signalling (Tokumaru et al., 2005, Niyonsaba et al., 2005, Niyonsaba et al., 2001, Zheng et al., 2007, Di Nardo et al., 2007).

The release of chemoattractants and cytokines is an important mechanism of cutaneous immune responses. Some dermal macrophages are located nearby to postcapilliary venules where they produce neutrophil-attracting chemokines (Abtin et al., 2014). Besides professional immune cells, keratinocytes play an important regulatory role in skin immune responses by producing various cytokines (Nestle et al., 2009). Keratinocytes constitutively synthesise proIL-1α, -β and IL-1Ra, but do not secrete these

pro-inflammatory cytokines under steady state conditions (Feldmeyer et al., 2007). Stimulation of the inflammasome, a multiprotein complex within the cytoplasm that functions as an intracellular PRR, for example by UV radiation, cleaves pro-IL-1β into the active form which is released (Feldmeyer et al., 2007). Depending on the stimulus, keratinocytes can also produce other cytokines, including IL-6, IL-10, IL-18 and TNF (Albanesi et al., 2005) and chemokines, such as CCL20, CXCL9, CXCL10 and CXCL11 to recruit effector T cells, and CXCL1 and CXCL8 to recruit neutrophils (Tokura et al., 2008).

Macrophages, neutrophils and mast cells can all participate in the direct killing of microorganisms by phagocytosis and reactive oxygen species (ROS) production (Forman and Torres, 2002). Dermal macrophages have high phagocytic ability and express a unique set of genes that support specific roles in killing microorganisms and scavenging degradation intermediates of self-macromolecules (Tamoutounour et al., 2013). They are involved in the recruitment of neutrophils to sites of infection (Abtin et al., 2014) and act as sentinels of tissue homeostasis and recruit other cells in response to injury (Minutti et al., 2017).

Dermal mast cells are crucial for protective responses against bacteria, as *P. aeruginosa*-infected mast cell-deficient mice exhibited strikingly larger lesions compared to wildtype (WT) mice, and the presence of mast cells was associated with mast cell degranulation, enhanced neutrophil recruitment and increased bacterial clearance (Siebenhaar et al., 2007). A different study reported that injection of *S. aureus*-derived peptidoglycan into the skin resulted in TLR2-dependent and mast cell-dependent vasodilation and inflammation, therefore contributing to innate immune responses (Supajatura et al., 2002). The recruitment of neutrophils by skin mast cells was critically important for the induction and development of cutaneous granuloma formation, which is crucial for the protective response against many intracellular pathogens (von Stebut et al., 2003). Indeed, mast cells were crucial for the control of *Leishmania major* infection, via recruitment of T cells and DC and skewing of the cytokine response towards Th1 (Maurer et al., 2006).

Priming of adaptive T cell immunity

LC and dermal cDC share the capability of migration to skin-draining lymph nodes (LN) where they efficiently prime naïve T cells.

Protective T cell responses generated by dermal DC are well defined: CD11b^{+} cDC prime CD4^{+} Th2 cells after cutaneous vaccination (Kumamoto et al., 2013, Gao et al., 2013, Murakami et al., 2013), while dermal CD103^{+} cDC promote antigen-specific Th1 responses after challenge with *Candida albicans* (Igyarto et al., 2011a) and are required

for the induction of humoral immune responses, leading to production of IgG2a and IgG2b (Nagao et al., 2009). Dermal CD103^{+} cDC efficiently cross-present exogenous antigen (Henri et al., 2010) and are critical for the priming of CD8^{+} T cells in murine infection models of *L. major*, *C. albicans*, Vaccinia virus and herpes simplex virus (HSV), and gene gun immunisation (Stoecklinger et al., 2011, Brewig et al., 2009, Bedoui et al., 2009, Igyarto et al., 2011a, Seneschal et al., 2014).

The need to extricate themselves from the keratinocyte network and cross the basement membrane means that LC are slower to arrive in draining LN than dermal cDC after topical activation, suggesting that dermal cDC will dominate in priming of T cells (Kissenpfennig et al., 2005). This is supported by experiments investigating the role of LC in viral infections. LC were shown to be dispensable for induction of immune responses to HSV (Allan et al., 2003, Allan et al., 2006). Indeed, LC do not appear to be required for the priming of CD8^{+} T cells against any viral infection tested, but may rather shuttle epidermal viral antigen to dermal DC, via uptake of apoptotic LC by bystander dermal DC at the site of infection and/or transfer to cDC in the LN (Cunningham et al., 2010, Allan et al., 2003, Kim et al., 2015). It still remains unclear to what extent migrating tissue APC directly prime T cells in the LN, or transport antigen which is subsequently handed over to LN-resident cells (Heath and Carbone, 2009, Belz et al., 2004).

LC may, however, play a direct role in the activation of CD4^{+} T cells. LC were necessary and sufficient to drive CD4^{+} Th17 responses in a cutaneous infection model of *C. albicans* (Igyarto et al., 2011a) by a mechanism in which detection of the PAMP Dectin-1 by LC resulted in production of the Th17-inducing cytokine IL-6 (Kashem et al., 2015). LC have additionally been shown to drive the pathogenesis of inflammatory skin diseases, including poison ivy dermatitis and psoriasis, by the presentation of lipid antigen on CD1a to activate Th17 cells (Kim et al., 2016).

LC are also important in activating humoral immunity and have been shown to be critical for the development of follicular helper T cells (Tfh) and germinal centres (Zimara et al., 2014, Yao et al., 2015, Levin et al., 2017). Moreover, LC facilitated IgG1 responses to gene gun-immunised bacterial antigens (Nagao et al., 2009) and experimental staphylococcal scalded skin syndrome (Ouchi et al., 2011) and were essential in the induction of IgE upon epicutaneous sensitisation with protein antigens (Nakajima et al., 2012).
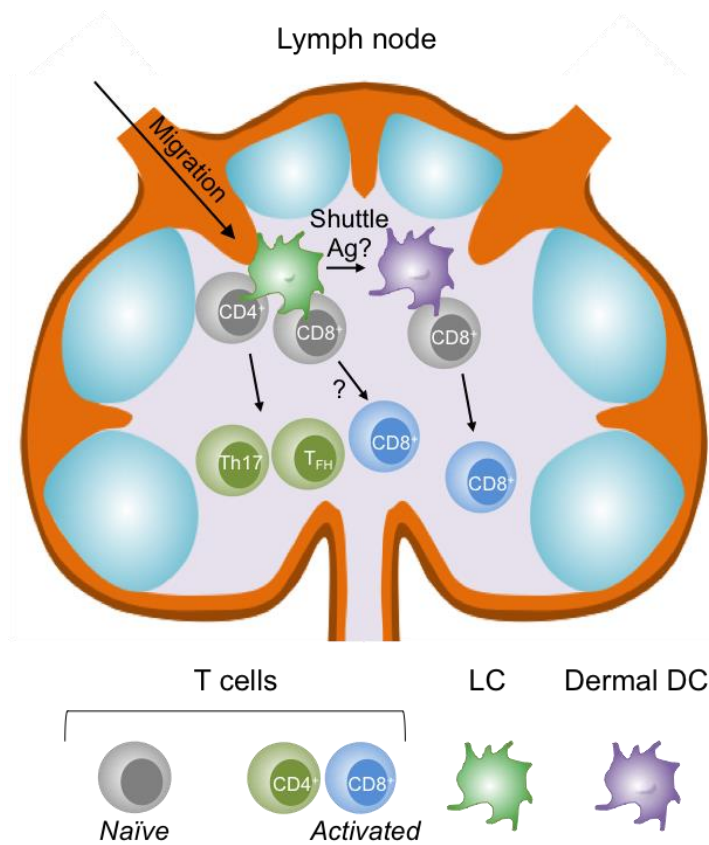
Figure 1.4 The migratory functions of LC

LC migrate to the skin draining LN where they prime naïve T cells. LC are known to drive CD4+ T cell responses and prime Th17 cells. The role of LC in CD8+ T cell responses is less clear. LC may directly prime CD8+ T cells, or there is evidence to suggest that LC shuttle antigen to other LN or dermal DC.

Priming of immunological tolerance

The ability to mount effective protective immune responses in the skin is critical for survival, but equally as important is immunological ignorance to harmless antigens, known as immunological tolerance. This is the default setting of the skin, and prevents inflammatory responses to self-antigens, commensal microbes and environmental antigens. Tolerance is partially maintained by the infiltration of CD4+ regulatory T cells (Treg) into the skin throughout life. A wave of Treg has been shown to populate the skin in the early neonatal period in a skin bacterial colonisation model in mice (Scharschmidt et al., 2015), suggesting the importance of crosstalk between Treg and the skin microbiome for the maintenance of a tolerogenic environment.
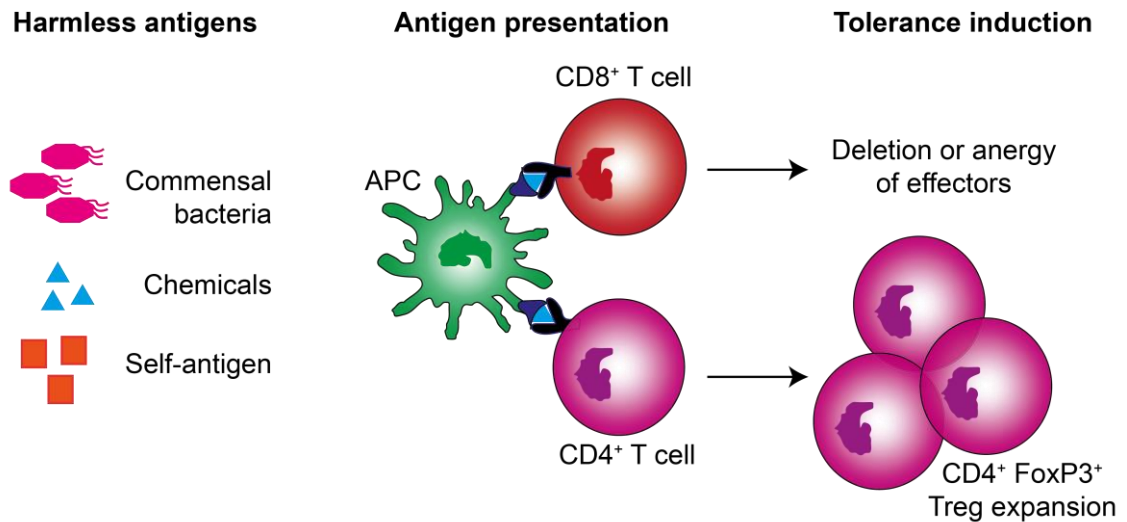
Figure 1.5 Immunological tolerance

Harmless antigen (including foreign antigens from commensal microorganisms and chemicals, and self-antigen derived from the host) is presented to T cells by APC in LN. Tolerance is induced by the deletion or anergy of antigen-specific effector T cells combined with the expansion of antigen-specific regulatory T cells.

Dermal DC prime Treg in LN. *In vivo* imaging revealed close localisation of migrating dermal DC with LN Treg clusters in the steady state (Liu et al., 2015), supporting previous studies demonstrating a role for RelB+CD103+ dermal DC (Azukizawa et al., 2011) or human CD141+ dermal DC (Chu et al., 2012) in the maintenance of skin tolerance via the induction of Treg. Homeostatic NF-κB signalling by dermal DC was shown to be required for Treg conversion and induction of tolerance (Baratin et al., 2015).

A number of studies over the last decade have also led to the concept that LC are important in maintaining tolerance via Treg in the skin. LC have been shown to maintain local tissue tolerance to skin self-antigen in an autoimmunity model (King et al., 2015) and prime autoantigen-specific Treg to prevent autoimmunity (Kitashima et al., 2018). Additionally, LC induced tolerance following ovalbumin (OVA) immunisation (Flacher et al., 2014), which was later demonstrated to require non-activated steady state LC to expand antigen-specific Treg (Strandt et al., 2017).

Shklovskaya et al. have suggested that LC are pre-committed to immune tolerance induction, as LC failed to induce effector/memory T cells or translocate RelB to the nucleus, a biochemical requirement for immunogenicity (Shklovskaya et al., 2011). LC are also negative regulators of the anti-Leishmania response, as absence of LC led to reduced Treg migration into the skin, an enhanced Th1 response and less IL-10, resulting in attenuated disease (Kautz-Neu et al., 2011).

In models of skin sensitisation and tolerance, interaction between migrating LC and CD8+ T cells in draining LN led to the induction of deletion and/or anergy in effector CD8+ T cells, whilst it was suggested that LC directly induced the expansion of LN ICOS+FoxP3+

Treg (Gomez de Aguero et al., 2012), possibly by a process involving IL-10 and cognate interactions with CD4^{+} T cells (Igyarto et al., 2009). LC have additionally been shown to induce Treg in response to irradiation (Price et al., 2015) and epidermal autoantigen, the latter potentially by a mechanism involving IL-2 receptor signalling by LC (Kitashima et al., 2018).

The above models have assumed direct cognate interaction of LC with CD4^{+} T cells in LN but have not tested a potential tolerising function of LC *in situ* in the skin. Seneschal et al. revealed that resting human LC selectively and specifically induced the activation and proliferation of skin-resident Treg *in vitro* and were in close proximity to proliferating Treg *in vivo* (Seneschal et al., 2012).

At present, it is unclear whether there is redundancy between LC and dermal DC in maintaining a tolerant environment in the steady state, or whether the different cells have distinct roles.

<u>Protection against cancer</u>

Skin cancer, including both melanoma and non-melanoma, is the most common type of malignancy in the Caucasian population (Whiteman et al., 2016, de Vries and Coebergh, 2004). UV radiation is the most important cause of skin cancer, while ionising radiation, chemical carcinogens, reduced immunity and genetics are also risk factors. DNA damage, gene mutations, immunosuppression, oxidative stress and inflammatory responses are generated in response to environmental or chemical carcinogens, leading to tumour development. However, the skin has many immune mechanisms in place to prevent tumourigenesis. Immune surveillance leads to the removal of stressed cells by phagocytosis and scavenging of ROS. NK cells have direct anti-tumour cytotoxicity, mediated by mechanisms involving perforin/granzyme B, TRAIL and FasL (Johnsen et al., 1999, Lee et al., 1996, Zamai et al., 1998). γδ T cells also exert direct anti-tumour cytotoxicity; mice lacking γδ T cells were highly susceptible to multiple regimens of cutaneous carcinogenesis (Girardi et al., 2001). Human skin-derived γδ T cell clones exerted cytotoxic responses against melanoma cell lines (Bachelez et al., 1992). Human dermal γδ T cells have been shown to express NKG2D that stimulated cell lysis (Bergstresser et al., 1993, Bauer et al., 1999), and skin-derived activated γδ T cells produced perforin and induced Fas-mediated cytotoxicity (Ebert et al., 2006).

<u>Wound healing</u>

As a protective barrier to the external environment, the skin is constantly exposed to potential injury. The integrity of the skin is essential in maintaining physiological homeostasis and therefore wound healing is a crucial process for survival. As such, wound healing is a conserved evolutionary process and is composed of three spatial and

temporal overlapping phases: coagulation and inflammation, tissue formation and tissue remodelling (Martin, 1997, Singer and Clark, 1999, Baum and Arpey, 2005, Liu and Velazquez, 2008, Seifert et al., 2012, Richardson et al., 2013). Wound healing requires a concerted effort of resident damage-sensing cells (e.g. dermal macrophages; Minutti et al., 2017) and recruited immune cells (e.g. neutrophils and monocytes; Kreisel et al., 2010, Niethammer et al., 2009, Wood, 2012).

Other cells play an important role in wound healing, including DETC (Jameson et al., 2002), Vγ4 T cells (Cai et al., 2011, Gray et al., 2013, MacLeod et al., 2013), peripheral nervous system glia (Parfejevs et al., 2018), mast cells (Weller et al., 2006) and ILC2 (Rak et al., 2016). ILC2 are present in healthy human and murine skin and are increased in number during cutaneous inflammation (Kim et al., 2013, Roediger et al., 2013, Salimi et al., 2013). Studies have shown that the alarmin IL-33 is rapidly increased following wounding (Yin et al., 2013a, Yin et al., 2013b) and promotes an ILC2 response to facilitate re-epithelialisation and wound closure (Rak et al., 2016).

LC have also been implicated in wound healing, as an increased number of LC in the epidermis of diabetic foot ulcers correlated with healing outcome (Stojadinovic et al., 2013).

1.3.   Developmental plasticity of cutaneous myeloid cells

Tissues are populated with myeloid cells throughout life, under conditions of homeostasis and as a result of disease. The ontogeny of myeloid cells can differ between cell types and even within a cell type, under different environmental conditions or disease states. This plasticity may impact upon myeloid cell function.

Monocytes, macrophages, DC, neutrophils, basophils and eosinophils are collectively termed myeloid cells, a subset of mononuclear phagocytes that are derived from a common myeloid progenitor (CMP; Hume, 2006). The focus of this thesis is on the populations of monocytes, macrophages and DC in the skin.
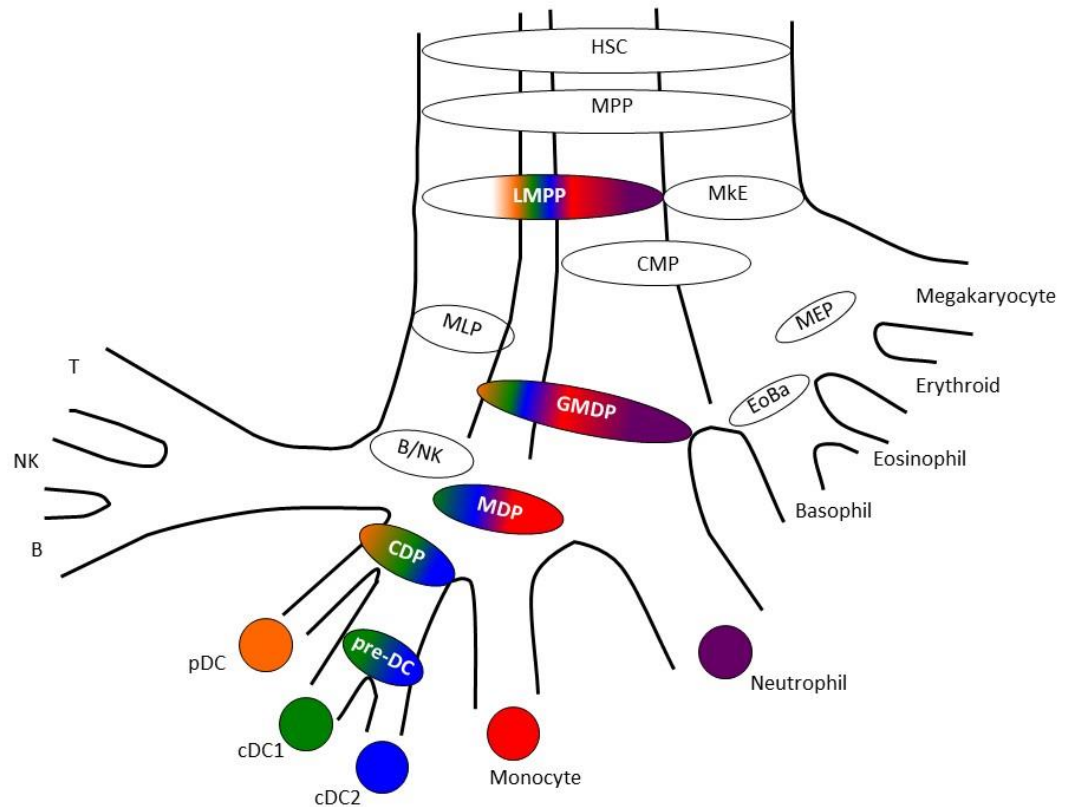
Figure 1.6 Myeloid cell development

The most significant early partitioning of cell fate occurs when the lymphoid primed multi-potent progenitor (LMPP) separates from megakaryocyte and erythroid potential (MkE). Lineage is primed in early progenitors so that most populations only contain cells with a single potential. MPP = multi-potent progenitor; CMP = common myeloid progenitor; MEP = megakaryocyte-erythroid progenitor; EoBa = eosinophil-basophil progenitor; MLP = multi-lymphoid progenitor; GMDP = granulocyte-monocyte DC progenitor; B/NK = B- and NK-cell progenitor; MDP = monocyte-DC progenitor; CDP = common DC progenitor. Adapted from (Collin et al, 2018).

1.3.1. Ontogeny of resident tissue macrophages

Numerous studies have elucidated the embryonic ontogeny of tissue macrophages and have begun to identify the precise haematopoietic developmental pathways from progenitor to mature macrophage. During embryonic development, there are three main successive waves of haematopoiesis. Around embryonic day 7.0 (E7.0) the first "primitive" wave is initiated by haematopoietic progenitors in the blood islands of the extra-embryonic yolk sac and produces early erythro-myeloid progenitors (EMP) that give rise to yolk sac macrophages (Ginhoux and Guilliams, 2016). Between E8.0 and E8.5 a second "transient definitive" wave occurs in the yolk sac homogenic endothelium, producing late EMP that migrate into the foetal liver once blood circulation is established from E8.5. The cells expand in the foetal liver and give rise to foetal liver monocytes that enter the circulation at E11.5-12.5. The third "definitive" wave of haematopoiesis arises with the generation of HSC in the para-aortic splanchnopleura region (P-sp) and aorta, gonads and mesonephros (AGM) region that colonise the foetal liver around E10.5. The

foetal liver becomes the major haematopoietic organ from E11.5, generating all haematopoietic lineages. Foetal liver monocytes emerge in the foetal liver around E12.5, are released into the blood from E13.5 and colonise all tissues except the brain around E14.5 (Hoeffel et al., 2012, Hoeffel et al., 2015).
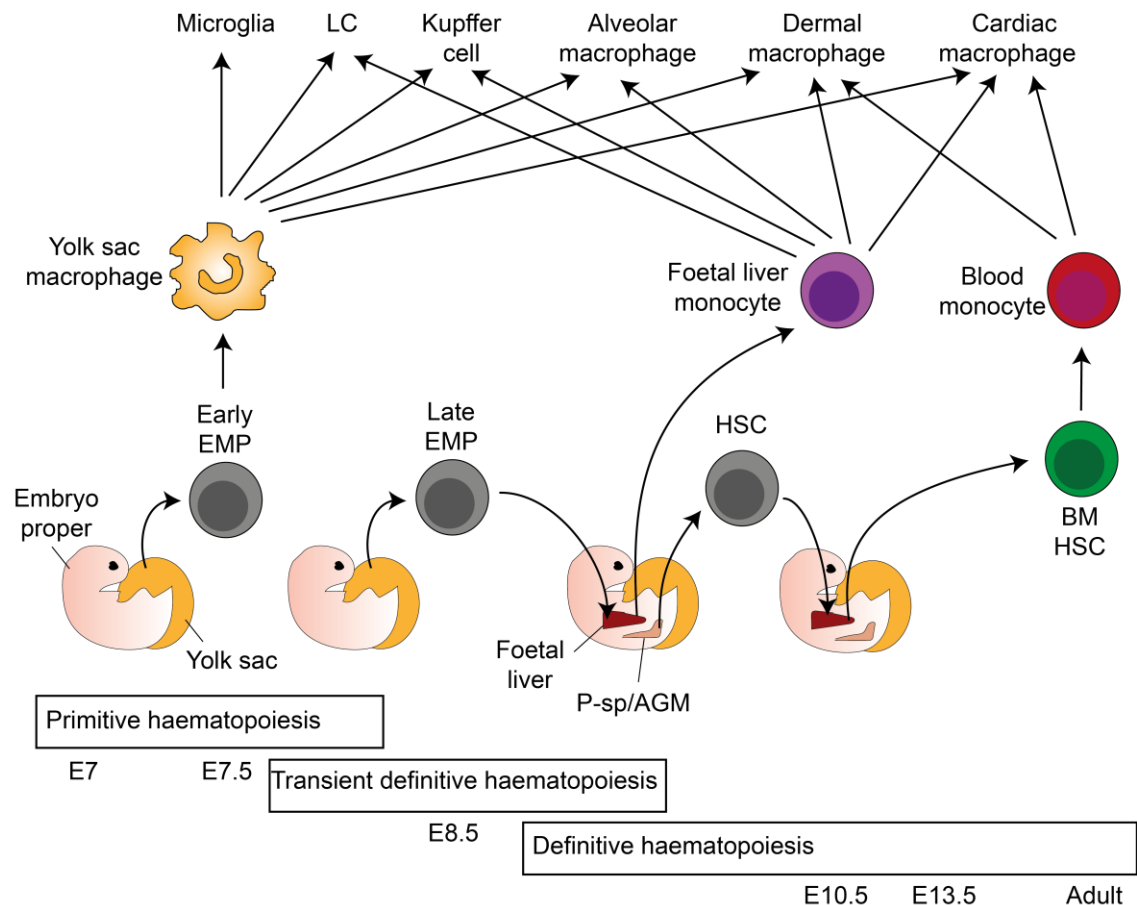
Figure 1.7 Embryonic haematopoiesis

Primitive haematopoiesis begins at embryonic day (E) 7-7.5 in the yolk sac with the emergence of early erythro-myeloid progenitors (EMP) from the yolk sac blood islands. These precursors give rise to yolk sac macrophages that seed tissue resident macrophages. Between E8-8.5, a second transient definitive wave is marked by late EMP arising from the yolk sac homogenic endothelium that seed the foetal liver. Here, the cells expand and give rise to foetal liver monocytes which seed tissue resident macrophage populations (excluding microglia) between E11.5-12.5. Definitive haematopoiesis begins when haematopoietic stem cells (HSC) are generated in the para-aortic splanchnopleura (P-sp) and the aorta, gonads, and mesonephros (AGM) regions and subsequently seed the foetal liver at E10.5. At E13.5, foetal liver HSC are released into the bloodstream and migrate into the bone marrow (BM) to give rise to adult monocytes. Adapted from (Franklin and Li, 2016).

PU.1 is required for differentiation of all macrophage populations and the transcriptional control of inducible genes in mature macrophages (Ghisletti et al., 2010, McKercher et al., 1996), and is considered a pioneering factor in the myeloid lineage throughout haematopoietic development (Italiani and Boraschi, 2015, Lavin et al., 2014a). Epigenetic profiling experiments have revealed that in macrophages there is an extensive overlap between PU.1 and enhancers, and it has been suggested that PU.1 is both necessary and sufficient to functionalise genomic regions as enhancers, thereby shaping the 3D landscape of the macrophage genome (Ghisletti et al., 2010). This

enables lineage-specific transcription factors to bind and orchestrate cell-type specificity (Heinz et al., 2010, Laslo et al., 2006). Several studies have revealed that macrophage populations exhibit distinct transcriptional and epigenetic signatures that are tissue-specific (Gautier et al., 2012, Gosselin et al., 2014, Lavin et al., 2014a) and indicate that distinct macrophage identities are imprinted and maintained by local cues from the environment. Indeed, a number of tissue-specific transcription factors have been identified that may work in combination with master regulators to establish the distinct chromatin landscape for each tissue macrophage population (Lavin et al., 2014a): *Gata6* for large peritoneal macrophages (Rosas et al., 2014, Okabe and Medzhitov, 2014), *Runx3* for LC (Fainaru et al., 2004), *Pparg* for alveolar macrophages (Schneider et al., 2014), *SpiC* for splenic red pulp macrophages (Kohyama et al., 2009), *Nr1h3* for splenic marginal zone macrophages (Guillen et al., 2013) and *Id3* for Kupffer cells (Mass et al., 2016). Recently, the transcription factor *Zeb2* has been implicated as a crucial regulator of the maintenance of tissue-specific macrophage identities (Scott et al., 2018).

1.3.2. Seeding and maintenance of dermal macrophages

Although all tissues are seeded by foetal macrophages at birth, gradual replacement of these cells by HSC can occur over time at specific sites. Adult dermal MHC II^{hi} macrophage populations contain almost no embryonic macrophages and are maintained by adult circulating precursors (Tamoutounour et al., 2013, Epelman et al., 2014, McGovern et al., 2014). The dermis is therefore considered an "open" macrophage niche, with fast steady-state recruitment of bone marrow (BM) monocytes (Ginhoux and Guilliams, 2016). Dermal macrophages are estimated to have a half-life of 4-6 weeks (Scott et al., 2014a, Tamoutounour et al., 2013).

Regardless of their origin, the major lineage regulator of dermal macrophages, and almost all macrophages, is the colony-stimulating factor 1 receptor (CSF-1R). Targeted ablation of *Csfr1* or *Csf1* causes severe depletion of macrophages in many tissues, including dermal macrophages (Dai et al., 2002, Ginhoux et al., 2006, Cecchini et al., 1994).

1.3.3. Ontogeny of dermal cDC

Macrophage-DC progenitors (MDP) develop from HSC in the BM, from which Flt3-dependent common DC progenitors (CDP) give rise to plasmacytoid DC (pDC) and pre-cDC that circulate in the blood and may enter tissues (Maraskovsky et al., 2000, McKenna et al., 2000). cDC develop from pre-cDC at these sites (Liu et al., 2009). The sites and precise pathways of pre-DC development have not been established, but pre-committed pre-cDC1 and pre-cDC2 populations have been identified in BM (Schlitzer et al., 2015, Grajales-Reyes et al., 2015).

Myeloid cDC1 development is dependent on GATA2, PU.1, GFI1, Id2, IRF8 and basic leucine zipper transcription factor (Batf3) (Collin et al., 2015, Onodera et al., 2016, Carotta et al., 2010, Ginhoux et al., 2009, Hacker et al., 2003, Grajales-Reyes et al., 2015, Tsujimura et al., 2003, Rathinam et al., 2005, Collin and Bigley, 2018). Myeloid cDC2 development is dependent on GATA2, PU.1, GFI1, ID2, ZEB2, RELB, IRF4, NOTCH2 and KLF4 (Collin et al., 2015, Murphy et al., 2016, Scott et al., 2016a, Briseno et al., 2017, Wu et al., 1998, Schlitzer et al., 2013).

cDC generally display a short half-life of approximately 3-6 days and are constantly replenished from conventional haematopoiesis (McKenna et al., 2000). In contrast to macrophages, DC identity in the steady state appears to be determined more by ontogeny than the tissue environment. DC populations cluster together according to transcription factor dependence (e.g. Batf3, IRF4), and not by tissue (Miller et al., 2012).

1.3.4. Ontogeny and differentiation of monocytes

As for macrophages, expression of CSF1R is a hallmark of monocytes; it is acquired during ontogeny and is conserved on circulating and infiltrating monocytes (Hume and MacDonald, 2012, MacDonald et al., 2005). Circulating monocytes are derived from common monocyte precursors (cMoP) in the BM (Hettinger et al., 2013, Kawamura et al., 2017) and can be divided into two subsets based on distinct chemokine receptor expression and the presence of specific surface molecules (van Furth and Cohn, 1968, Palframan et al., 2001, Geissmann et al., 2003). In mice, expression of CD11b and Ly6C identifies a subset of Ly6C^{hi} inflammatory monocytes that express high levels of the chemokine receptor CCR2 and low levels of the chemokine receptor CX$_3$CR1.

Monocytes are recruited to the dermis in the steady state (Tamoutounour et al., 2013), from where they may travel to LN and present antigen to T cells (Jakubzick et al., 2013). However, induction of a pro-inflammatory environment within the skin will significantly enhance recruitment of monocytes and neutrophils (Minutti et al., 2017). These monocytes are rapidly recruited to sites of infection and inflammation in a CCR2-dependent manner (Serbina et al., 2008, Kurihara et al., 1997, Kuziel et al., 1997). This subset corresponds to classical CD14^{++}CD16^{-} monocytes in humans (Ingersoll et al., 2010, Sunderkotter et al., 2004, Geissmann et al., 2003). A less prevalent subset of circulating monocytes express high levels of CX$_3$CR1 and low levels of CCR2 and Ly6C (Palframan et al., 2001, Geissmann et al., 2003). These monocytes are referred to as Ly6C^{low} monocytes and a major fraction has been shown to constantly patrol the blood vessel wall by adhering to and migrating along the luminal surface of endothelial cells (Geissmann et al., 2003, Auffray et al., 2007). While this subset has classically been defined as resident, it is now recognised that Ly6C^{low} monocytes can be rapidly recruited to sites of infection, even preceding Ly6C^{hi} monocytes (Auffray et al., 2007). This subset

corresponds to non-classical $CD14^+CD16^{++}$ monocytes in humans (Ingersoll et al., 2010, Geissmann et al., 2003, Sunderkotter et al., 2004).

Table 1.2 Markers and functions of monocyte subsets in human and mouse

Adapted from (Yang et al., 2014).

Species	Subsets	Surface markers	Chemokine receptors	Function
Human	Classical	$CD14^{++}\,CD16^-$	$CCR2^{hi}\,CX_3CR1^{low}$	Phagocytosis
	Intermediate	$CD14^{++}\,CD16^+$	$CCR2^{int}\,CX_3CR1^{hi}$ $CCR5^+$	Pro-inflammatory
	Non-classical	$CD14^+\,CD16^{++}$	$CCR2^{low}\,CX_3CR1^{hi}$	Patrolling
Mouse	$Ly6C^{hi}$	$CD11b^+\,CD115^+$ $Ly6C^{hi}$	$CCR2^{hi}\,CX_3CR1^{low}$	Phagocytosis and pro-inflammatory
	$Ly6C^{low}$	$CD11b^+\,CD115^+$ $Ly6C^{low}$	$CCR2^{low}\,CX_3CR1^{hi}$	Patrolling and tissue repair

Robust experimental evidence indicates that monocytes recruited to sites of infection are innate effectors of the inflammatory response to pathogens, mediating pathogen killing through cytokine release, phagocytosis and production of ROS, nitric oxide and myeloperoxidase (Serbina et al., 2008, Barbalat et al., 2009). It was previously thought that $Ly6C^{hi}$ monocytes preferentially differentiate into M1 inflammatory macrophages and $Ly6C^{low}$ monocytes preferentially differentiate into M2 anti-inflammatory macrophages during early inflammation (Auffray et al., 2007). However, it is now recognised that monocyte and macrophage subsets are highly plastic and that environmental cues are important in monocyte differentiation. In the skin, $Ly6C^{hi}$ monocytes are recruited after injury to facilitate skin repair and wound angiogenesis (Willenborg et al., 2012, Rodero et al., 2014). Similarly, $Ly6C^{hi}$ macrophages are recruited to allergic skin and acquire an M2-like phenotype in response to basophil-derived IL-4 and exert an anti-inflammatory function (Egawa et al., 2013).

It is now established that $Ly6C^{hi}$ monocytes can alternatively differentiate into monocyte-derived DC (moDC) or TNF-α/inducible nitric oxide synthase (iNOS)-producing DC (Tip-DC) under inflammatory conditions in peripheral tissues (Randolph et al., 1999, Shi and Pamer, 2011, Leon et al., 2007, Plantinga et al., 2013, Qu et al., 2004, Tamoutounour et al., 2013, Serbina et al., 2003). In the skin, this has been demonstrated during infection (Leon et al., 2007, Schmid and Harris, 2014, De Trez et al., 2009), contact hypersensitivity responses (Tamoutounour et al., 2013) and inflammatory diseases (Haniffa et al., 2015, Zaba et al., 2009).

The differentiation of monocytes to moDC versus macrophages is regulated by transcription factors and cues from the microenvironment. One study revealed that all monocyte subsets express a partial macrophage transcriptomic signature, but local environmental cues can drive monocyte fate towards moDC or macrophage (Goudot et al., 2017). The authors hypothesised that these different fates could be driven by local signals owing to stochastic heterogeneity in chromatin accessibility (Goudot et al., 2017, Buenrostro et al., 2015). Critical transcription factors identified thus far include MafB for restricting differentiation into self-renewing macrophages (Bakri et al., 2005, Sieweke and Allen, 2013), and IRF4 and BLIMP-1 for driving moDC differentiation (Goudot et al., 2017). The aryl hydrocarbon receptor (AhR) has also been implicated in driving moDC fate (Goudot et al., 2017).

1.3.5. The contribution of monocyte infiltration to disease pathogenesis

The pathogenesis of many cutaneous inflammatory diseases is crucially linked to the recruitment and infiltration of monocytes that become pathogenic cells in the inflamed dermis. Maintenance of psoriasiform skin inflammation has been shown to critically depend on recruitment of monocytes and differentiation to macrophages that became activated and released TNF-α (Wang et al., 2006, Stratis et al., 2006). Macrophages were also activated in an imiquimod-induced model of psoriasis (Morimura et al., 2016, Nakai et al., 2017). Psoriasis-like inflammation has been shown to require moDC producing the critical cytokines TNF-α and IL-1β (Singh et al., 2016). Other studies have described inflammatory Tip-DC in lesional psoriatic skin, (Lowes et al., 2005), CD11c$^+$CD1c$^-$ cells and "slanDC" that prime naïve T cells to produce Th1 and Th17 cytokines (Zaba et al., 2009, Hansel et al., 2011). The ontogeny of these inflammatory DC has not been defined and it is possible that they could be moDC or inflammatory monocytes (Segura and Amigorena, 2013).

Application of chemical contact allergens – such as 2,4-dinitro-1-fluorobenzene (DNFB) – to the skin of mice is used to experimentally model allergic contact dermatitis. In this model, epicutaneous sensitisation resulted in the extravasation of large numbers of Ly6C^{hi} monocytes into the dermis and the generation of inflammatory moDC (Tamoutounour et al., 2013). Furthermore, during atopic dermatitis, macrophages have been found to dominate the dermal mononuclear infiltrate (Akdis et al., 2006, Kiekens et al., 2001). Monocyte infiltration during GVHD has been reported in several studies. Infiltration of monocytes and subsequent differentiation into CD163$^+$ macrophages was described as a predictive factor for refractory GVHD and poor prognosis (Nishiwaki et al., 2009), while a separate study reported increased intermediate monocytes in patients with acute GVHD that promoted the induction of a subset of Th17 cells that were resistant to glucocorticoids (Reinhardt-Heller et al., 2017). In preclinical models of chronic GVHD,

donor-derived M2-like macrophages were increased in the skin and contributed to dermal sclerosis in a CSF-1 dependent manner (Alexander et al., 2014, Du et al., 2017). Recipient radio-resistant dermal macrophages may also persist following transplant-related conditioning and GVHD and have been shown to directly contribute to pathology (Haniffa et al., 2009).

1.4. LC: the resident macrophage population of the epidermis

1.4.1. Seeding and maintenance of epidermal LC

In contrast to dermal macrophages, epidermal LC are seeded by embryonic progenitors and are not replaced by BM monocytes during life (Yona et al., 2013, Merad et al., 2002).

Given their dendritic morphology and immunostimulatory function, LC have classically been defined as DC, and were considered the paradigm for DC activation and migration (Schuler and Steinman, 1985, Romani et al., 1989, Larsen et al., 1990).

However, within the last 10 years, fate-mapping models have revealed that LC are derived predominantly from foetal liver monocytes, with a minor contribution from yolk sac progenitors. LC are seeded initially from a first wave of yolk sac-derived progenitors before E7.5 that are recruited to the skin around E10.5 and are largely replaced by a second wave of foetal liver monocytes arising at E8.5 - E9.5 that are recruited to the skin between E13.5 and E16.5 (Schulz et al., 2012, Hoeffel et al., 2012). LC have more recently been shown to originate from a *Mafb*-expressing progenitor, supporting their macrophage origin (Wu et al., 2016). MafB was shown to regulate lineage-specific enhancers of self-renewal genes in proliferating resident macrophages, thus implicating MafB in LC self-renewal (Soucie et al., 2016).

Chorro et al. reported that the embryonic LC niche was seeded by a single wave of $CX_3CR1^+CD45^+Langerin^-$ precursors, which entered the skin around E18 and differentiated into $CX_3CR1^{low}CD45^+Langerin^+MHC\ II^+$ LC shortly after birth, before undergoing a massive burst of proliferation between postnatal day 2 (P2) and P7 to fill the niche (Chorro et al., 2009). This could potentially be a mechanism for filling the niche quickly to avoid influx of haematopoietic progenitors. Indeed, a similar proliferative burst is detectable in other resident macrophage populations during neonatal development, including peritoneal macrophages (Davies et al., 2011) and microglia (Kierdorf et al., 2013).

Parabiotic mice studies revealed that the epidermal network of LC is maintained throughout life by self-renewal, independently of circulating precursors (Merad et al.,

2002). When LC were depleted in the steady state, under mild conditions of inflammation, the LC network repopulated without recruitment of BM-derived precursors.

Multicolour fate mapping analysis provided evidence that the adult epidermal LC network is constituted by adjacent proliferative units composed of "dividing" LC and their terminally differentiated daughter cells, and not mature coequal LC endowed with proliferative capabilities (Ghigo et al., 2013). The authors proposed a model whereby immature LC are responsible for the maintenance of LC proliferative units.

Evidence from human studies corroborates the view that LC persist independently from BM cells and are maintained through self-renewal throughout life. LC from human skin grafted onto xenogeneic hosts incorporated BrdU (Czernielewski and Demarchez, 1987) or stained for Ki67 (Hemmerling et al., 2011) indicating proliferation, while LC in a human hand allograft remained of donor origin and were long-lived (Kanitakis et al., 2011). Patients with GATA2 or IRF8 mutations, transcription factors required for definitive haematopoiesis and terminal differentiation of monocytes and DC, respectively, have persisting LC and tissue macrophages, providing further evidence of independence from definitive haematopoiesis (Collin et al., 2015, Hambleton et al., 2011).
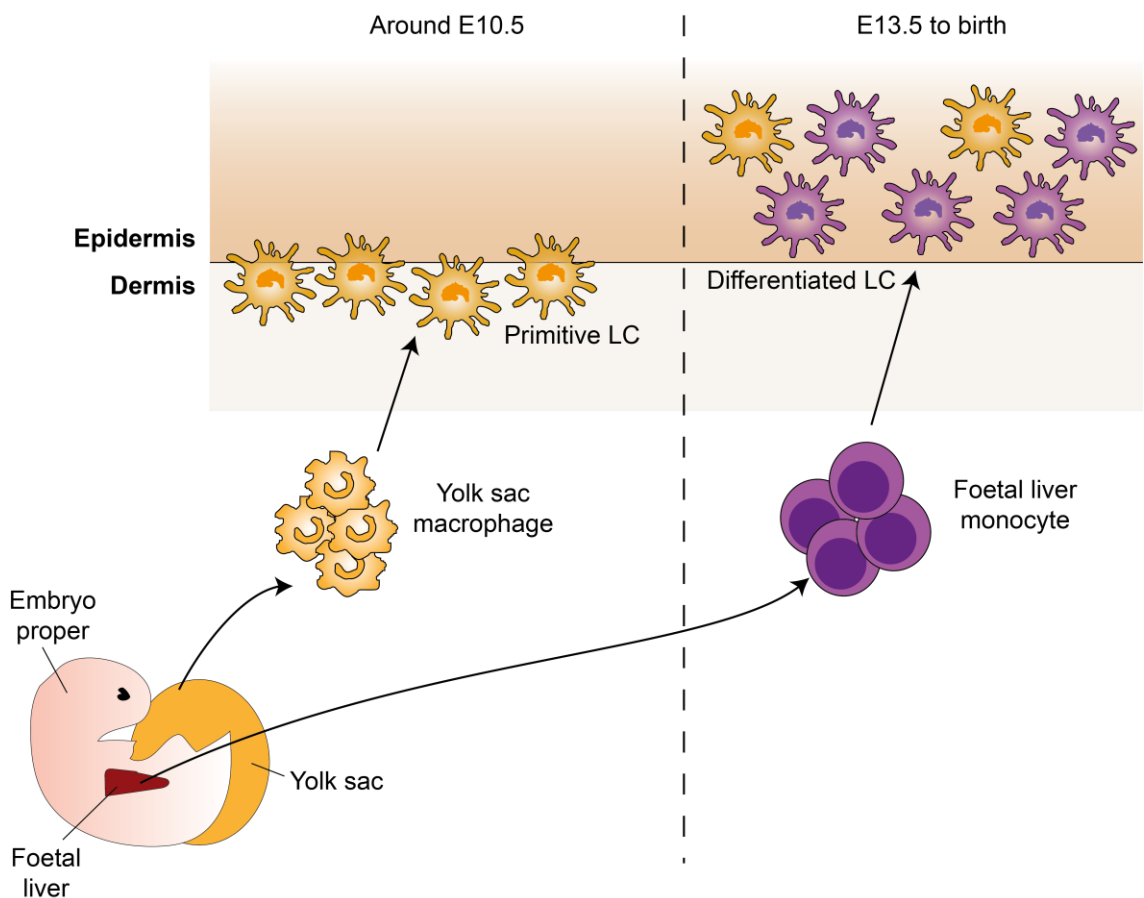
Figure 1.8 Ontogeny of LC during the steady state

LC are seeded initially from a first wave of yolk sac-derived progenitors before E7.5 that are recruited to the skin around E10.5 and are largely replaced by a second wave of foetal liver monocytes arising at E8.5 - E9.5 that are recruited to the skin between E13.5 to birth. Adapted from (Otsuka et al., 2018).

1.4.2. Growth factor control of LC

Elucidation of the embryonic origin of LC and their reassignment as resident tissue macrophages is also consistent with the essential requirement for CSF-1R signalling for LC development, evidenced by the lack of LC in *Csf1r*-deficient mice (Dai et al., 2002, Ginhoux et al., 2006). However, while dermal and other macrophage populations were lost in *Csf1*-deficient mice, LC were only mildly reduced at birth and numbers were normal in adulthood (Cecchini et al., 1994, Witmer-Pack et al., 1993). This finding suggested the existence of an additional ligand for CSF-1R that could partly compensate for the lack of CSF-1 in driving the differentiation of LC. As such, IL-34 was identified as an alternative ligand for CSF-1R (Lin et al., 2008) and the use of *Il34*-deficient mice revealed that keratinocyte-derived IL-34 was crucial for the embryonic development of LC (Wang et al., 2012). The absence of IL-34 strongly reduced the number of LC precursors within the skin rudiment at a time point when increased *Il34* expression was detected in the epidermis of WT embryos, indicating that LC precursors required IL-34 locally for their survival and/or differentiation into LC (Wang et al., 2016).

TGF-β1 is produced by both LC and keratinocytes and has been shown to be essential for LC homeostasis by maintaining immature LC in the epidermis (Kaplan et al., 2007, Kel et al., 2010). Congruent with this, LC are absent in mice lacking TGF-β, or the TGF-β-controlled transcription factors Id2 or Runx3 (Borkowski et al., 1996, Hacker et al., 2003, Fainaru et al., 2004). Canonical TGF-β1-ALK5-Smad3 signalling is not critical for LC development, as deletion of the classical TGF-β receptor chain TGFβR1/ALK5 did not affect seeding of the LC network; LC networks were only affected postnatally (Xu et al., 2012, Kel et al., 2010, Zahner et al., 2011, Li et al., 2016). Downstream mechanisms of classical TGF-β signalling include the expression of the adhesion molecules EpCAM, TROP2, E-Cadherin and β-catenin (Yasmin et al., 2013), and expression of Axl, a member of the TAM (Tyro3, Axl, Mer) family of receptors for apoptotic cells that binds to its epidermal ligand Gas 6 and maintains TLR hyporesponsiveness (Bauer et al., 2012).

The alternative TGF-β family signalling molecule bone morphogenetic protein 7 (BMP7) was later described to be an instructive factor for human embryonic LC development (Yasmin et al., 2013). In contrast to TGF-β, BMP7 signals through the ALK3 receptor and phosphorylation of Smad 1/5/8. BMP7 expression precedes TGF-β1 induction in the foetal epidermis, coinciding with the time point when LC precursors are first detectable in human embryonic skin (Schuster et al., 2009). BMP7 has also been shown to promote translocation of mucosal LC precursors to the epithelium by inducing mesenchymal-to-epithelial transition (MET) programming, as seen in other tissues (Zeisberg et al., 2005, Capucha et al., 2018).

1.4.3. Replacement of LC after pathology

During mild inflammation and disease, LC are partially lost from the epidermis and are repopulated from self-renewing epidermal precursors, similar to the steady state (Chorro et al., 2009, Merad et al., 2002). However, in mice and humans, UV exposure leads to recruitment of LC precursors from the BM (Merad et al., 2002, Baadsgaard et al., 1987, Meunier et al., 1995, Kennedy Crispin et al., 2013, Achachi et al., 2015). Clinically, GVHD is the only disease that results in complete turnover of the resident LC population. Studies have shown that recipient LC are replaced by donor-derived cells within a few weeks after allogeneic bone marrow transplantation (BMT) (Stingl et al., 1980, Katz et al., 1979, Merad et al., 2002), which induces GVHD and the skin inflammation required for recruitment of BM-derived precursors (Merad et al., 2002). LC survive conditioning, but succumb to killing by allogeneic T cells, possibly due to high levels of HLA on their surface. In contrast, when T cells were absent from the graft, GVHD was not induced and LC remained of recipient origin (Merad et al., 2002, Li et al., 2011).

In keeping with the macrophage origin of LC, monocytes have long been thought to be the precursors of repopulating LC. Recruitment of replacement cells to the epidermis requires CCR2 and CCR6, suggesting infiltration of monocytic cells (Merad et al., 2004). Repopulation of LC after inflammation is highly dependent on CSF1R signalling, but LC replacement after UV irradiation was delayed, but not aborted, in *Csf1*-deficient mice (Ginhoux et al., 2006), suggesting that IL-34 could be driving the differentiation of monocyte-derived LC in the absence of CSF-1. In fact, studies revealed that IL-34 was not critical for recruitment of monocyte-derived LC, as the defect of LC in *Il34*-deficient mice was partially compensated for by monocyte-derived LC (Wang et al., 2012, Greter et al., 2012). A later study revealed that the development of monocyte-derived LC depended on neutrophil-derived CSF-1 (Wang and Colonna, 2014). As for embryonic-derived LC, IL-34 is critical for the maintenance of monocyte-derived LC, as monocyte-derived LC developed in *Il34*-deficient mice but were not maintained (Greter et al., 2012).

However, while it is clear that monocytes are recruited to the epidermis under conditions of LC destruction, some work has questioned whether monocytes, which are non-proliferating cells with a short half-life outside the BM, can differentiate into *bona fide* LC, with full functionality and a long half-life. Rather, Sere et al., have described LC repopulation in two waves: a fast and transient wave of "short-term" LC transcriptionally similar to Gr-1^{hi} monocytes and independent from ID2, followed by a second wave of "long-term" LC with high EpCAM and Langerin expression that were dependent on ID2 (Sere et al., 2012). The existence of different LC precursors is supported by a study by Nagao et al., which reported the generation of a subpopulation of LC from Gr-1^{hi} monocyte-derived precursors, but not full reconstitution of the niche (Nagao et al., 2012).

In support of the notion of an alternative precursor, CD1c^{+} DC in human blood have been shown to have the potential to differentiate into LC-like cells (Milne et al., 2015) and LC within the oral epithelia were also maintained by influx of Flt3-dependent DC precursors (Capucha et al., 2015).

Emerging evidence indicates that the environment is dominant over origin in programming tissue resident macrophages. Lavin et al. demonstrated that after complete replacement of the embryonic-derived tissue macrophage compartment with BM-derived progenitors, the replacement macrophages displayed a phenotype more similar to their embryonic counterparts than to the transplanted macrophages in other tissues (Lavin et al., 2014). Although the authors did not analyse LC, these data suggested that the epidermal environment may be key in orchestrating LC differentiation from monocytes. Indeed, Sere et al. reported that long-term BM-derived LC were transcriptionally similar to embryonic derived LC (Sere et al., 2012).
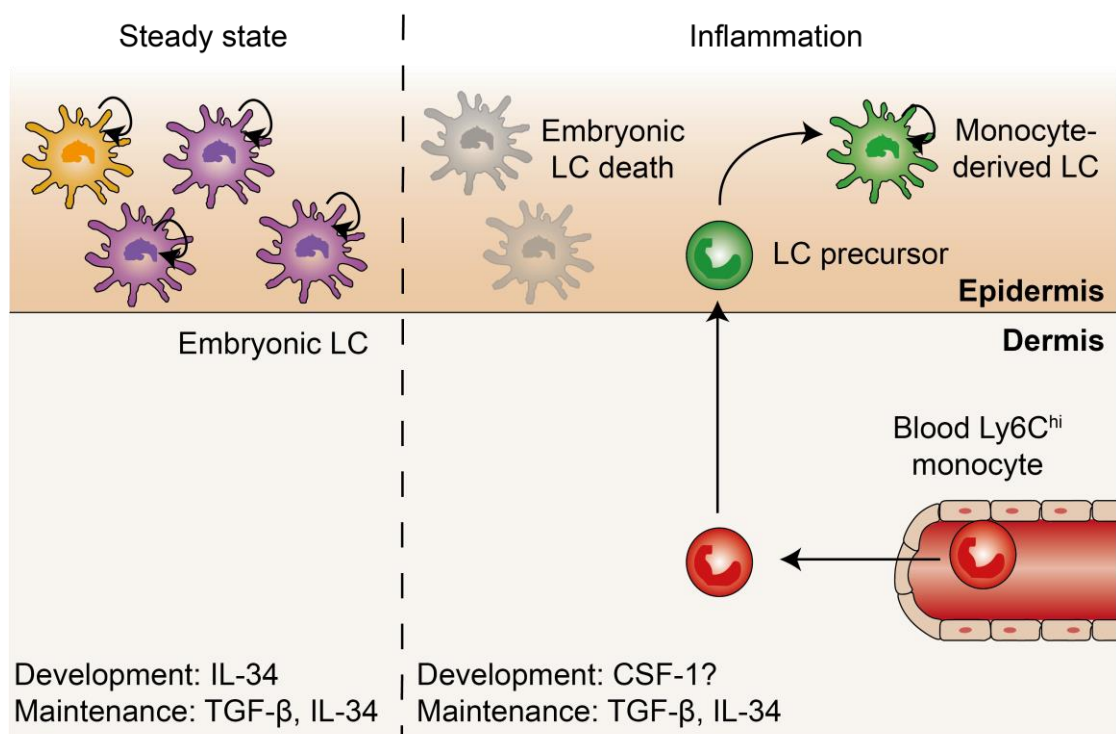
Figure 1.9 Replacement of LC after pathology

In the steady state, embryonic-derived LC require IL-34 and TGF-β for their homeostasis. After pathology and conditions of cutaneous inflammation, embryonic LC undergo cell death and are replaced by a monocyte-derived precursor. CSF-1 has been implicated as a critical cytokine in the development of monocyte-derived LC, however this is still unclear.

1.4.4. Functional implications for LC turnover in disease

Cutaneous GVHD

Persistence of LC as the dominant population of DC-like cells after transplant conditioning led to the assumption that they played an important role in the direct presentation of allo-antigen to donor T cells. Murine studies have reported that persisting recipient LC prime allo-responses and can trigger GVHD (Merad et al., 2004, Durakovic et al., 2006). Our lab has shown that recipient LC were necessary for licensing CD8$^+$ donor T cells in the epidermis to induce epithelial injury in an MHC-mismatched model of acute GVHD (Bennett et al., 2011). Similarly, in three independent models of MHC-matched minor antigen-mismatched acute GVHD, CD8$^+$ T cell pathogenicity was dependent on LC-mediated upregulation of a Notch-dependent T cell gene cluster that is critical for pathology and was also conserved in human patients at the onset of acute GVHD (Santos et al., 2018b). However, one study that used LC-deficient mice that specifically only lack epidermal LC (Lang-DTA mice; Kaplan et al., 2005), as recipients in allogeneic BMT models, reported that recipient LC could be deleted without consequence when other APC populations can efficiently present host antigens (Li et al., 2011). This model constantly provides contradicting results to other LC-depletion models, for example the requirement for LC in skin graft rejection (Obhrai et al., 2008, Fernandes et al., 2011). These data are difficult to interpret as it is possible there are as-yet-undefined changes to baseline immunity in the long-term in the absence of LC (Kaplan, 2017).

In humans, recipient LC can persist long-term following allogeneic HSCT, especially following transplantation involving T cell depletion or reduced intensity conditioning (Collin et al., 2006). The persistence of recipient LC does not predict clinical or histological cutaneous acute GVHD (Mielcarek et al., 2014) or chronic GVHD (Andani et al., 2014), although this does not exclude a role for this population in initiating inflammation. A clinical trial of UV light treatment during allogeneic HSCT concluded that depletion of LC by broadband UVB decreased the risk of acute GVHD (Kreutz et al., 2012), and patients with a genetic immunodeficiency syndrome caused by mutations in GATA2 who lack DC but retain LC and macrophages, were reported to experience GVHD after allogeneic HSCT (Bigley et al., 2011, Dickinson et al., 2011, Cuellar-Rodriguez et al., 2011). While murine studies strongly suggest a role for recipient LC in mediating GVHD pathogenicity, further studies are required to determine any associations in human patients.

<u>Psoriasis</u>

The strongest evidence for how LC differentiation and/or repopulation may impact on disease is for psoriasis. The density of LC in psoriasis has been long debated with reports detecting stable (Guttman-Yassky et al., 2007, Martini et al., 2017, Czernielewski et al., 1985, Gommans et al., 1987, Gunther et al., 2012), increased (Fujita et al., 2011, Baker et al., 1985, Komine et al., 2007) or decreased (Bos et al., 1983, Glitzner et al., 2014, Lisi, 1973) densities of LC in psoriasis-afflicted epidermis. In lesional epidermis, resident LC displayed impaired migrational capacity (Cumberbatch et al., 2006, Shaw et al., 2010), elevated expression of CXCL9, CXCL10 and CCL20 (Fujita et al., 2011) and increased IL-23 production in response to TLR3 stimulation (Sweeney et al., 2016). Impaired migration of LC was shown to be driven by an altered keratinocyte secretome induced by IL-17 (Eaton et al., 2018). However, it is unclear whether resident LC contribute to initiation of disease.

More importantly, however, is the role for replacement cells. During active psoriasis, monocytes are recruited to the epidermis and become LC-like and their persistence is required for continued pathology in the skin (Martini et al., 2017). Therefore, during psoriasis, recruited cells contribute to the perpetuation of the disease, whereas during GVHD, forcing the replacement of LC may be beneficial.

1.4.5. The known unknowns of LC replacement

It has been established that severe loss of LC from the epidermis causes replacement from the BM by a monocyte-derived CCR2-dependent precursor. However, it remains to be defined how similar these replacement cells are to LC in the steady state, in terms of both phenotype and function. Furthermore, the cytokine requirements for development and maintenance of repopulating LC are ill-defined, as are the environmental cues that trigger differentiation of monocytic cells into LC-like cells in the epidermis, compared to macrophages or moDC in the dermis. Moreover, while Sere et al. have suggested that cells of unknown origin may differentiate to become cells which are long-lived and transcriptionally similar to *bona fide* LC, it is not known how this process may be disturbed within a chronic inflammatory disease environment, nor how other myeloid cells contribute to the pathogenic response. We will seek to address some of these questions in this thesis.

1.5. Project rationale

GVHD remains the second leading cause of death following allogeneic haematopoietic stem cell transplantation. The skin, gut and liver are the principle target organs in acute GVHD, with cutaneous manifestations the most common and often the presenting sign of the disease. During the course of the disease, the immune environment of the skin is drastically altered resulting in a significantly disrupted myeloid compartment. The full effect of these changes on long-term patient immune function has not previously been characterised.

In the clinic, patients who are recovering from GVHD can suffer from ongoing skin infections (Ferrara et al., 2009). This has consistently been ascribed to the immunosuppressive drug regimen, however it is increasingly possible that the changes to the cutaneous immune compartment play a role. Furthermore, greater consideration is being given to the concept that tissue tolerance regulates GVHD severity (Wu and Reddy, 2017). Indeed, levels of circulating Treg are inversely correlated with acute or chronic GVHD (Zorn et al., 2005, Rieger et al., 2006, Rezvani et al., 2006, Miura et al., 2004) and there is evidence that Treg suppressive function is impaired in acute GVHD (Ukena et al., 2011).

Given these clinical observations, the primary focus of this thesis is to understand how the skin myeloid compartment is altered after GVHD and the consequences of these changes on skin immunity and function.

Secondarily, our acute GVHD model permits the study of LC replacement *in vivo*. Our knowledge of LC differentiation is lacking: firstly, because the LC network is established at birth and is thus technically difficult to study, and secondly, because LC are never physiologically depleted *in vivo*. We can use our acute GVHD model to force the replacement of LC by monocyte-derived cells and study their differentiation.

In this thesis we have used a combinatorial approach of flow cytometric analysis, transcriptomic and proteomic profiling, functional assessment and a reductionist *in vitro* model in a murine model of acute GVHD. This approach has allowed us to interrogate the cellular and cytokine composition of GVHD skin, delineate functional consequences and further define myeloid differentiation.

1.6.   Research hypotheses and aims

Hypotheses

1. Damage to the skin induced by GVHD leads to changes in the absolute number and/or frequency of myeloid cells.
2. LC are replaced by monocyte-derived cells during GVHD, generating donor LC that are transcriptionally distinct and causative in loss of cutaneous immune function.
3. Damage to the skin induced by GVHD results in an overtly inflammatory environment that blocks the differentiation of LC.
4. GVHD-mediated changes to the skin result in long-term loss of cutaneous immune function.
5. Monocyte-derived LC have different growth factor requirements to LC in the steady state.
6. Monocyte-derived LC have an "inflammatory" gene expression profile and potentiate disease.

Aims

1. Define the cellular changes in the skin as a result of GVHD pathology.
2. Identify cytokines in the skin that are upregulated during GVHD.
3. Analyse gene expression for various cellular populations to:
    a. validate a putative LC precursor population;
    b. define whether donor LC are transcriptionally similar to recipient LC; and
    c. further define the differentiation pathway of LC.
4. Identify functional defects of mice following GVHD, by assessing:
    a. the ability to invoke hapten-mediated tolerance to contact hypersensitivity;
    b. the humoral immune response following epicutaneous immunisation with protein antigen; and
    c. the response to cutaneous fungal pathogen challenge.
5. Optimise a BM culture protocol to model myeloid cell differentiation *in vitro.*
6. Further define the requirements for TGF-β and IL-34 for LC differentiation *in vitro.*
7. Validate LC differentiation pathways identified from transcriptional analyses *in vivo.*

Chapter 2 Materials and Methods

2.1.   *In vivo* experiments

2.1.1.   Mice

C57BL/6 TCR-transgenic anti-HY MataHari (Valujskikh et al., 2002), C57BL/6 Langerin.DTREGFP (Langerin-DTR; Kissenpfennig et al., 2005), C57BL/6J and B6.SJL mice were bred in house by UCL Biological Services under specific pathogen-free conditions. Animals used as recipients for BMT were 8-12 weeks old, and donors were 8-20 weeks old. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the Ethics and Welfare Committee of the Comparative Biology Unit, Royal Free and University College London Medical School, and by the UK Home Office.

2.1.2.   BMT

Recipient mice were lethally irradiated (11 Gy total body irradiation, split into two doses over a period of 48 hours, at day -2 and day 0) and reconstituted 4 hours later with 5 x 10^6 BM cells, 2 x 10^6 CD4$^+$ T cells and 1 x 10^6 CD8$^+$ T cells, administered by intravenous injection through the tail vein. Isolation of CD4$^+$ and CD8$^+$ T cells from spleen and LN was performed using Manual MACS Cell Separation Technology (QuadroMACS Separator, LS columns, CD4 [L3T4] MicroBeads, CD8a [Ly-2] MicroBeads; Miltenyi Biotec, Germany), according to the manufacturer's instructions. C57BL/6 (CD45.2$^+$/Thy1.2$^+$), B6.SJL (CD45.1$^+$/Thy1.2$^+$) and Langerin-DTR (CD45.2$^+$/Thy1.2$^+$) male mice were used as recipients. C57BL/6 (CD45.2$^+$/Thy1.2$^+$), B6.SJL (CD45.1$^+$/Thy1.2$^+$) and Langerin-DTR (CD45.2$^+$/Thy1.2$^+$) female mice were used as BM and CD4$^+$ T cell donors. MataHari (CD45.1$^+$/Thy1.1$^+$) female mice were used as CD8$^+$ T cell donors. Male recipients transplanted with female bone marrow without T cells, or female recipients transplanted with female bone marrow with T cells, were used as BMT controls.

2.1.3.   CHS model

Contact hypersensitivity (CHS) to 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich, UK) was determined by the mouse ear swelling test (Garrigue et al., 1994). Mice were sensitised epicutaneously on day 0 by application of 25 µl 0.5% DNFB in acetone/olive oil (4:1 v/v) onto 2 cm^2 shaved abdominal skin. Mice were challenged on day 5 with a topical application of 10 µl of a non-irritant concentration of 0.2% DNFB in acetone/olive oil to each side of the right ear. The left ear was treated with acetone/olive oil alone.

Mice that were ear challenged without previous sensitisation served as non-specific inflammation controls. To induce cutaneous tolerance, mice were painted 7 days prior to sensitisation by application of 100 µl 1% 2,4-dinitrothiocyanobenzene (DNTB; Lancaster Synthesis, USA) in acetone/olive oil onto 2 cm^2 shaved flank skin. Ear thickness was measured in a blinded fashion before and for 6-8 consecutive days after the challenge, using a digital caliper. Ear swelling was calculated as (T-T_0 of the right ear) - (T-T_0 of the left ear), where T_0 and T represent the values of ear thickness before and after the challenge, respectively.

2.1.4.   *Candida albicans* challenge model

Mice were first anaesthetised by isofluorane inhalation, shaved on the back with an electric clipper, and chemically depilated with Nair hair remover (Church & Dwight, USA) for 2 min. The skin was washed with 70% isopropanol and the stratum corneum was removed with 15 light strokes with 220 grit sandpaper (3M, USA). *Candida albicans* (strain ATCC10231X) was kindly provided by Prof. Timothy McHugh (UCL Centre for Clinical Microbiology) and was grown in YPD medium (Sigma-Aldrich, UK) at 37°C shaking until the OD_{600} reached 1.5-2.0. After washing with sterile PBS, 2 x 10^8 *C. albicans* in 50 µl of sterile PBS was applied onto the skin. The inoculum was plated in serial dilutions onto YPD plates (Sigma-Aldrich, UK) for retrospective colony counting. Mice were sacrificed prior to infection and on days 2,4,5 and 7 after infection. The infected area was cleansed with povidone-iodine (Alfa Aesar, USA), and a 2.0 cm^2 section of skin was homogenised in sterile PBS containing 1% Penicillin-streptomycin (Life Technologies, USA) using a tissue homogeniser. Tissue homogenates were plated in serial dilutions on YPD plates and incubated at 37°C. Colony counts were obtained 24 – 48 h later.

2.1.5.   Epicutaneous immunisations

The stratum corneum was removed from both sides of the ear by application and removal of Scotch™ tape (3M, USA) eight times. 24 h later, 25 ul of OVA protein (Sigma-Aldrich, UK) in PBS (4 mg/ml) was applied to both sides of the ear with a cotton bud. The application of protein to the skin was repeated on the next 2 consecutive days. PBS without antigen applied to tape-stripped skin was included as a control. The mice were sacrificed 10 days after the initial immunisation and the serum collected and assayed for OVA-specific total IgG, IgG1 and IgG2c, and non-specific IgE.

2.1.6.   EdU pulsing

Mice were injected with a single dose of 100 µg EdU i.p. (Invitrogen, USA) and were sacrificed 4 h later. Blood and ears were harvested, processed and assessed for EdU

incorporation by flow cytometry. Mice not injected with EdU were used as EdU negative controls.

2.2. *Ex vivo* cell isolation

2.2.1. Skin

Ears were excised, split into dorsal and ventral sides and incubated on 2.5 mg/ml dispase II (Roche, Switzerland) at 4°C for 15 h to permit separation of epidermis and dermis. Epidermal sheets were peeled carefully away from the dermis. Epidermal cell suspensions were generated using a GentleMACS tissue dissociator (Miltenyi Biotec, Germany). Dermis was minced into small pieces and digested with 250 U/ml collagenase IV (Worthington, USA) and 800 U/ml DNase I (AppliChem, USA) at 37°C, 220 rpm for 1 h. Dermal cell suspensions were then generated using a GentleMACS tissue dissociator. In some experiments, whole skin was processed in the same way as dermis but without pre-digestion with dispase II.

2.2.2. Secondary lymphoid organs

Spleens were mechanically disrupted in 1ml ACK lysing buffer (Gibco, USA) for 1 min using a syringe plunger. The cells were filtered through 40 μm nylon mesh and quenched with 9 ml complete RPMI medium (RPMI 1640 (Lonza, Switzerland) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies, USA), 1% L-glutamine (2 mM; Life Technologies, USA), 1% Penicillin/Streptomycin (100 U/ml; Life Technologies, USA) and 2-beta-mercaptoethanol (50 μM; Sigma-Aldrich, UK)).

For isolation of T cells, LN were mechanically disrupted in MACS buffer (1% heat-inactivated fetal calf serum and 1 mM EDTA (Sigma-Aldrich, UK) in PBS) using a syringe plunger. The cells were filtered through 70 μm nylon mesh. For isolation of APC, LN were teased apart with needles in HBSS (Lonza, Switzerland), and subsequently digested with 4000 U/ml collagenase IV in PBS at 37°C for 30 min. The cells were then mechanically disrupted in MACS buffer using a syringe plunger and filtered through 70 μm nylon mesh.

2.2.3. Bone marrow

Tibias and femurs were harvested from mice and flushed with 5 ml RPMI using a 20 ml syringe and a 27G needle. Cells were pipetted to generate a single cell suspension and passed through 40 μM nylon mesh.

2.2.4. Cell counting

Total viable cell concentration of the cell suspensions was determined using a CASY®1 Model TT cell counter and analyser system (Schärfe-System, Germany). Absolute number of specific cell populations was determined by flow cytometry using CountBright™ Absolute Counting Beads (Life Technologies, USA), as per the manufacturer's instructions.

2.3. *In vitro* cultures

2.3.1. BMLC culture

BM was harvested and red blood cells were lysed by resuspending the cell pellet in 1ml ACK lysing buffer for 1 min. Cells were then washed with complete RPMI medium. Cells were counted and resuspended at 2.5×10^6 cells/ml. 1 ml of cell suspension was plated per well in tissue-culture-treated 24 well plates and supplemented with 20 ng/ml recombinant GM-CSF (Peprotech, USA) and 5 ng/ml recombinant TGF-β (Peprotech, USA). Cultures were incubated at 37°C. Half of the medium was removed at day 2 and replaced by fresh pre-warmed medium supplemented with GM-CSF (20 ng/ml) and TGF-β (5 ng/ml). The culture medium was entirely discarded at day 3 and replaced by fresh pre-warmed medium supplemented with GM-CSF (20 ng/ml) and TGF-β (5 ng/ml). Cells were harvested on day 4 or 6. BMDC and BMMac were generated by excluding TGF-β from the culture.

2.3.2. Addition of growth factors

BMLC cultures were supplemented for the length of culture with 8 µg/ml IL-34 (Generon, UK), 100 µg/ml BMP7 (R&D Systems, USA) and/or 10 µg/ml CSF-1 (Biolegend, USA).

2.3.3. EdU pulsing

Cultures were pulsed with 10 µM EdU beginning on day 2 or day 5. After 24 hours, the media was replaced with fresh pre-warmed medium containing the relevant cytokines. Cells were harvested on day 6 and assessed for EdU incorporation by flow cytometry. One well of cells not pulsed with EdU was used as an EdU negative control.

2.4. Flow cytometry

2.4.1. Staining of extracellular markers

Cells were routinely stained for flow cytometry analysis (typically $0.5\text{-}1.5 \times 10^6$ cells). Cells were washed once in MACS buffer and subsequently resuspended in 100 µl MACS

buffer containing CD16/CD32 Fc Block antibody (BD Biosciences, Germany) for 30 min at 4°C. Cells were then resuspended in 100 ul MACS buffer containing the monoclonal antibodies of interest in the appropriate dilutions (see Table 2.1 for details), for 30 min at 4°C. For biotinylated antibodies, extracellular staining was followed by 20 min staining in 100 µl MACS buffer containing a fluorochrome-conjugated streptavidin antibody in the appropriate dilution. Cells were washed twice in MACS buffer and resuspended in a final volume of 200µl MACS buffer.

Table 2.1 Extracellular marker antibodies for flow cytometry

Marker	Fluorochrome	Clone	Supplier	Dilution
B220	BV786	RA3-6B2	BD Biosciences	1:400
CCR2	APC	475301	R&D systems	1:25
CD103	APCR700	M290	BD Biosciences	1:100
CD115	APC	AFS98	eBioscience	1:100
CD115	Biotin	AFS98	eBioscience	1:25
CD117	APC	2B8	BD Biosciences	1:100
CD11b	FITC	M1/70	BD Biosciences	1:800
CD11b	V450	M1/70	eBioscience	1:400
CD11b	Biotin	M1/70	eBioscience	1:100
CD11c	APC	HL3	BD Pharmingen	1:200
CD11c	V450	HL3	BD Pharmingen	1:100
CD127	PE-Cy7	A7R34	eBioscience	1:100
CD135	PerCP-eFluor710	A2F10	eBioscience	1:100
CD19	APC-Cy7	1D3	BD Biosciences	1:400
CD24	FITC	M1/69	Biolegend	1:1000
CD24	BV711	M1/69	BD Biosciences	1:1000
CD24	PE	M1/69	Biolegend	1:200
CD25	BV786	PC61	BD Biosciences	1:200
CD25	FITC	eBio7D4	eBioscience	1:100
CD3	APC-Cy7	145-2C11	BD Biosciences	1:50
CD4	BUV737	GK1.5	BD Biosciences	1:200
CD4	PE	GK1.5	eBioscience	1:800
CD4	APC	GK1.5	eBioscience	1:800
CD40	PE	3/23	BD Biosciences	1:200
CD45	V450	30-F11	Biolegend	1:100
CD45	BV711	30-F11	Biolegend	1:15,000
CD45.1	APC780	A20	eBioscience	1:100
CD45.1	Biotin	A20	eBioscience	1:400
CD45.1	BV605	A20	BD Biosciences	1:100
CD45.1	BV650	A20	BD Biosciences	1:400
CD45.2	APC-Cy7	104	BD Biosciences	1:100
CD45.2	Biotin	104	eBioscience	1:400
CD45.2	PerCP-Cy5.5	104	eBioscience	1:200
CD64	PE	x54-5.7.1	Biolegend	1:200
CD69	PE-Cy7	H1.2F3	eBioscience	1:100
CD70	PE	FR70	eBioscience	1:200

CD8	BUV395	53-6.7	BD Biosciences	1:100
CD80	PE	16-10A1	Biolegend	1:200
CD86	PE	GL1	eBioscience	1:2000
DEC205	PE-Cy7	205yekta	eBioscience	1:100
EpCAM	APC	G8.8	eBioscience	1:800
EpCAM	PerCP-eFluor710	G8.8	eBioscience	1:400
γδ TCR	PE	eBioGL3	eBioscience	1:5000
ICOS	BUV395	C398.4A	BD Horizon	1:100
LY-6C	PE-Cy7	AL-21	BD Biosciences	1:400
LY-6G	APC-Cy7	1A8	BD Biosciences	1:50
LY-6G	Biotin	RB6-8C5	eBioscience	1:100
MerTK	PE	2B10C42	Biolegend	1:100
MHC II	APC780	M5/114.15.2	eBioscience	1:800
MHC II	PE	AF6-120.1	BD Pharmingen	1:800
MHC II	V500	M5/114	BD Biosciences	1:800
MHC II	Biotin	AF6-120.1	BD Pharmingen	1:1000
NK1.1	APC-Cy7	PK136	Biolegend	1:200
NK1.1	Biotin	PK136	BD Pharmingen	1:100
TCRβ	AlexaFluor700	H57-597	BD Biosciences	1:100
Thy1.1	PE-Cy7	HIS51	eBioscience	1:10,000
Thy1.1	FITC	HIS51	eBioscience	1:800
Thy1.1	APC	HIS51	eBioscience	1:800
Thy1.2	FITC	53-2.1	BD Pharmingen	1:100
Thy1.2	V500	53-2.1	BD Biosciences	1:1000
Vβ8.3	FITC	1B3.3	BD Biosciences	1:100

2.4.2. Staining of cytoplasmic markers

Cells were first stained for surface molecules as above, and then were fixed and permeabilised using Cytofix/Cytoperm kit (BD Biosciences, Germany) according to manufacturer's instructions. Cells were then resuspended in 100 µl Perm/Wash buffer containing the monoclonal antibodies of interest in the appropriate dilutions (see Table 2.2) for 45 min at 4°C. Cells were washed twice in Perm/Wash buffer and resuspended in a final volume of 200 µl MACS buffer.

Table 2.2 Cytoplasmic marker antibodies for flow cytometry

Marker	Fluorochrome	Clone	Supplier	Dilution
CD207	PE	eBioL31	eBioscience	1:400

2.4.3. Staining of intranuclear markers

Cells were first stained for surface molecules as above, and then were fixed and permeabilised using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience, USA) according to manufacturer's instructions. Cells were then resuspended in 100 µl

Perm buffer containing the monoclonal antibodies of interest in the appropriate dilutions (see Table 2.3) for 45 min at 4°C. Cells were washed twice in Perm buffer and resuspended in a final volume of 200 µl MACS buffer.

Table 2.3 Intranuclear marker antibodies for flow cytometry

Marker	Fluorochrome	Clone	Supplier	Dilution
FoxP3	APC	FJK-165	eBioscience	1:100
Ki67	eFluor660	SolA15	eBioscience	1:100

2.4.4. Click-iT EdU staining

For EdU-incorporation experiments, cells were stained using the Click-iT Plus EdU Flow Cytometry Assay Kit (Invitrogen, USA), according to manufacturer's instructions.

2.4.5. Viability staining

Dead cells were excluded from analyses using 2 µl propidium iodide (PI; BD Biosciences, Germany) on live cells immediately prior to analysis, except for when cells were fixed or stained with a monoclonal antibody conjugated to PerCP-Cy5.5. In some experiments, dead cells were excluded from fixed samples using Fixable Viability Dye eFluor™ 450 (eBioscience, USA) or Live/Dead™ Fixable Red Dead Stain Kit (Life Technologies, USA).

2.4.6. Flow cytometry analysis

All analyses were performed on an LSRFortessa (BD Biosciences, Germany). Data were analysed with FlowJo software v10.0 (TreeStar Inc., USA).

2.5. Transcriptomic analysis

2.5.1. Fluorescence-activated cell sorting (FACS)

Samples were stained for extracellular markers as above and resuspended in 1 ml MACS buffer. Propidium iodide was used to exclude dead cells. FACS was performed on an Aria IIu (BD Biosciences Germany). Cell populations were sorted into PBS supplemented with 2% FCS at 4°C for further culture, or directly into RLT buffer (Qiagen, USA) if performing RNA extraction. Purity of sorted populations was assessed after sorting and was routinely greater than 95%.

2.5.2. Preparation of nucleic acids

RNA extraction was carried out on sorted cell populations using an RNeasy Micro Kit (Qiagen, USA), according to manufacturer's instructions. cDNA was synthesised with a

QuantiTect Reverse Transcription Kit (Qiagen, USA), according to manufacturer's instructions.

2.5.3. Quantitative real-time PCR

Quantitative reverse transcription PCR (qRT-PCR) was run on a CFX96 Touch Real-Time PCR detection system (Bio-Rad, USA), using a Quantifast SYBR Green PCR kit (Qiagen, USA), according to manufacturer's instructions. Primers were obtained for a selection of cellular receptors, transcription factors and *Gapdh* and are shown in Table 2.4. The expression of mRNAs was normalised to *Gapdh* mRNA by calculating $2^{-\Delta Ct}$.

Table 2.4 qRT-PCR primers

Target	Forward primer (5'-3')	Reverse primer (5'-3')
Tlr1	CCCACAATGAGCTAAAGGTGATCT	GGCATTAAAGGAGAGGTCCAAA
Tlr2	CGGAGGTAGAGTTCGACGACTG	CACTACGTCTGACTCCGAGGG
Tlr3	AAGTTATTCGCCCTCCTCTTGA	CCCAGCAAAGCTTCTCTGTGA
Tlr4	AAATGCCAGGATGATGCCTC	CTGGTGTCAGGCAGGAGAAGA
Tlr5	CCAGTTGCTGGAGCTGGG	GGCAGGTTTCTGAAAGCCC
Tlr6	CGAAGCTGACTTTCCTGGGA	CAAGTGAGCAACTGGGAGCA
Tlr7	ATATCCCAGAGGCCCATGTG	GGAATGCCCTCAGGGATTTC
Tlr9	TCCAACATCACCCGCCTC	GGACGAAGTCGGAGTTGTGC
Dectin1	AGGTTTTTCTCAGTCTTGCCTTC	GGGAGCAGTGTCTCTTACTTCC
Dectin2	TCCACAAGGTAATGGCAAATGG	CTATTGAAACACACCGCTCTTCT
Id2	AAAACAGCCTGTCGGACCAC	CTGGGCACCAGTTCCTTGAG
Runx3	CAGGTTCAACGACCTTCGATT	GTGGTAGGTAGCCACTTGGG
Tgfbr1	TCAGCTCTGGTTGGTGTCAG	ATGTGAAGATGGGCAAGACC
Bmp1ra	GAAAGCAGCAGGTGAAAGTC	CTATAATGGCAAAGCAATGG
Gapdh	ACCTGCCAAGTATGATGACATCA	GGTCCTCAGTGTAGCCCAAGAT

2.5.4. RNA Sequencing

cDNA amplification from total RNA was performed using the SMART-seq® v4 Ultra® Low Input RNA Kit (Clontech, USA). Paired-end sequencing libraries were prepared from the amplified cDNA according to the Nextera® XT DNA library prep protocol (43 bp reads, approx. 23 million reads/sample), and sequenced using an Illumina NextSeq 500 (Illumina, USA).

Each analysis was performed in duplicate or triplicate using independently sorted cells from independent experiments.

RNA sequencing was performed in collaboration with UCL Genomics.

2.5.5. RNA Sequencing analysis

Library pre-processing

FASTQ Toolkit, version 1.0.0 (BaseSpace, Illumina), was used for adapter trimming of the reads. Alignment and mapping of all libraries were performed using TopHat

Alignment, version 2.0.0, and Cufflinks Assembly & DE, version 2.0.0 (BaseSpace, Illumina, USA).

Dataset quality control

The dataset was filtered to remove genes where two or more samples in each group had an FPKM count of <1.

Gene expression analysis

Gene expression levels were calculated using the Cufflinks Assembly & DE, version 2.0.0 (BaseSpace, Illumina, USA), employing a geometric library normalisation method and a fragment bias correction algorithm.

Differential gene expression was calculated using a threshold of fold change > 2 and false discovery rate (FDR) < 0.05.

To analyse the similarity between the samples, we used the principle component analysis algorithm, using R studio prcomp function from the stat package.

Gene Ontology (GO) overrepresentation analysis

The Web-based Gene Set Analysis Toolkit (WebGestalt), a suite of tools for functional enrichment analysis, was used to identify overrepresented GO annotation categories and translate gene lists into functional profiles. Enrichment of GO terms and associated p-values were calculated based on hypergeometric distribution statistics, adjusting the false discovery rate using the Benjamini-Hochberg procedure.

2.6.   Protein analyses

2.6.1.   *Ex vivo* cytokine analysis

Ex vivo cytokine production was assessed by incubating cell suspensions with 10 µg/ml brefeldin A (Sigma-Aldrich, UK) for 2 h at 37°C, followed by flow cytometry staining and analysis.

2.6.2.   Serum immunoglobulin ELISA

Mice were sacrificed 10 days after epicutaneous immunisation with OVA or PBS by cardiac puncture and the sera prepared for specific antibody determinations. For IgG, IgG1 and IgG2c antibodies, 96 well Maxisorb plates (Thermo Fisher, USA) were coated with 25 µg OVA protein per well in carbonate-bicarbonate buffer at 4°C overnight. After washing in PBS supplemented with 0.05% Tween-20, plates were blocked in 1% normal goat serum (Sigma-Aldrich, UK) at 37°C for 90 min. Appropriately diluted sera (100 µl in PBS) were added and the plates incubated at 37°C for 90 min. After washing, alkaline

phosphatase-conjugated goat anti-mouse IgG Fc (Sigma-Aldrich, UK), rat mAb to mouse IgG1 (Zymed, USA) or rat mAb to IgG2c (BD, Germany) were added for 1 h at 37°C. The alkaline phosphatase substrate pNPP (Sigma-Aldrich, UK) was then added and absorbance measured at 405 nm. Total IgE was measured by an IgE capture method. Sera to be tested were added to Maxisorb microtiter plate wells coated with 1ug/ml rat monoclonal anti-mouse IgE (BD, Germany).

2.6.3. Proteomic screen

Ears were excised, split into dorsal and ventral sides and incubated on 2.5 mg/ml dispase II (Roche, Switzerland) at 37°C for 45 min to permit separation of epidermis and dermis. Epidermal sheets were peeled carefully away from the dermis. The epidermis and dermis pooled from 2 mice per group were then floated separately on 1ml RPMI supplemented with 2% FCS at 37°C for 16 h. The supernatant was then harvested and assayed for the presence of 111 cytokines using the Proteome Profiler Mouse XL Cytokine Array (R&D Systems, USA), according to manufacturer's instructions.

2.7. Statistical analyses

Apart from RNA sequencing data, which were analysed with aforementioned programs and methodologies, statistical analysis was performed using GraphPad Prism version 6.00 for Mac OSX (GraphPad Software, USA).

The data were typically expressed as mean ± standard deviation, or mean ± standard error of the mean, where indicated. Significance was assessed using a student's t-test, one-way ANOVA or two-way ANOVA, as indicated in each figure legend. A p-value of less than 0.05 was taken to indicate a significant difference between groups; only statistically significant differences are marked in the figures. Sample sizes, n, and number of independent experiments, are indicated in the figure legends.

Chapter 3 Characterising the changes to the cutaneous immune environment following BMT

The immune environment of physiological skin has been well characterised (Nestle et al., 2009). Numerous lymphoid and myeloid populations are involved in a delicate interplay to maintain homeostatic functions, including protection against external insults and tolerance to harmless foreign antigens. As such, the skin is a tightly regulated environment and dysregulation and imbalance in the various cellular populations can lead to skin pathology.

Studying the skin under insult and during pathophysiology allows us to examine mechanisms of cutaneous immune activation and dysregulation and use this to further our knowledge of basic skin immunology. In parallel, there is a clear clinical benefit to studying disease. Patients with acute GVHD frequently go on to suffer from chronic GVHD which can affect the skin, and it is not known to what extent this continued autoimmune-like pathology is due to ongoing changes to the cutaneous immune environment. Elucidating the mechanisms behind dysregulation can reveal potential targets and lead to the development of novel treatments to improve patient quality of life or even cure disease.

We have established a murine single minor mismatch model of acute GVHD where allogeneic MataHari CD8$^+$ T cells (expressing a transgenic TCR specific for the male antigen (H-Y)) and are co-transferred with female polyclonal CD4$^+$ T cells and BM at the time of BMT (Fig. 3.1A; Toubai et al., 2012, Santos et al., 2018b). Following BMT, MataHari T cells are primed in the lymph nodes (LN) and infiltrate GVHD target organs, including the skin, where they cause pathology that replicates acute GVHD (Santos et al., 2018b). This model is considered sub-lethal as the majority of mice do not die as a result of GVHD. At 10 weeks post-BMT, allogeneic transplant recipients do not present with overt GVHD; this timepoint was chosen as a post-GVHD timepoint when mice have resolved the disease. This model therefore provides an excellent setting with which to investigate the impact of pathological T cells on the cutaneous myeloid compartment.

This model has been described as of mild to moderate severity, affecting predominantly the skin, lung, small intestine and the liver. The onset of the clinical signs of GVHD is at day 4-5 post-transplant, and it progresses gradually for 2-3 weeks. The severity of clinical GVHD is scored by assessing weight loss, posture (hunching), activity (reduction of movement, not resisting handling), fur texture (piloerection), eyes (closing of the eyes) and diarrhoea.

Allogeneic BMT and GVHD may alter the cutaneous cellular compartment in two ways. Firstly, alloreactive donor T cells are cytotoxic towards immune cells, resulting in destruction of that population. Secondly, the ensuing inflammatory environment may alter recruitment and differentiation of immune cells in the damaged tissue. Our lab has recently described the role of LC in activating enhanced effector function and survival by allogeneic T cells within the epidermis, and thereby driving GVHD pathology (Santos et al., 2018b). However, cytotoxic T cells kill the resident LC network leading to replacement by donor-derived cells and turn-over of patient LC (Stingl et al., 1980, Katz et al., 1979, Merad et al., 2002, Collin et al., 2006) and could therefore be self-limiting for disease (Santos et al., 2018b, Merad et al., 2004, Bennett et al., 2011, Collin and Jardine, 2014).
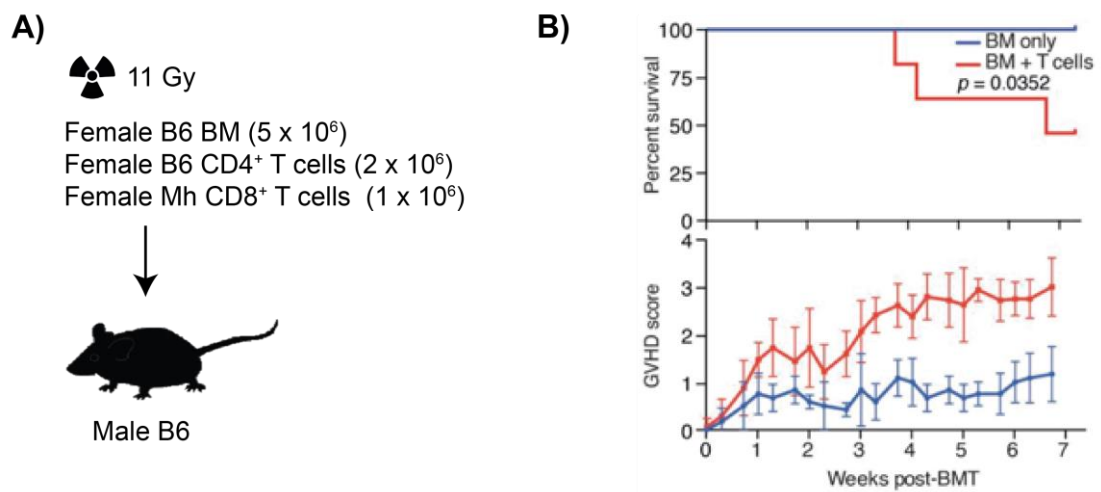
Figure 3.1 The acute GVHD model

(A) Experimental setup of the acute GVHD model. Irradiated male recipients receiving female BM without T cells are used as a control. **(B)** Kaplan Meier survival curve (log-rank Mantel-Cox test) and clinical GVHD score over time (mean $\pm$ SD). BM only (N = 6), BM + T cells (N = 11). Figure reproduced with permission from (Santos et al., 2018b).

LC are an epidermal population of radioresistant, self-renewing APC that depend on TGF-β for their maintenance, via the transcription factors Runx3 and Id2. We have investigated LC turn-over during GVHD in the lab and have demonstrated that the network is replaced by recruited myeloid cells in a CCR2-dependent manner. BM-derived monocytes differentiate into donor EpCAM+Langerin+ LC via a putative intermediate which can be defined by expression of EpCAM in the absence of Langerin (CD11b+MHC II+EpCAM+CD207low; Bennett and Ferrer, unpublished). However, the cellular signals that control the differentiation of monocytes into long-term LC have not been established.
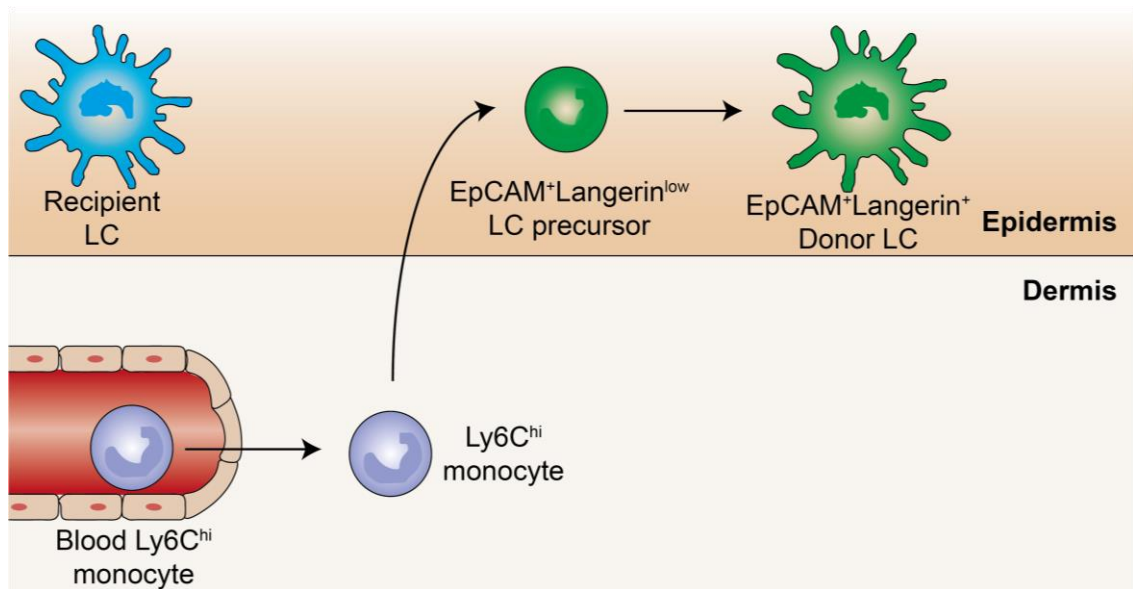
Figure 3.2 LC differentiation during GVHD

During GVHD, donor-derived blood monocytes differentiate into donor LC via a putative intermediate defined by the high expression of EpCAM and low expression of Langerin.

In contrast to the epidermis, it is less clear what the long-term impact of BMT and T cell pathology may be on dermal immune populations. Some dermal immune cells have been described as radioresistant, including a subset of dermal DC and Vγ6 γδ T cells (Bogunovic et al., 2006, Cai et al., 2014). Many other dermal immune cells, including macrophages, are short-lived and depend on continuous replenishment by precursors from the circulation (Tamoutounour et al., 2013, Scott et al., 2014). These cells are lost following irradiation and are replaced by donor-derived cells. However, recipient dermal macrophages have been shown to persist in patients following GVHD and may contribute to pathology (Haniffa et al., 2009). Additionally, BMT and T cell pathology may lead to the recruitment of new populations of cells not present in the steady state. Altered recruitment of monocytes and changes in the balance of dermal myeloid cells, or changes to the function of cells in the GVHD environment, could drastically affect immune regulation.

Myeloid infiltration during GVHD has been reported in several studies. Infiltration of CD163$^+$ macrophages was described as a predictive factor for refractory GVHD and poor prognosis (Nishiwaki et al., 2009), while a separate study reported increased intermediate monocytes in patients with acute GVHD that promoted the induction of a subset of Th17 cells that were resistant to glucocorticoids (Reinhardt-Heller et al., 2017). In preclinical models of chronic GVHD, donor-derived M2-like macrophages infiltrated the skin and contributed to dermal sclerosis in a CSF-1 dependent manner (Alexander et al., 2014, Du et al., 2017).

In this chapter, we hypothesised that:

1. damage to the skin induced by GVHD results in long-lasting changes in the absolute number and/or frequency of myeloid cells;
2. damage to the skin induced by GVHD results in an overtly inflammatory environment;
3. LC are replaced by monocyte-derived cells during GVHD, generating donor LC that are transcriptionally distinct from LC in the steady state.

Therefore, we aimed to:

1) perform a systematic flow cytometric characterisation the cellular immune environment of the skin during GVHD;
2) characterise the inflammatory cytokine milieu during GVHD;
3) use transcriptional profiling to infer myeloid cell development and functional characteristics within GVHD skin; and,
4) establish whether skin immune homeostasis was restored following GVHD.

Unless otherwise stated, irradiated male CD45.2$^+$ recipients received female donor CD45.1$^+$ BM, alone (BMT, to control for effects of transplantation) or in combination with T cells (BMT+T cells) and analysed the epidermal and dermal compartments over time.

3.1. Recipient LC are lost from the epidermis during GVHD and are replaced by donor-derived cells

To investigate changes to the skin immune environment during and after GVHD, we used flow cytometry to analyse the skin compartments at different time points in our GVHD model. We identified LC as CD11b$^+$MHC II$^+$EpCAM$^+$CD207$^+$ cells using the gating strategy shown in Figure 3.3A. Recipient and donor LC could be further segregated by the expression of CD45.2 and CD45.1, respectively. 3 weeks after BMT, LC remained primarily of recipient origin, in line with their known resistance to irradiation (Merad et al., 2002, Price et al., 2015). However, when T cells were present in the graft (BMT+T cells), LC were replaced by donor-derived cells and achieved mixed chimerism. Previous work in the lab has demonstrated that full donor chimerism was reached by 4 weeks post-transplant, and repopulating LC were maintained in the epidermis for at least 10 weeks (Fig. 3.3B, courtesy of Dr. Ivana Ferrer). The infiltration of allogeneic MataHari T cells into the epidermis between weeks 1-2 coincided with the appearance of donor LC. The number of donor LC in the epidermis peaked at 4 weeks post-transplant (2.62 ± 2.12 x 10^5 cells/g) and remained constant up to 10 weeks (1.94 ± 1.88 x 10^5 cells/g). Notably, the total number of LC at 10 weeks post-transplant, regardless of origin, were similar in the BMT and BMT+T cell recipients, and moreover were significantly decreased compared to untreated controls (BMT+T cells: 2.20 ± 1.88 x 10^5 c.f. Untreated: 6.46 ± 2.09 x 10^5 cells/g). We assessed a later timepoint of 18 weeks post-transplant (Fig. 3.3C) and revealed that the total LC number of transplanted mice, regardless of T cell transfer, remained lower than untreated controls. Additionally, we could still detect MataHari T cells in the epidermis of BMT+T cell mice at this timepoint. In summary, we identified replacement of the recipient LC network by donor-derived cells following GVHD. BMT led to a long-term reduction in epidermal LC number compared to the steady state. This suggested that, despite the radioresistance of LC, irradiation and/or transplantation led to a permanent alteration of the LC network.
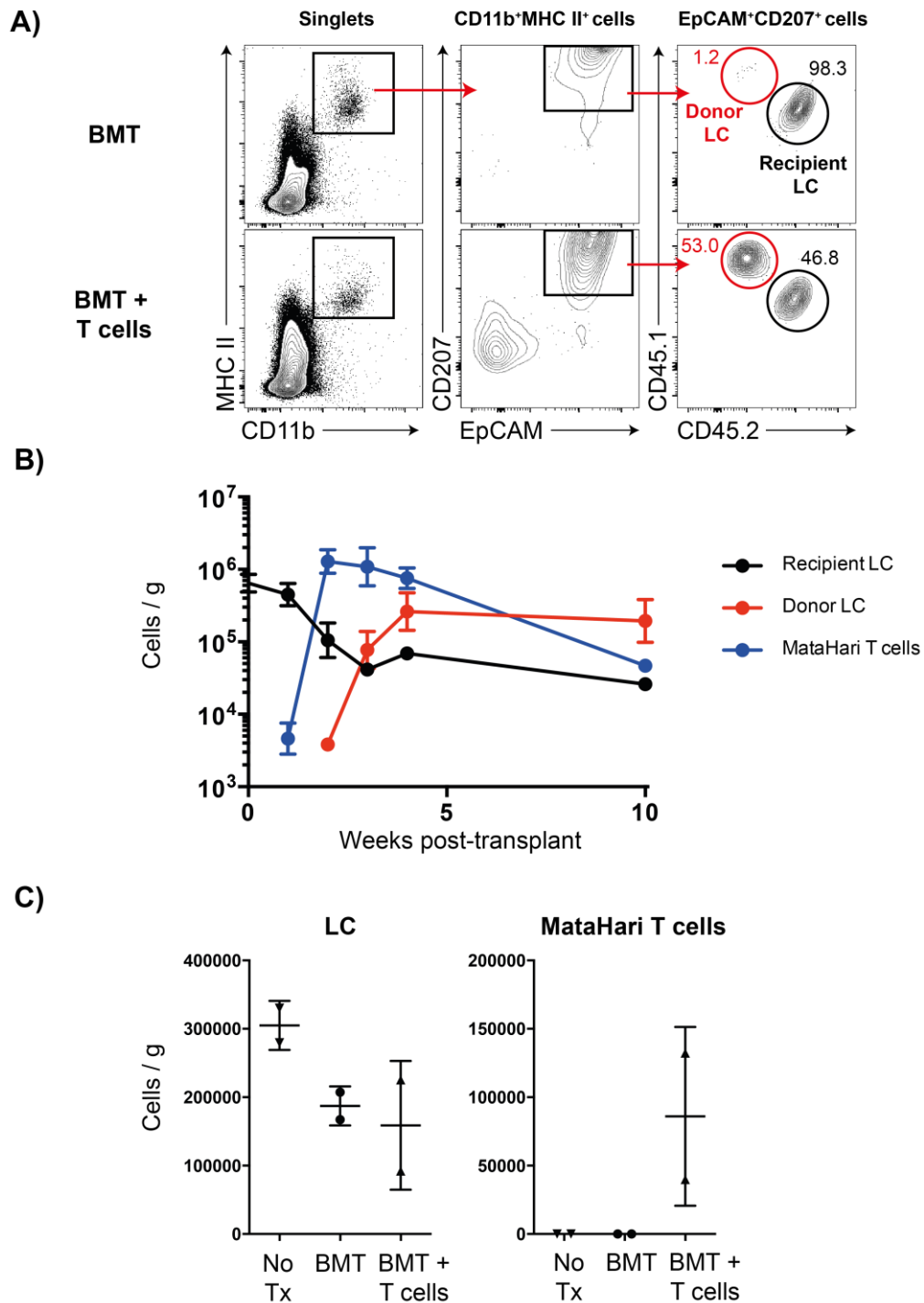
Figure 3.3 Recipient LC are lost from the epidermis during GVHD and replaced by donor-derived cells

(A) Gating strategy used to identify donor and recipient LC based on congenic markers. **(B)** Longitudinal analysis of absolute cell number normalised to ear weight of recipient LC, donor LC and MataHari T cells in the epidermis of mice transplanted with BMT + T cells (mean ± SD). N = 7-15. Data from two independent experiments. **(C)** Absolute cell number normalised to ear weight of total LC and MataHari T cells in the epidermis of untransplanted mice (No Tx) and mice transplanted with BMT or BMT + T cells, harvested at 18 weeks after transplantation (mean ± SD). N = 2. Data from one experiment.

3.2. Alteration of the composition of the dermal myeloid cell compartment after BMT

Analysis of the epidermis demonstrated T cell-dependent recruitment of BM-derived cells to replenish the LC niche. To determine the impact of allogeneic T cells on the dermal immune compartment, we initially focused on changes to monocyte-derived cells. For this, we utilised a flow cytometry gating strategy, shown in Figure 3.4A, that had previously been described (Tamoutounour et al., 2013). This strategy facilitated the identification of $CCR2^+$ cells, which could be further segregated into monocytes (P1), $Ly6C^{hi}$ moDC (P2) and $Ly6C^{low}$ moDC (P3), and $CCR2^-$ cells, which could be further segregated into MHC II^{low} macrophages (P4) and MHC II^{hi} macrophages (P5). P1 → P2 → P3 represented a developmental series referred to as the monocyte waterfall (Tamoutounour et al., 2012), following the differentiation of extravasated $Ly6C^{hi}$ monocytes into $Ly6C^{low}$ moDC, via a $Ly6C^{hi}$ intermediate.

We transplanted irradiated male recipient mice with female donor BM, alone (BMT) or in combination with T cells (BMT+T cells), and analysed the P1-P5 populations in the dermis over 10 weeks by flow cytometry (Fig. 3.4B, C, D). We analysed total cell populations, irrespective of recipient or donor origin. In untransplanted mice (steady state), all five populations were present in the dermis, with larger contributions from P3 (35.5 ± 10.3%), P4 (25.3 ± 18.8%) and P5 (17.2 ± 4.31%), compared to P1 (7.8 ± 0.9%) and P2 (11.6 ± 2.7%). This suggested low levels of recruitment of blood monocytes into steady state skin. After transplantation however, the relative balance of P1-P5 populations was altered. Our data demonstrated an increased frequency of P1 and P2 in both the BMT and BMT+T groups from 2 weeks post-transplant compared to steady state. These frequencies had not returned to steady state levels by 10 weeks post-transplant. The contribution of P3 to the overall P1-P5 gate was reduced between weeks 1 to 3 post-transplant in both the BMT and BMT+T groups and recovered 10 weeks post-transplant in both groups. However, when we assessed the frequency of P3 within the total live cells in the dermis, we only detected a reduced frequency at 1 week post-transplant in both groups, with the frequency returning to steady state levels from week 2 onwards. Our data suggested an increased frequency of P3 in the BMT+T group at 10 weeks post-transplant, compared to steady state and the BMT group.

Analysis of the P4 and P5 populations was more complicated as we observed high Ly6C expression by $CCR2^-$ cells after transplantation (Fig. 3.4B). Similar upregulation of Ly6C was detected in an imiquimod-induced model of psoriasis (Terhorst et al., 2015), and consistent with this paper, we decided to maintain our P4 and P5 gating strategy, excluding the $CCR2^-Ly6C^{hi}$ cells from our analyses. Our data demonstrated a similar frequency of P4 in the steady state and in the BMT group after transplantation. In

contrast, a reduced frequency of P4 was detected in the BMT+T group following transplantation, with the frequency not recovering to steady state levels by 10 weeks post-transplant. The frequency of P5 was reduced compared to steady state in both the BMT and BMT+T groups up to 4 weeks post-transplant. By 10 weeks post-transplant, both groups had recovered the frequency of P5 to above steady state levels, with a greater frequency of P5 in the BMT+T group.

In summary, we have detected continued extravasation of monocytes from the blood, even at 10 weeks post-transplant, irrespective of T cell-mediated pathology. Between weeks 2 to 4, these cells did not appear to differentiate into macrophages since we detected a low frequency of P4 and P5 cells. However, we could have underestimated the macrophage population owing to the exclusion of Ly6C^{hi} cells. In any case, it was unclear whether these monocyte-derived cells were entering the dermis and undergoing apoptosis, differentiating into non-macrophage cells or alternatively en route to the epidermis. The increase in frequency of P5 macrophages at 10 weeks post-transplant in both groups suggested that monocytes differentiated into macrophages at this later timepoint. Despite very few obvious differences between BMT and BMT+T cell recipients, we detected an increased frequency of P3 and P5 at 10 weeks post-transplant in the BMT+T cell recipients, suggesting that the GVHD environment permitted the accumulation of these cells. Together, these data demonstrated that irradiation and BMT were sufficient to cause changes to the dermal environment that led to long-term alterations in the recruitment and differentiation of monocytic cells.
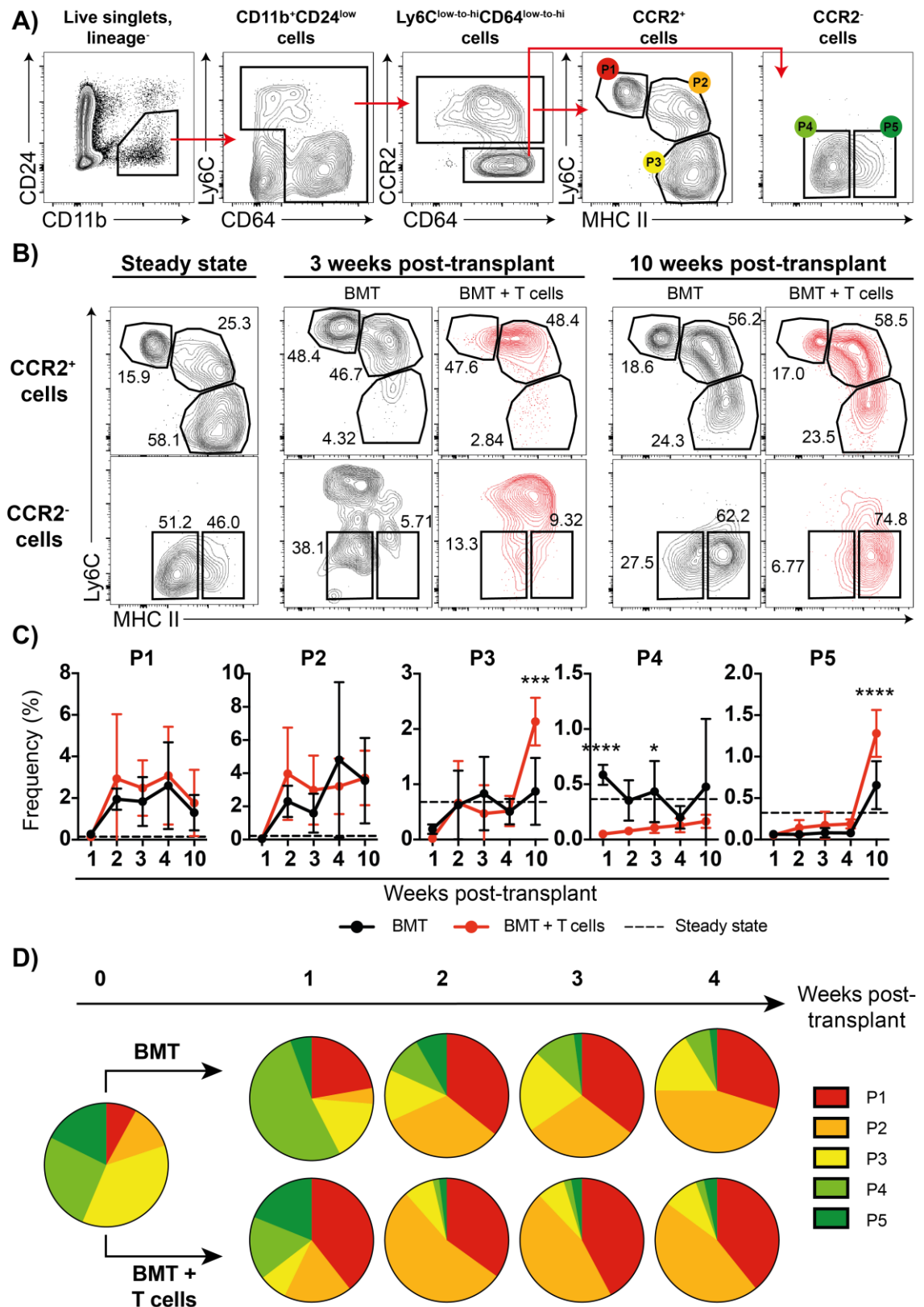
Figure 3.4 Dermal myeloid cell infiltration during GVHD is T cell independent and ongoing at 10 weeks post-transplant

(A) Gating strategy of dermal cells used to identify monocytes (P1), Ly6C^{hi} (P2) and Ly6C^{lo} moDC (P3), and MHC II^{lo} (P4) and MHC II^{hi} (P5) macrophages. Lineage stain consisted of CD3, CD19, NK1.1 and Ly6G. **(B)** Representative contour plots of CCR2$^+$ and CCR2$^-$ cells in the dermis of untransplanted mice (steady state) and transplanted mice at 3 and 10 weeks after transplantation with BMT or BMT + T cells. Numbers indicate the frequency of the individual P1 – P5 populations from gated CCR2$^+$ or CCR2$^-$ populations. **(C)** The frequencies of P1 – P5 over 10 weeks post-transplant are shown as a percentage of total live dermal

cells (mean $\pm$ SD). The dashed line indicates the frequency of the population at steady state. **(D)** Pie charts showing the mean frequencies of P1 – P5 over 4 weeks following transplant, as a percentage of the total Ly6C$^{lo-to-hi}$CD64$^{lo-to-hi}$ gate. N = 5-8, data from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.0001. Statistics calculated using a 2 way ANOVA with Sidak's multiple comparisons test.

3.3.   Defining long-term changes to dermal immune cells after BMT

In subsequent experiments we extended our analyses to determine the impact of BMT and GVHD on other dermal immune cells. For these experiments we used a syngeneic control (i.e. BMT with MataHari T cells into a female recipient). Here, MataHari T cells are not primed due to the absence of male alloantigen, and do not enter peripheral tissues. To capture the range of immune cells in the dermis, we adapted published gating strategies to design the flow cytometry panels shown in Table 3.1 and Figure 3.5 (Tamoutounour et al., 2013, Malissen et al., 2014).

Table 3.1 Phenotyping markers for identification of dermal immune cell populations

Cell type	Phenotype
CD11b$^+$ cDC2	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^-$CD45$^+$MHC II$^+$CD11b$^+$CD24^{low}CD64$^-$Ly6C$^-$
CD103$^+$ cDC1	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^-$CD45$^+$MHC II$^+$CD24$^+$CD11b$^-$
DN cDC	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^-$CD45$^+$MHC II$^+$CD24$^-$CD11b$^+$
Migrating LC	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^-$CD45$^+$MHC II$^+$CD24$^+$CD11b$^+$
Neutrophils	CD3$^-$CD19$^-$NK1.1$^-$Ly6G$^+$CD11b$^+$
γδ T cells	CD3$^+$γδTCR$^+$
ILC2	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^-$Thy1$^+$CD103$^+$
NK cells	CD3$^-$CD19$^-$Ly6G$^-$NK1.1$^+$
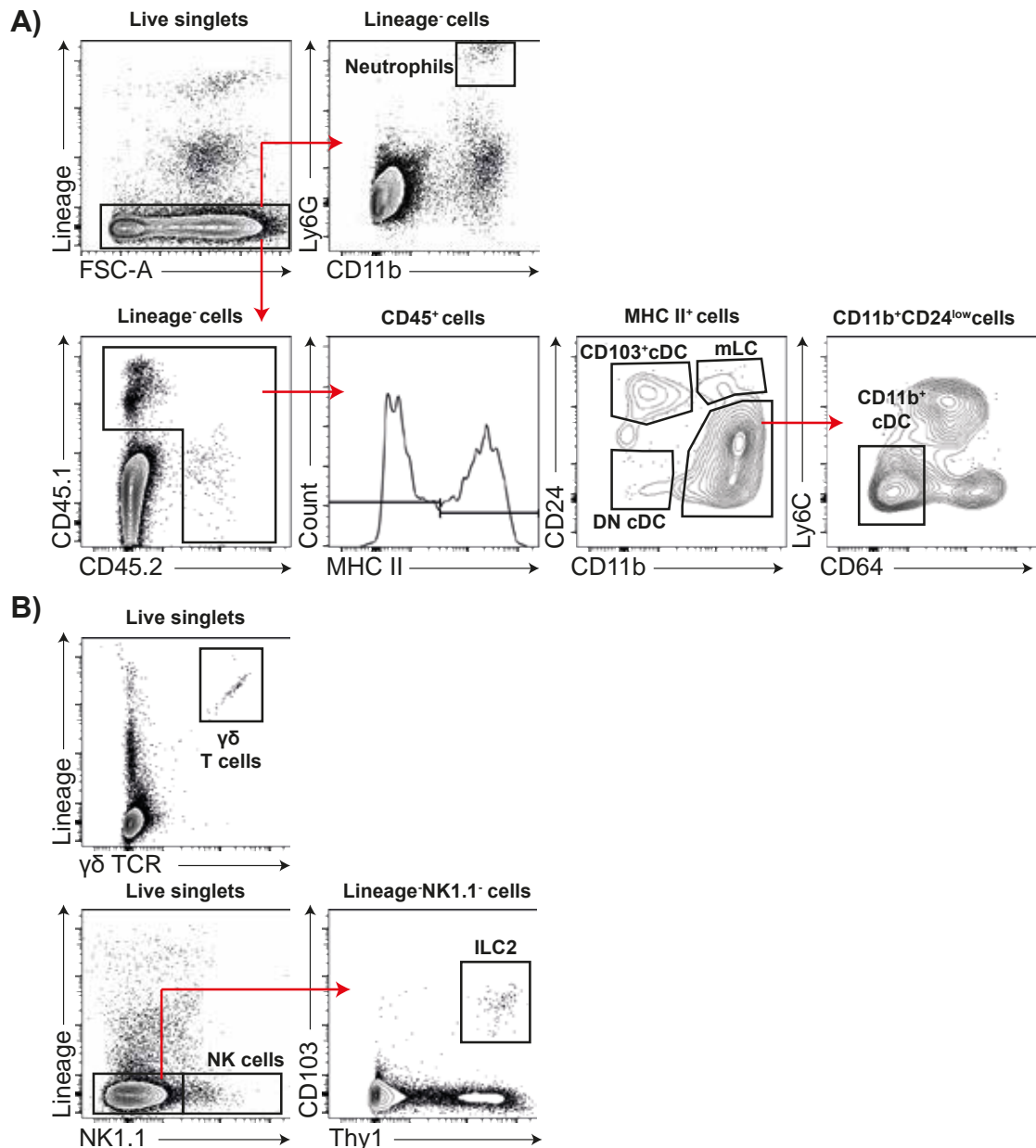
Figure 3.5 Gating strategies to identify different immune cells in the dermis

(A) Gating strategy used to identify neutrophils, CD103+ cDC, DN cDC, mLC and CD11b+ cDC from the dermis. Lineage stain consisted of CD3, CD19 and NK1.1, and gates were set according to lineage fluorescence-minus-one (FMO) controls. **(B)** Gating strategy used to identify γδ T cells, NK cells and ILC2 from the dermis. Lineage stain consisted of CD3, CD19 and Ly6G.

As previously, we analysed total cell populations, irrespective of recipient or donor origin. We detected a reduction in the number of CD11b+ cDC (cDC1) and CD103+ cDC (cDC2) in allogeneic transplant recipients compared to syngeneic transplant controls, suggesting a prolonged T cell-dependent effect on the cDC compartment (Fig. 3.6A). The DN cDC population was reduced in all irradiated mice. This is a small and poorly defined cDC compartment and was therefore difficult to interpret. We could not detect a significant difference in the number of migrating LC between the groups, however there was a trend towards a lower number of migrating LC in the allogeneic setting.

Influx of neutrophils into tissues is commonly associated with tissue inflammation. However, we did not detect differences in the number of neutrophils between groups. Similarly, NK cells were not differentially recruited to the dermis in this model. By contrast, irradiation had a dramatic impact on both dermal γδ T cells and ILC2, neither of which had recovered 10 weeks post-transplant (Fig. 3.6B).

In summary, these data demonstrated that, in addition to the continued recruitment of blood LyC6$^+$ monocytes into the dermis 10 weeks after BMT, irradiation and transfer of alloreactive T cells resulted in long-term changes to dermal cDC, γδ T cells and ILC2 populations.
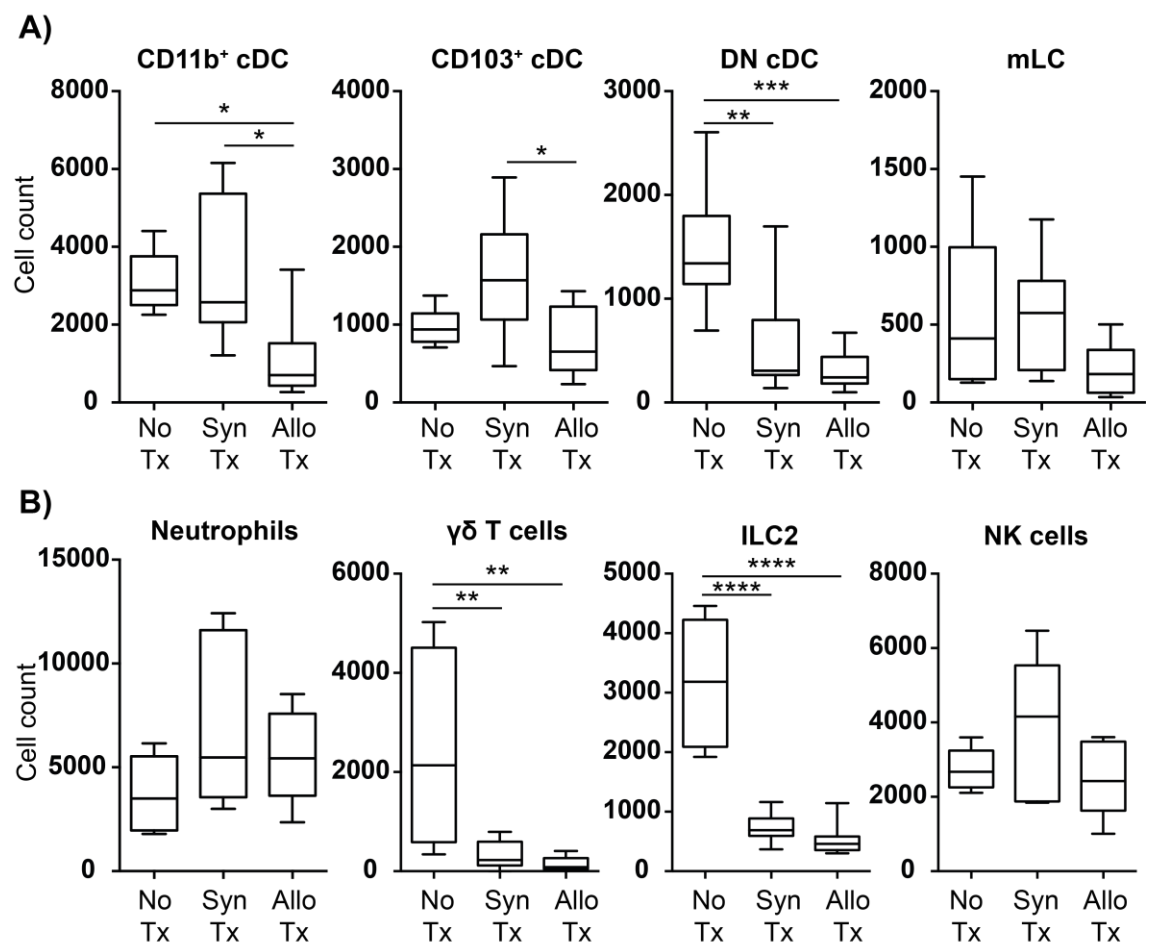
Figure 3.6 CD11b$^+$ and DN cDC are lost from the dermis of mice following GVHD

Absolute numbers of dermal cell populations from untransplanted (No Tx), syngeneic transplanted (Syn Tx) and allogeneic transplanted (Allo Tx) mice at 10 weeks post-transplant (mean ± SD). **(A)** Absolute numbers of DC subsets. **(B)** Absolute numbers of neutrophils, γδ T cells, ILC2 and NK cells. N = 6-9, data from two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001. Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test.

3.4.   Proteomic profiling of the epidermis during GVHD

Our data demonstrated that irradiation and T cell-mediated pathology resulted in lasting changes to the epidermal and dermal environments that potentially altered both

differentiation and recruitment of myeloid cells. Therefore, to characterise the extrinsic environment, we analysed the cytokine milieu of the respective compartments during ongoing GVHD. At 3 weeks post-transplant, we harvested skin, separated the epidermis and dermis by dispase II incubation, and floated the tissues separately on RPMI (2% FCS). After 16 h, we collected the supernatant and analysed protein production using a Proteome Profiler™ Array (R&D Systems, USA) to screen a large number of defined inflammatory proteins.

Of the total 111 proteins analysed, 28 were upregulated (fold change ≥ 2) in the epidermis of GVHD mice compared to BMT only controls (Fig. 3.7A). These included chemokines (CXCL1, CXCL2, CXCL5, CXCL10, CCL21), cytokines (G-CSF, HGF, Flt-3L, GM-CSF, LIF, IL-27 p28), cell adhesion molecules (VCAM-1, P-selectin) and inflammatory proteins (CRP, C5a), among others. We did not detect a striking inflammatory signature from GVHD epidermis at this timepoint (e.g. no significant overexpression of GVHD-associated pro-inflammatory cytokines, TNF-α, IL-1β and IL-6). This may be due to the sub-lethal pathology elicited in this minor H antigen-mismatched model, compared to MHC-mismatched models of GVHD. The broad upregulation of chemokines and cytokines suggested activation of tissue repair mechanisms following tissue injury, as has been described for GM-CSF (Mann et al., 2001), G-CSF (Gardner et al., 2014), HGF (Bevan et al., 2004), LIF (Banner et al., 1998) and IL-27 (Yang et al., 2017). CXCL2 and CXCL1 were the most highly upregulated proteins on the membrane, suggesting higher leukocyte recruitment to GVHD epidermis compared to BMT controls. CCL21 expression by lymphatic endothelial cells has been reported as the major mechanism responsible for LC and DC emigration from the skin to the draining lymphatics (Saeki et al., 1999, Engeman et al., 2000, Eberhard et al., 2004). Upregulation of CCL21 in peripheral tissues, however, is likely to recruit CCR7^{+} effector T cells (Lo et al., 2003). It is important to note that TGF-β and IL-34, critical cytokines in LC development and homeostasis, were missing from the membrane.
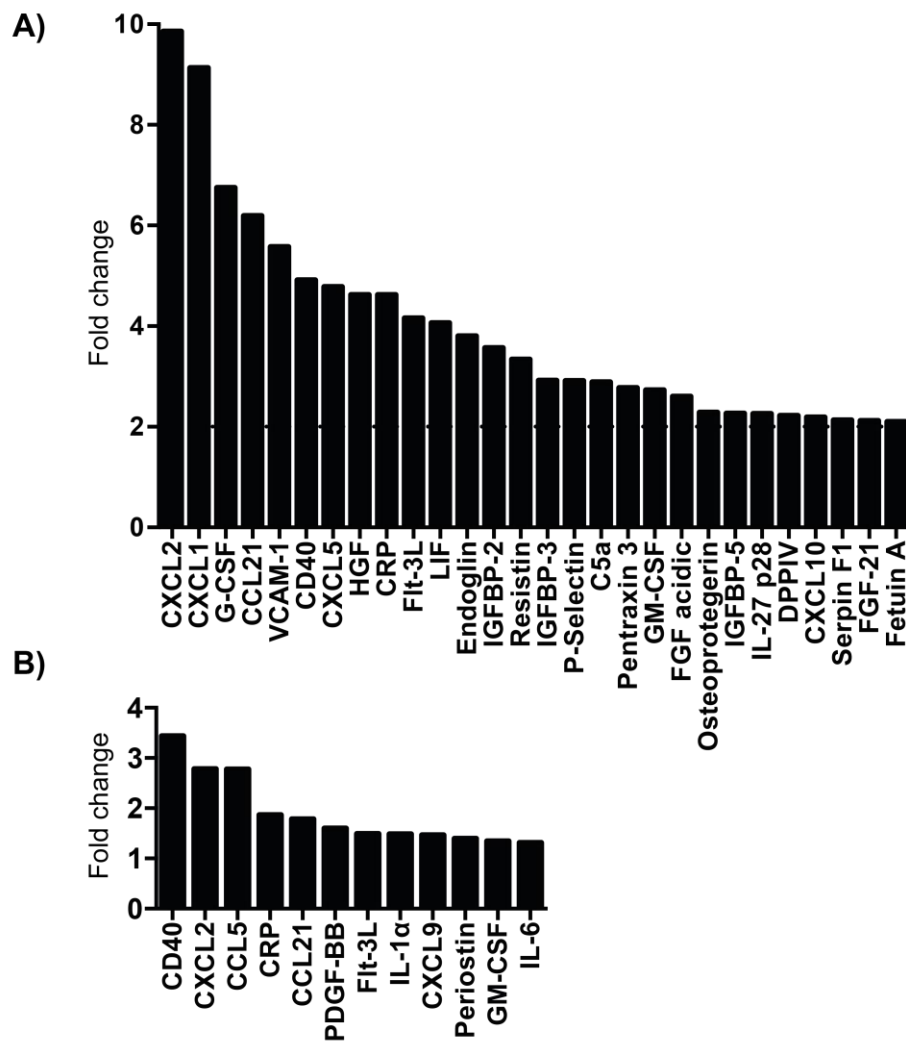
Figure 3.7 Proteomic profiling of GVHD skin

Ear skin was harvested at 3 weeks post-transplant and floated on dispase II for 45 min at 37°C to separate the epidermis and dermis. The epidermis and dermis were then floated separately on RPMI supplemented with 2% FCS for 16 hours, and a membrane-based antibody array was utilised to determine the relative levels of 111 proteins in the supernatant. Proteins upregulated in the **(A)** epidermis (fold change >2) and **(B)** dermis (fold change >1.2) of GVHD mice compared to BMT controls. N=1, data from one experiment.

In the dermis, only 20 proteins were upregulated (with a fold change greater than 1.2) in GVHD mice compared to BMT controls (Fig. 3.7B). This reflected that our model of acute GVHD is sub-lethal and does not present with severe pathology. The upregulated proteins included the leukocyte chemoattractants CXCL2, CCL5 and CXCL9, associated with upregulated expression during acute GVHD (Bouazzaoui et al., 2009, Kittan and Hildebrandt, 2010), and IL-1α and IL-6, characteristic proinflammatory cytokines of the "cytokine storm" of GVHD (Hill et al., 1997, Hill, 2009). Other proteins upregulated in the dermis of GVHD mice included periostin, an extracellular matrix protein involved in tissue remodelling following injury, and the growth factors Flt-3L and GM-CSF, known to be produced locally at sites of tissue inflammation (Ramos et al., 2014, Dehlin et al., 2008, Kay et al., 1991, Williamson et al., 1988, Al-Saffar et al., 1996, Carrieri et al., 1998).

Together, our data indicated that GVHD epidermis in this model was not an overtly inflammatory environment, and instead one undergoing active tissue repair. In comparison, GVHD dermis in this model was pro-inflammatory.

3.5. Transcriptional analysis of immune cells following GVHD

It is known from the literature and previous work in the lab that following BMT with MataHari T cells, extravasated monocytes enter the dermis and can differentiate into dermal monocytes, moDC or macrophages, or enter the epidermis and differentiate into long-lived LC, via an EpCAM$^+$ precursor intermediate (Fig. 3.8). Therefore, we aimed to determine the local signals and genetic control that allowed this plasticity within different skin sub-compartments. To this end, we compared the transcriptional profiles of monocyte-derived cells in the blood, dermis and epidermis. It has previously been shown that microglia, a self-renewing macrophage population in the brain, may be replaced by functionally similar monocyte-derived cells after pathology that retain the expression of a "monocyte-signature" (Cronk et al., 2018). Therefore, we also investigated whether monocyte-derived LC could be similarly distinguished from resident cells.
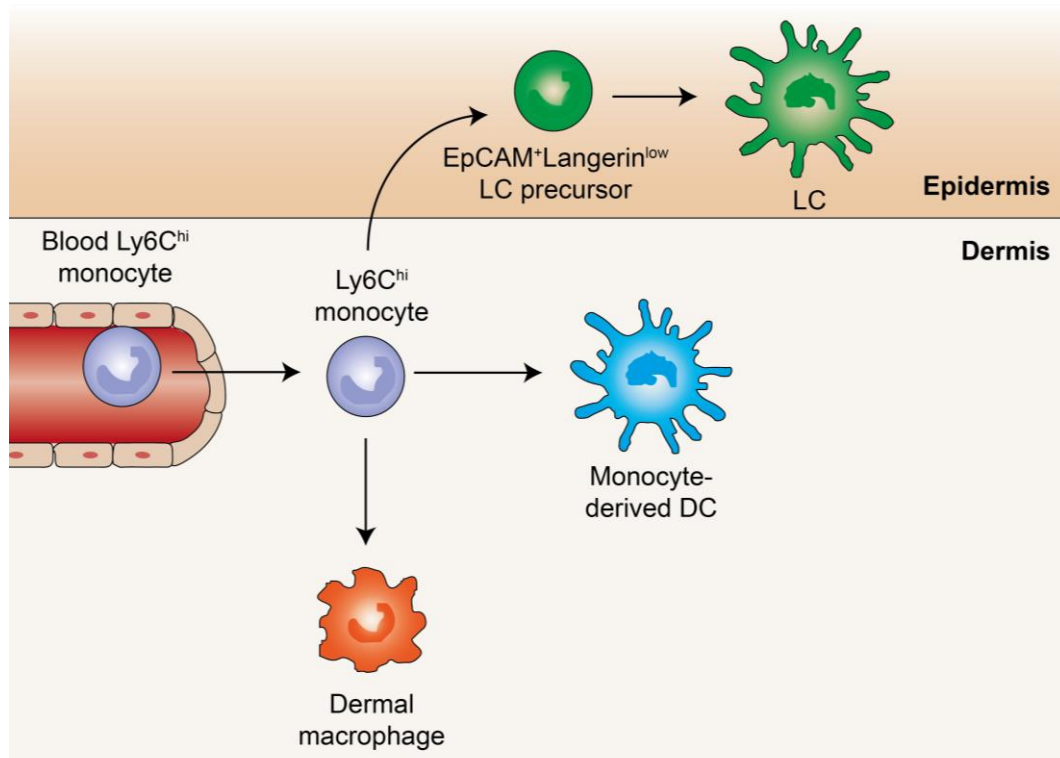
Figure 3.8 During GVHD, extravasated monocytes enter the dermis and epidermis where they can follow distinct differentiation pathways

In the dermis, Ly6C^{hi} monocytes differentiate into dermal macrophages, monocyte-derived DC or remain as monocytes and traffic to LN. In the epidermis, a monocyte-derived progenitor differentiates into LC, via an EpCAM^{+}Langerin^{low} intermediate.

Specifically, we hypothesised that:

1. donor LC were transcriptionally distinct from recipient LC, owing to their distinct cellular origin;

2. blood monocytes were transcriptionally distinct from dermal monocytes, and both in turn were transcriptionally distinct from recipient and donor LC; and,

3. the putative LC precursor previously identified in the lab would have an intermediate transcriptional profile between blood monocytes and donor LC.

To test these hypotheses, we performed BMT with MataHari T cells and isolated the different populations by FACS (see Table 3.2). As Langerin is an intracellular protein, and therefore cannot be used to sort live cells, we used Langerin-DTR/EGFP (Lang-DTR) mice to label donor or recipient LC. In the first instance, we transplanted irradiated male CD45.1 recipients with congenic BM and polyclonal CD4^{+} T cells from Lang-DTR donors, along with MataHari CD8^{+} T cells. At 3 weeks post-transplant, we purified donor LC and the putative LC precursor population from the epidermis based on phenotype, congenic markers and EGFP expression, using the gating strategy shown in Figure 3.9A. Low expression of CD24 was used to distinguish LC precursors from CD24^{hi}EpCAM^{+}EGFP^{-} recipient LC, based on previous data from our lab showing that the precursor population do not upregulate CD24 (Bennett and Ferrer, unpublished). We additionally sorted CD11b^{+}CD115^{+}Ly6C^{hi} cells (activated monocytes) from the dermis (Fig. 3.9B) and Ly6C^{hi} monocytes from the blood (Fig. 3.9C). Conversely, to sort recipient LC, we transplanted irradiated male Lang-DTR recipients with wild type (WT) congenic BM and polyclonal CD4^{+} T cells, along with MataHari CD8^{+} T cells. At 3 weeks post-transplant, we purified recipient LC from the epidermis based on phenotype, congenic markers and EGFP expression. By isolating cells at the 3 week timepoint we were therefore able to investigate host and donor LC within the same epidermal environment. Additionally, donor LC were purified from mice 10 weeks post-transplant and untreated control LC were purified from age-matched (i.e. approximately 20 weeks old) untransplanted mice. All samples had a purity of at least 95%. RNA was extracted from the sorted samples and cDNA generated and sequenced by UCL Genomics.

Table 3.2 RNA sequencing samples

Sorted population	Timepoint post-alloBMT	Origin	Tissue
Donor LC	3 weeks 10 weeks	Donor	Epidermis
Recipient LC	3 weeks	Recipient	Epidermis
LC precursor	3 weeks	Donor	Epidermis
Activated dermal monocytes	3 weeks	Donor	Dermis
Blood monocytes	3 weeks	Donor	Blood
Untreated LC	3 weeks (age-matched) 10 weeks (age-matched)	Primary recipient (no irradidation).	Epidermis
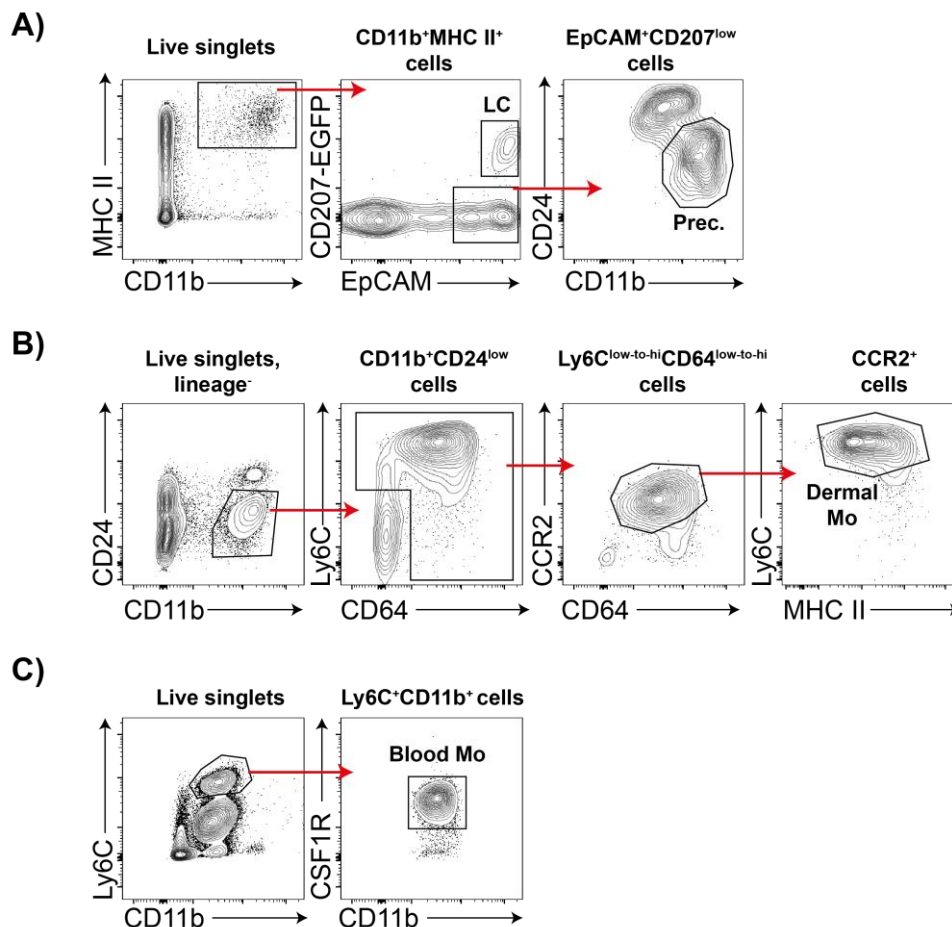
Figure 3.9 Gating strategies used to sort cell populations for subsequent RNA sequencing

(A) Epidermal gating strategy to sort LC and LC precursors (Prec.). **(B)** Dermal gating strategy to sort dermal monocytes (Mo). **(C)** Blood gating strategy to sort blood monocytes.

3.5.1.   Donor LC are transcriptionally similar to LC from untreated mice

Principal component analysis (PCA) of normalised expression revealed that blood monocytes and dermal monocytes clustered away from epidermal cells (Fig. 3.10A). LC precursors formed a distinct cluster, at in intermediary point between blood monocytes and the various LC populations. All LC clustered together along the horizontal axis (PC1,

comprising the largest fraction of sample variance), and away from LC precursors, suggesting similarity between host and donor EpCAM$^+$Langerin$^+$ cells collected at different timepoints. Individual LC samples were separated on the vertical axis (PC2), but did not segregate according to cell type, suggesting heterogeneity within replicates. However, donor LC at 3 and 10 weeks post-transplant clustered more closely with untreated LC than recipient LC. One possibility was that recipient LC were in the process of being eliminated by MataHari T cells 3 weeks post-transplant, and therefore expressed a distinct transcriptional signature based on activation of stress and cell death pathways. However, we could not detect the up-regulation of genes associate with cell death in these cells.

Hierarchical clustering also demonstrated that LC samples clustered together (Fig. 3.10B). The dendrogram showed two major branches, with donor, recipient and untreated LC forming sub-branches on one of the major branches, and blood monocytes, dermal monocytes and LC precursors forming sub-branches on the other major branch. The putative LC precursors clustered most closely with blood monocytes, in accordance with their monocytic origin.

A) PC2 (13.2% variance) vs PC1 (21.5% variance)

Legend:
- Blood monocyte
- Dermal monocyte
- LC Precursor — 3 weeks post-tx
- Donor LC
- Recipient LC
- Donor LC - 10 weeks post-tx
- Age-matched untreated LC (3 weeks)
- Age-matched untreated LC (10 weeks)

B) Dendrogram

C)

	BMo	DMo	Prec.	DLC
DMo	1706			
Prec.	1967	1751		
DLC	2578	3000	1702	
ULC	2690	2613	2215	597

D) Venn diagram

LC Precursor: 332
Untreated LC: 220
Donor LC: 206
30, 462, 113, 324

E)

LC Precursor

Gene	log₂(FC)	q
AA467197	9.73	4.58E-03
Cd63	8.50	2.78E-04
Epcam	7.91	2.78E-04
Krt14	7.83	2.78E-04
Cxcl16	7.81	2.78E-04
Cdh1	7.57	2.78E-04
Ly6d	7.10	5.26E-03
Ccl24	6.98	2.78E-04
C1qa	6.79	2.78E-04
Cd72	6.79	2.78E-04
Itgax	6.57	2.78E-04
Krt10	6.55	2.78E-04

Donor LC

Gene	log₂(FC)	q
Krt14	8.58	2.36E-04
Cd207	8.14	2.36E-04
Cldn1	8.00	2.36E-04
H2-M2	7.88	2.36E-04
Ly6d	7.80	2.27E-03
Lpar3	7.80	2.36E-04
Epcam	7.79	2.36E-04
Mmp9	7.79	2.36E-04
F830045P16Rik	7.76	2.36E-04
Hpgds	7.72	2.36E-04
Cdh1	7.69	2.36E-04
P2rx2	7.65	2.36E-04

Untreated LC

Gene	log₂(FC)	q
Krt14	10.68	2.51E-04
Ly6d	10.00	4.67E-03
Mmp9	8.08	2.51E-04
H2-M2	7.89	2.51E-04
Krt10	7.68	2.51E-04
Cd63	7.65	2.51E-04
Epcam	7.64	2.51E-04
P2rx2	7.62	2.51E-04
Ltc4s	7.62	2.51E-04
Lpar3	7.59	2.51E-04
F830045P16Rik	7.56	2.51E-04
Cd207	7.54	2.51E-04

Figure 3.10 LC precursors cluster between blood monocytes and LC, while donor and untreated LC are transcriptionally similar

(A) Principle component analysis (PCA) analysis of gene expression of the various sorted populations. **(B)** Dendrogram from unsupervised hierarchical clustering of the various sorted populations. **(C)** Pairwise differential expression analysis was performed between blood monocytes (BMo), dermal monocytes (DMo), LC precursors (Prec.), donor LC at 10 weeks post-transplant (DLC) and age-matched untreated LC (ULC). Green colouring indicates fewer differentially expressed genes while red colouring indicates more differentially expressed genes in a pair. Differentially expressed genes had fold change ≥ 2, q < 0.05. **(D)** Overlapping and differentially expressed genes that are significantly upregulated by LC precursors, donor LC at 10 weeks post-transplant and age-matched untreated LC, compared to blood monocytes (fold change ≥ 2, q < 0.05). **(E)** Top 12 overexpressed genes of LC precursors, donor LC at 10 weeks post-transplant and age-matched untreated LC compared to blood monocytes. The log₂(fold change) and q values are provided. N = 2-3, from independent experiments.

To gain insight into the biology of the different cell populations, we performed pairwise differential gene expression testing between blood monocytes, dermal monocytes, putative LC precursors, donor LC (10 weeks post-transplant) and age-matched untreated LC (Fig. 3.10C). Donor LC showed the greatest differential expression with dermal monocytes, with 3000 differentially expressed genes (fold change ≥ 2, q < 0.05). The top three differentially expressed genes upregulated by donor LC were *Epcam* ($\log_2$ fold change = 9.05, q = 0.0002), *Ptgs1* ($\log_2$ fold change = 8.59, q = 0.0002) and *H2-M2* ($\log_2$ fold change = 8.29, q = 0.0002). Blood monocytes were most similar to dermal monocytes, with 1706 differentially expressed genes, and least similar to untreated LC with 2690 differentially expressed genes. Our data demonstrated an increase in the number of differentially expressed genes along the LC differentiation pathway, with precursors differing from blood monocytes by 1967 genes, donor LC by 2578 genes and untreated LC by 2690. Donor LC differed the least from untreated LC, with 597 differentially expressed genes between the two populations.

We compared the genes upregulated by LC precursors, donor LC and untreated LC in comparison to blood monocytes and found a significant degree of overlap: 462 genes (27.4%) were commonly upregulated by all three subsets; 324 genes (19.2%) were upregulated by only untreated LC and donor LC; 206 (12.2%) genes were upregulated by only LC precursors and donor LC (Fig. 3.10D). Indeed, when we assessed the twelve most upregulated genes by each subset, *Epcam*, *Krt14*, *Ly6d* were common to all three lists (Fig. 3.10E). Donor LC and untreated LC shared nine of the twelve most upregulated genes in comparison to blood monocytes, including *Cd207 (Langerin)*, *H2-M2*, *Lpar3*, *Mmp9*, *F830045P16Rik* and *P2rx2*. The shared upregulation of Langerin was in accordance with the use of this marker to define LC. Intriguingly, LC precursors upregulated CD63, a quintessential marker of endosomes and antigen presentation in human LC, suggesting that LC precursors acquire some LC-like properties before expressing Langerin. Together, our data demonstrated increased differential gene expression during LC differentiation, resulting in donor LC that were transcriptionally similar to untreated LC. Therefore, these data suggested that, in contrast to the development of monocyte-derived microglia in the brain, the epidermal environment instructs the differentiation of monocytes into LC that are transcriptionally similar to the resident cells seeded before birth. The commonality of upregulated genes by LC precursors, untreated LC and donor LC in comparison to blood monocytes further provided strong evidence for EpCAM[+]Langerin^{low} cells being the true precursors for donor LC in this setting.

3.5.2. Entry of monocytes into the dermis or epidermis leads to activation of distinct biological pathways

To better understand the factor controlling differentiation of monocytes within the dermal and epidermal environments, we used an overrepresentation analysis to detect enriched biological pathways (Fig. 3.11A, B, C; Huang da et al., 2009). Initially, we analysed the 332 overexpressed genes that were specifically upregulated by LC precursors (but not by untreated LC or donor LC) in comparison to blood monocytes. This, however, did not generate any significantly enriched pathways, and so we continued our analysis on the total overexpressed genes compared to blood monocytes.

Genes over-expressed by LC precursors in comparison to blood monocytes were enriched for gene ontology (GO) pathway terms such as "DNA replication" (q = 6.35 x 10^{-11}), "Cell cycle" (q = 9.65 x 10^{-11}) and "Mismatch repair" (q = 3.64 x 10^{-8}). This suggested that LC precursors switched on a proliferative transcriptional program. The differentially expressed genes expressed by donor LC compared to blood monocytes were similarly enriched for proliferative GO pathway terms, in accordance with the high proportion of common upregulated genes with LC precursors. In addition, differentially expressed genes were enriched for GO pathway terms such as "Th1 and Th2 cell differentiation" (q = 1.25 x 10^{-2}) and "Th17 cell differentiation" (q = 3.60 x 10^{-2}), indicative of LC function in lymphocyte differentiation (Bittner-Eddy et al., 2016, Aliahmadi et al., 2009, Kim et al., 2016, Kobayashi et al., 2015, Igyarto et al., 2011), but also "Fatty acid metabolism" (q = 9.11 x 10^{-3}) and "Peroxisome" (q = 2.12 x 10^{-2}), pathways involved in LC homeostasis (Varga et al., 2011, Dubrac et al., 2007).

Differentially expressed genes of dermal Ly6C^{+}MHCII^{+} monocytes in comparison to blood monocytes were enriched for GO pathway terms such as "TLR signalling pathway" (q = 3.36 x 10^{-5}), "Phagosome" (q = 5.44 x 10^{-4}) and "Th1 and Th2 cell differentiation" (q = 6.24 x 10^{-3}). This was in accordance with their function as innate effector cells when recruited to inflamed sites (Shi and Pamer, 2011). Activated monocytes/moDC have been shown to directly control effector and memory T cell function in the skin and other mucosal sites (McLachlan et al., 2009, Wakim et al., 2008, Iijima et al., 2011). It would be interesting to further investigate whether dermal monocytes directly alter allogeneic dermal T cell function in our model.

It was striking how different the GO pathway terms were for the epidermal cells compared to the dermal cells. This analysis suggested that the basement membrane separated two very different immune environments: the proliferative epidermis with active cell cycle control and DNA damage repair, and the protective innate immunity environment of the dermis.

A) LC Precursor
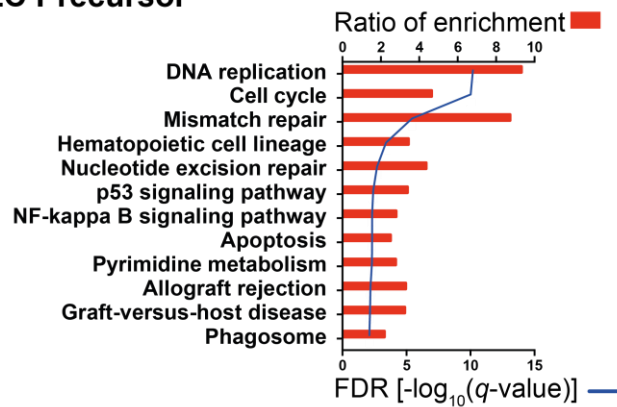
B) Donor LC
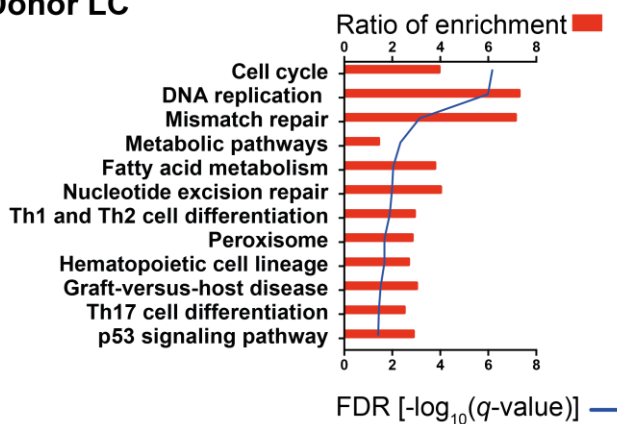
C) Dermal monocyte
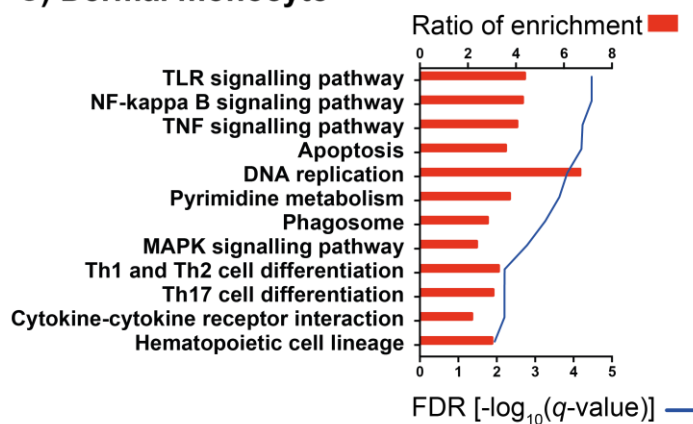
Figure 3.11 LC precursors switch on a proliferative program

Graphs showing the ratio of enrichment (bars) and FDR q values (line) for pathways predicted by WebGestalt to be overexpressed by LC precursors **(A)**, donor LC at 10 weeks post-transplant **(B)** and dermal monocytes **(C)** compared to blood monocytes. Overexpressed genes had fold change ≥ 2, $q < 0.05$.

3.5.3. Defining transcriptional regulators of LC differentiation after allogeneic BMT

The data presented from the over-representation analysis suggested that entry of monocytes into the epidermis and differentiation into LC was associated primarily with activation of cell cycle pathways. Differentiated LC are largely quiescent in the steady state due to TGF-β-dependent expression of Runx3 but may undergo low levels of proliferation to maintain cell number, or higher levels of proliferation under inflammatory

79

conditions (Ghigo et al., 2013, Chorro et al., 2009). Therefore, we further interrogated our transcriptional data to investigate the genes that control LC turnover in our GVHD model, and which were driving the dominant proliferative programme identified above. To this end, we designed a panel of genes that had previously been identified as cell cycle genes within a pro-proliferative gene network of the B cell lineage *in vivo* (Cohen et al., 2013). Monocytes and untreated LC expressed low levels of these genes, in agreement with the limited proliferation of these cells (Chorro et al., 2009, Patel et al., 2017). However, in support of the overrepresentation analysis, this signature was present in LC precursors but downregulated in donor LC (Fig. 3.12A).

A core panel of genes associated with self-renewal has previously been defined in BM macrophages (Soucie et al., 2016). Therefore, we hypothesised that differentiation of donor LC from precursors would be associated with expression of genes associated with this programme. Analysis of this gene set demonstrated that the expression of self-renewal genes was highly variable between cell types but that the genes expressed by LC were largely distinct from the genes expressed by blood monocytes (Fig. 3.12B). LC precursors expressed *Akt1, Brca1, Suz12, Myc, Terf1* and *Prune,* whereas donor LC expressed *Suz12*, *Nfya*, *Cebpz* and *Cited2*. Untreated LC expressed a broader set of self-renewal genes, including *Cited2*, *Asb6*, *Ddx18*, *Myc*, *Klf5*, *Nfyb*, *Terf1* and *Eed*. This analysis suggested the activation of a proliferative program in LC precursors, which was subsequently muted after differentiation into LC.

It has been proposed that migration of LC from the epidermis requires epithelial-to-mesenchymal transition (EMT), whereas the reverse, mesenchymal-to-epithelial transition (MET), is required for LC residency (Hieronymus et al., 2015). Therefore, we hypothesised that an EMT gene program would be switched on in LC precursors and subsequently downregulated on differentiation into mature LC (Capucha et al., 2018). Indeed, we detected expression of the EMT gene *Vim*, encoding vimentin in LC precursors, but not in donor LC (Fig. 3.12C). In contrast donor LC expressed *Zeb1*, which was not detected in LC precursors.

In summary, while the self-renewal capacity of LC and the requirement for ID2 is well established, the wider gene program that controls self-renewal has not been well defined. We assessed a self-renewal panel that was identified in BM macrophages and validated in peritoneal macrophages; the indication that the panel wasn't broadly expressed by untreated LC suggested that the core set of genes required for self-renewal in LC was distinct to the genes required in BM and peritoneal macrophages.

Despite this, it was clear that donor LC had a distinct gene expression profile to untreated LC. This hinted that donor LC were acquiring a self-renewal programme, but it was not fully established. Additionally, we should consider that repopulating cells may be more

heterogeneous than resident cells, with LC at different stages of their development and cells that have recently repopulated the epidermis. It is unknown whether all repopulating cells will persist as long-term LC, and therefore this heterogeneity could be reflected in the gene expression analysis.
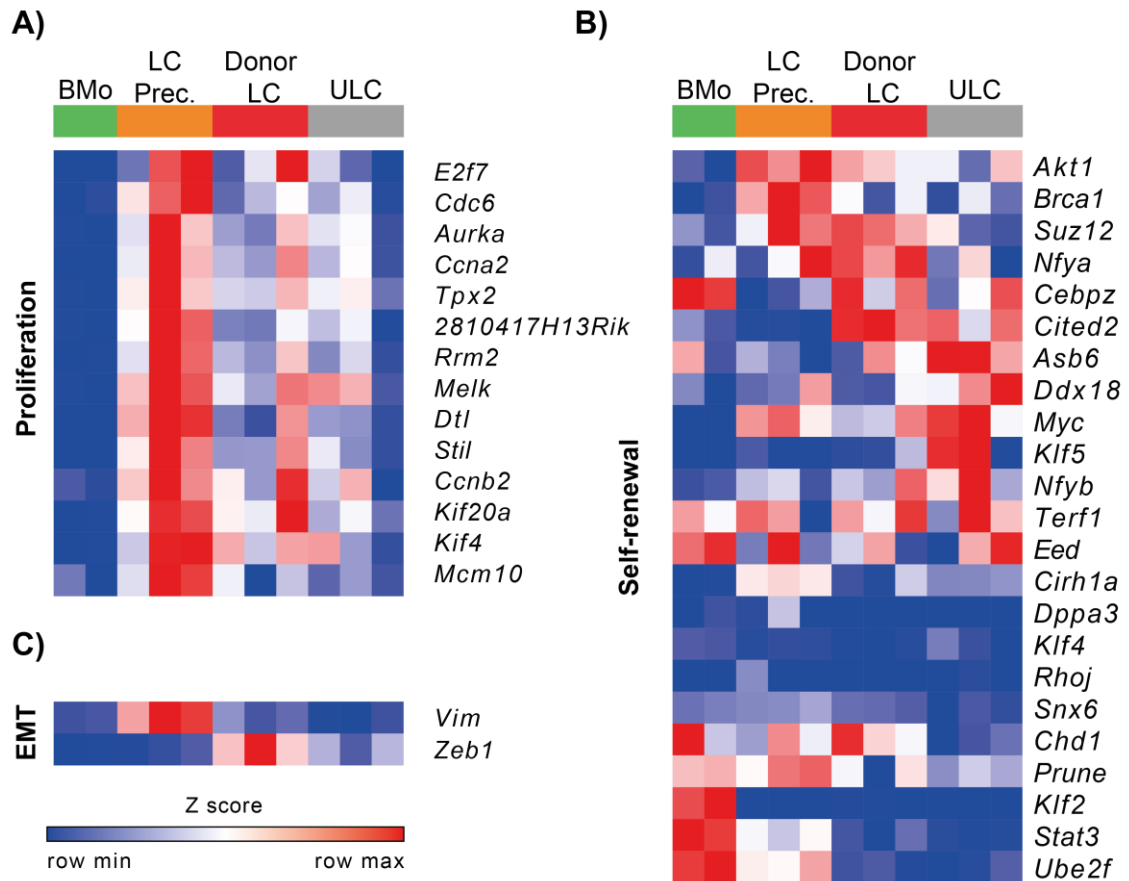
Figure 3.12 LC precursors switch on a proliferative program and donor LC acquire a self-renewal program

Heat maps of the relative expression of panels of genes associated with **(A)** proliferation, **(B)** self-renewal and **(C)** epithelial-to-mesenchymal transition by blood monocytes (BMo), LC precursors (Prec.), donor LC at 10 weeks post-transplant and age-matched untreated LC (ULC).

A core of essential transcription factors has been shown to be essential for the differentiation of quiescent, self-renewing LC in the epidermis, including PU.1 (*Spi1*), RUNX3, ID2 and CBF-β (*Cbfb*) (Chopin et al., 2013, Fainaru et al., 2004, Hacker et al., 2003, Sere et al., 2012, Tenno et al., 2017).

We analysed the relative expression of *Spi1*, *Runx3*, *Id2* and *Cbfb* by blood monocytes, LC precursors, donor LC at 10 weeks post-transplant and age-matched untreated LC (Fig. 3.13A). All subsets expressed similar levels of *Spi1,* in accordance with its role in myeloid cell differentiation. *Id2* and *Runx3* expression was upregulated in LC precursors over blood monocytes and was further upregulated by donor LC. *Id2* was expressed the

highest in untreated LC, whereas the expression of *Runx3* in untreated LC was similar to LC precursors. The expression profile of *Cbfb* mirrored that of *Runx3*. Together, our data provided evidence that, in the GVHD environment, LC precursors and donor LC were acquiring key transcription factors that are required for the development of long-term, persistent LC. Additionally, this suggests a previously unreported role for *Cbfb* in the development of BM-derived LC.

Next, we assessed the relative expression of key cytokine receptors reported to be involved in LC development. The development of embryonic LC and BM-derived LC is highly dependent on CSF1R signalling via binding of IL-34, and *Csf1R*-deficient mice lack LC (Dai et al., 2002, Ginhoux et al., 2006). *Csf1r* was similarly expressed by all subsets (Fig. 3.13B), in accordance with the requirement for CSF1R signalling by mononuclear phagocytes (Hume and MacDonald, 2012, Nakamichi et al., 2013).

BMP7 has been identified as an instructive factor for human embryonic LC generation and binds to its receptor BMPR1A (Yasmin et al., 2013). Similar to *Id2* expression, we detected progressive upregulation of *Bmpr1a* expression along the differentiation pathway of blood monocytes to LC precursors and subsequently to donor LC (Fig. 3.13B). Untreated LC had the highest expression of *Bmpr1a*. This provided strong evidence that BMP7 is instructive for BM-derived LC development.

TGF-β1 produced by both LC and keratinocytes has been shown to be essential for LC homeostasis by maintaining immature LC in the epidermis (Kaplan et al., 2007, Kel et al., 2010, Borkowski et al., 1996). However, TGF-β1 signalling is not critical for LC development, suggesting that functional responsiveness to TGF-β1 is acquired on differentiation to mature LC. We therefore assessed the relative expression of genes that indicated responsiveness to TGF-β in the epidermal environment (Fig. 3.13C). Our data revealed high *Tgfbr1* expression by LC precursors, relative to the other cell types. Blood monocytes, LC precursors and donor LC expressed similar levels of *Tgfbr2*, upregulated in comparison to untreated LC. *Smad7* was overexpressed by LC precursors, donor LC and untreated LC compared to blood monocytes, and similarly, expression by untreated LC was lower compared to LC precursors and donor LC. Together, these data indicated that LC precursors were signalling via TGF-βR1 and TGF-βR2, the former being potentially specific to precursor differentiation. Surprisingly, TGF-β signalling was dominant in the precursors rather than the mature cells, suggesting that TGF-β responsiveness was acquired prior to LC differentiation.

The vitamin D_3 receptor (VDR) has been reported to be induced by TGF-β1 during LC lineage commitment and repressed during IL-4-dependent moDC differentiation (Gobel et al., 2009). Additionally, the VDR regulates the tissue resident macrophage response to injury (Song et al., 2016). In accordance with these data, we detected high relative

expression of *Vdr* by LC precursors, with subsequent downregulation on differentiation into mature LC (Fig. 3.13D). This suggested that vitamin D_3 signalling was induced during lineage commitment but was repressed during differentiation. Indeed, vitamin D_3 has a negative effect on LC antigen presentation (Dam et al., 1996); repression may be required to facilitate LC function.

Finally, we assessed expression of other genes that have been implicated in LC differentiation or maintenance (Fig. 3.13D). The late endosomal/lysosomal adaptor and mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) activator/regulator complex 2 (LAMTOR2) have been reported to regulate TGF-β1-mediated homeostasis of LC (Sparber et al., 2014, Sparber et al., 2015). Our data demonstrated that blood monocytes expressed *Lamtor2* the highest, and that *Lamtor2* expression progressively increased from LC precursors to donor LC and subsequently to untreated LC. This suggested that LC precursors and donor LC were acquiring key transcription factors that are required for the maintenance of persistent LC.

Interferon regulatory factor 8 (IRF8) is required for MDP to CDP and cMoP to monocyte transitions (Kurotaki et al., 2014, Sichien et al., 2016). Although once implicated in the development and migration of LC (Schiavoni et al., 2004), several studies have reported that LC generation is largely independent of IRF8 (Hashimoto et al., 2011, Chopin et al., 2013). We detected an inverse expression profile of *Irf8* to *Id2* and *Bmpr1a*, with more expression by blood monocytes and stepwise reduction of expression through LC precursors, donor LC and the least expression by untreated LC (Fig. 3.13D). These data supported IRF8-independent generation of embryonic and BM-derived LC.

KLF4 is a monocyte/macrophage lineage identity transcription factor reported to be inhibited during monocyte-derived LC differentiation (Jurkin et al., 2017). In agreement, we demonstrated that *Klf4* was downregulated in LC precursors and donor LC compared to blood monocytes (Fig. 3.13D). *Klf4* expression by untreated LC was similar to expression by blood monocytes, in accordance with no reported role for *Klf4* in embryonic LC development.

In summary, we have revealed that LC precursors and donor LC were switching on genes essential for long-term LC development and maintenance (*Id2*, *Runx3* and *Cbfb*, *Bmpr1a, Smad7* and *Lamtor2*), and were repressing negative regulators of LC differentiation (*Klf4*). These data strongly suggested unreported roles for BMPR1A and CBFβ in the generation of repopulating LC after injury.
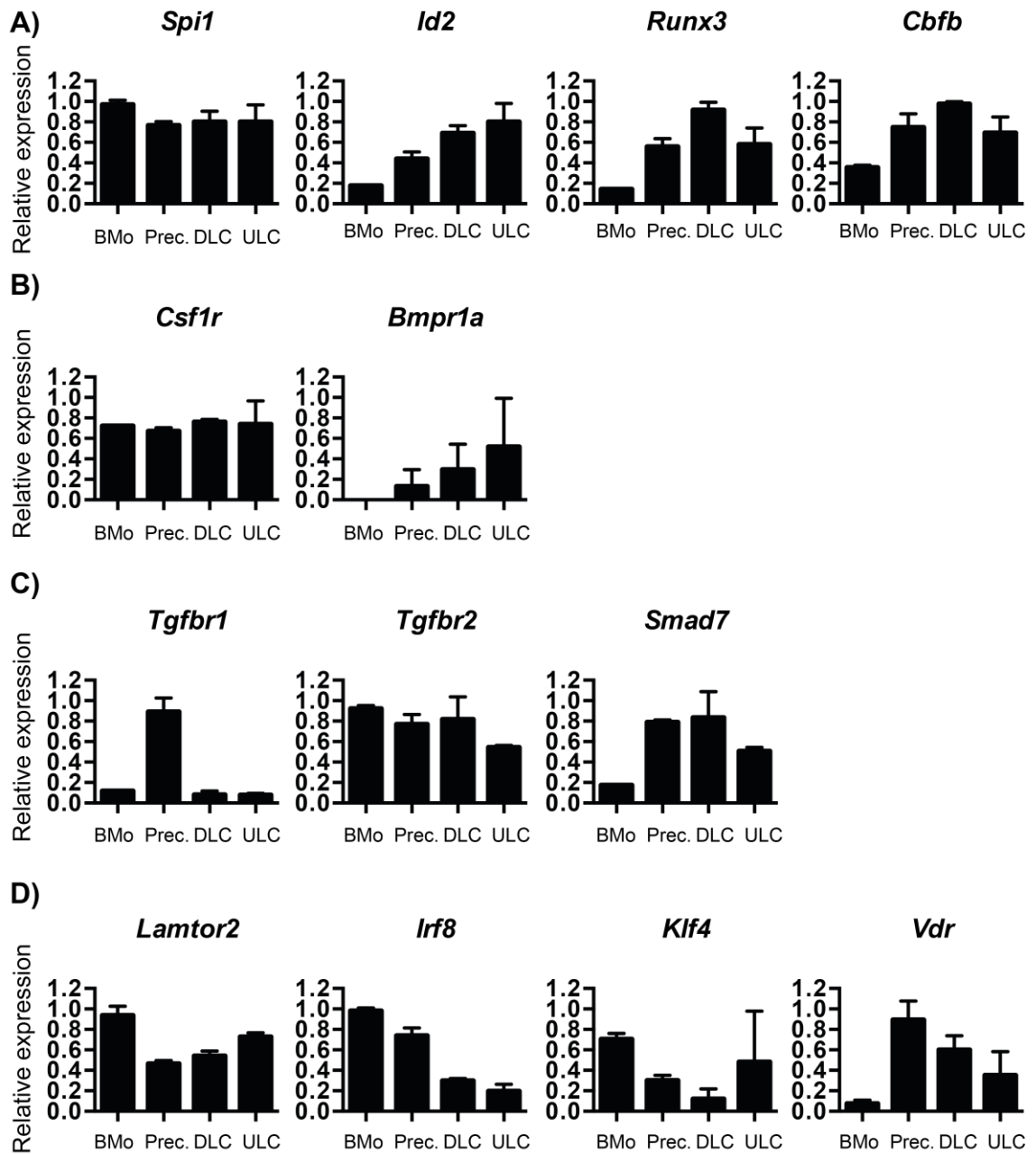
Figure 3.13 LC precursors and donor LC acquire gene expression critical for long-term LC development and maintenance

RNA sequencing gene expression profiles of blood monocytes (BMo), LC precursors (Prec.), donor LC at 10 weeks post-transplant (DLC) and 10 week age-matched untreated LC (ULC). **(A)** Transcription factors and **(B)** cytokine receptors involved in LC development. **(C)** TGF-β signalling genes involved in LC homeostasis. **(D)** Genes implicated in LC development. The y axis corresponds to the relative gene expression normalised to the maximal value observed in the analysed samples. Data are shown as mean ± SD, N = 2-3.

3.5.4. Distinct transcriptional programs are switched on during monocyte differentiation

The previous analyses provided insight into the differentiation of myeloid cells as they entered the skin after BMT. To investigate potential functional differences between the cells that could be tested in further experiments, we analysed the expression of defined

panels of genes that were related to distinct functions (Fig. 3.14): antigen uptake, antigen presentation, chemokines, cytokines, phagocytosis, extravasation and matrix metalloproteases (MMP; Schridde et al., 2017).

Blood monocytes displayed a strong antigen uptake and extravasation signature. *Cd1d1* (involved in lipid antigen presentation), *B2m* (a component of MHC I) and *Tapbp* (TAP binding protein, involved in peptide loading of MHC I), genes involved in antigen presentation, were also expressed. Negligible chemokine expression was detected, but we did detect expression of *Csf1*, *Il16*, *Flt3l* and *Tgfb1*.

LC precursors displayed a dominant antigen presentation signature, with expression of *B2m*, *Tapbp*, *Cd40*, *Cd80*, *Fcgrt*, *CD86* and *CD74*. We detected expression of *Ccl17*, a chemokine implicated in the process of establishing inflammatory infiltrate (Goebeler et al., 2001, Martin et al., 2003, Onoue et al., 2009, Riis et al., 2011), and *Ccl5*, a T cell and monocyte chemoattractant but additionally implicated in LC migration into the epidermis (Schall, 1991, Ouwehand et al., 2012, Ouwehand et al., 2010). A phagocytosis signature was also detected, including *Axl*, a downstream effector of TGF-β during LC differentiation and homeostasis (Bauer et al., 2012), *Cd9*, a tetraspanin involved in the regulation of cell adhesion, and *Itgb5* and *Itgav*, together encoding integrin αvβ5, expressed at low levels in resting epidermis and induced during wound healing and in other pathologies (Watt, 2002, Margadant et al., 2010, Jones et al., 1997). Compared to other cell types, LC precursors expressed a number of metalloproteinases (*Mmp12*, *Mmp13*, *Mmp14* and *Mmp25*). LC precursors expressed the proinflammatory cytokines *Il12b*, a subunit of IL-23 that has been implicated in organ-specific pathology in GVHD (Das et al., 2009), and *Mif*, encoding macrophage migratory inhibitory factor (MIF) where local and systemic expression is associated with the occurrence of acute GVHD (Lo et al., 2002).

Donor LC did not express clear gene signatures associated with chemokines, cytokines or extravasation, according to the panels tested. The antigen presentation genes *CD86*, *CD74* and *H2-DMa* (MHC II) were expressed by donor LC, as was *Stk4*, a gene involved in the inhibition of Th17 differentiation but also implicated in trafficking of LC to LN (Katagiri et al., 2009). Similar to LC precursors, we detected expression of *Cd9*. High relative expression of *Mmp9* was detected, which has previously been shown to be required for egress of LC out of the epidermis (Kobayashi, 1997, Ratzinger et al., 2002).

Dermal monocytes expressed a broad profile of chemokines, including *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8*, *Ccl11*, *Ccl12* and *Ccl19*, and a broad panel of cytokines, including *Flt3l*, *Tgfb1*, *Tgfb3*, *Cxcl1*, *Cxcl2*, *Cxcl10*, *Cxcl12*, *Il10*, *Il6* and *Tnf*. IL-6 and TNF-α are key pro-inflammatory cytokines in the pathogenesis of GVHD and suggested that the dermis was an inflammatory environment. A phagocytosis signature was detected, with

expression of *Cd81*, *Gas6*, *Mertk* and *Mrc1*. Dermal monocytes expressed *Mmp2,* an MMP that has been reported to be absent in healthy skin and elevated after skin injury (Jansen et al., 2007).

In summary, these analyses suggested that, in the context of GVHD, blood monocytes were poised for antigen presentation and entry into the dermis. The broad chemokine and cytokine expression by dermal monocytes suggested these cells were contributing to the inflammatory GVHD environment and, together with *Mmp2* expression, indicated tissue injury. Upon entry into the epidermis, putative LC precursors appeared to express genes that were consistent with preparation for tissue remodelling and residency in the epidermis. Maturation into donor LC was associated with an increase in the relative expression of genes associated with antigen presentation to T cells. Whilst gene expression analysis is by no means definitive, it provides direction for future mechanistic work. In the next chapter, we tested specific functions of cutaneous myeloid cells.
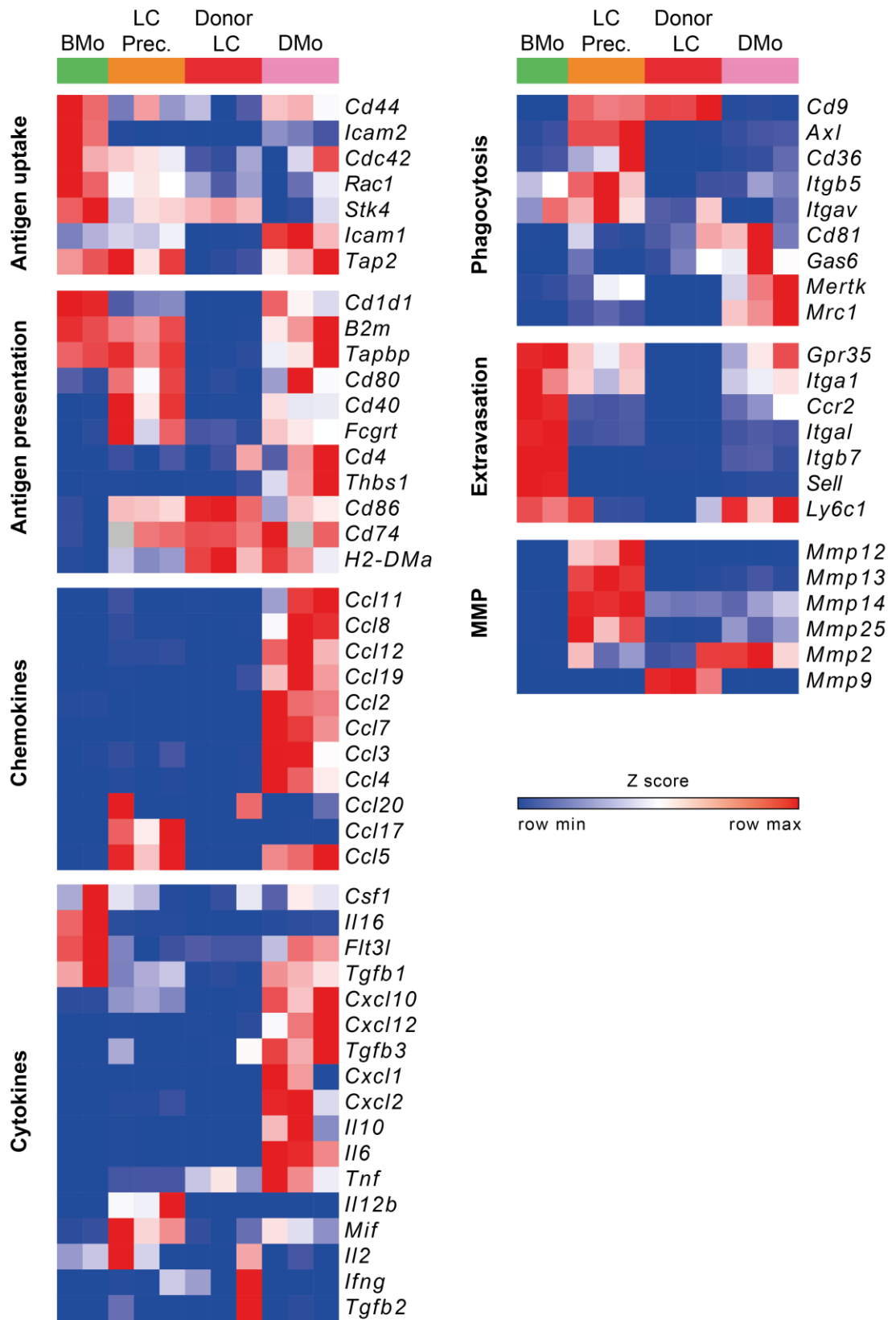
Figure 3.14 Blood monocytes, LC precursors, donor LC and dermal monocytes are transcriptionally distinct

Heat maps of the relative expression of panels of genes associated with antigen uptake, antigen presentation, chemokines, cytokines, phagocytosis, extravasation and matrix metalloproteases (MMP) by blood monocytes (BMo), LC precursors (Prec.), donor LC at 10 weeks post-transplant and dermal monocytes (DMo).

3.6.   Summary

This work aimed to characterise the changes to various myeloid cell populations in the skin after BMT and T cell-mediated pathology in our murine model of acute GVHD. We combined flow cytometric analysis of immune cell populations with transcriptional profiling to determine the long-term consequences of acute GVHD on the myeloid compartment.

Firstly, we demonstrated that irradiation and BMT in the absence of T cells had long-lasting effects on the cellular composition of the dermis. These data highlighted how irradiation can drastically impact the skin, and potentially other tissues. This is of particular importance as congenic BMT chimeras are regularly used as controls in experimental models and the impact of irradiation is often ignored.

Secondly, our data revealed that T cell mediated pathology led to continued recruitment of monocytes into the dermis that acquired a transcriptional profile consistent with innate immune function and production of IL-6.

Finally, we demonstrated that destruction of the LC network by T cells led to recruitment of monocytes that appeared to acquire the ability to proliferate before differentiating into repopulating LC. Transcriptional profiling indicated that repopulating cells were acquiring a gene program consistent with long-term, functional LC.

<u>Dermis</u>

We detected ongoing infiltration of monocytes into the dermis for at least 10 weeks following BMT, irrespective of T cells. Given their $Ly6C^{hi}$ phenotype, it could be assumed that these monocytes were inflammatory. Further phenotyping is required, and fate mapping of these cells would be useful, although there are no published $Ly6C^{+}$ monocyte-specific reporter mice. Other groups have utilised *Zbtb46*-GFP mice to trace DC, LC and moDC (Wu et al., 2016) and *Cx3cr1*-Cre mice to trace tissue resident macrophages, but no group has been able to faithfully trace the differentiation of inflammatory monocytes into multiple cell types (e.g. pro-inflammatory macrophages, anti-inflammatory macrophages, moDC and LC).

From our data it was unclear whether monocytes were entering the dermis and undergoing apoptosis, exiting the dermis or differentiating into other dermal cells. A significant population of $CCR2^{-}Ly6C^{hi}$ cells were excluded from our analyses, as in a previous report identifying similar cells (Terhorst et al., 2015). It is unclear what these cells were, and on investigation, we could not find any reports that have described this cell type. However, one group have described *in situ* reprogramming of inflammatory $CCR2^{hi}CX_3CR1^{low}$ monocytes into $CCR2^{low}CX_3CR1^{hi}$ monocytes in the liver, with a

progressive reduction of Ly6C staining over time (Dal-Secco et al., 2015). It is possible that we are capturing an intermediate population of cells that are differentiating from classical CCR2$^+$Ly6C^{hi} monocytes into CCR2$^-$Ly6C^{lo} macrophages; further cell tracing experiments are required to confirm this. Alternatively, CCR2 staining may be less accurate in conditions of inflammation (we used an antibody with the same clone as Terhorst et al.). In this case, we could be underestimating the P1-P3 populations after BMT. Testing different CCR2 antibodies after BMT would resolve this.

Our transcriptional analyses of dermal monocytes in the GVHD environment indicated that, on entry to the dermis, these cells became efficient APC and phagocytes, capable of interacting with T cells and influencing immune responses through cytokine and chemokine production. Our data implicated these cells as drivers of GVHD pathology, potentially through the production of IL-6. Further experiments are required to define the signal (e.g. DAMP) that is driving IL-6 production in GVHD dermis.

In any case, transcriptional profiling revealed that dermal monocytes were distinct to LC precursors and were unlikely to represent an intermediate in the LC differentiation pathway. However, our data has highlighted a plasticity of the myeloid compartment which could facilitate monocytes to adopt a cell fate dependent on the microenvironment. Lineage-tracing of cells using reporter mice (e.g. *Cx3cr1*-cre) and intradermal injection of purified cell populations could determine whether dermal monocytes have differential plasticity to differentiate into LC.

We noted several other changes in the dermis as a result of irradiation and BMT, including reduction of $\gamma\delta$ T cells and ILC2. Previous reports have suggested that ILC2 are radiosensitive and can be reconstituted postnatally after HSCT (Vely et al., 2016). Contrary to this, our data suggested that ILC2 were ablated during BMT but were not reconstituted in the skin. Dermal ILC2 are likely a recirculating population, since ILC2 are detected in the circulation (Salimi et al., 2013, Teunissen et al., 2014) and are increased in number in skin-draining LN during cutaneous inflammation (Salimi et al., 2013, Imai et al., 2013, Kim et al., 2013). We need to identify whether ILC2 were reconstituted in other tissues following BMT, as this would indicate that repopulation was specifically blocked in the skin. Nevertheless, ILC2 are a major source of IL-13 in the skin in the steady state (Roediger et al., 2013) and are involved in the initiation of type 2 immune responses and wound healing (Salimi et al., 2013, Kim et al., 2013, Imai et al., 2013, Roediger et al., 2013, Rak et al., 2016). Future work could investigate the functional consequences of loss of ILC2 on wound healing after BMT. Indeed, chronic GVHD patients can suffer from skin ulcers (Jachiet et al., 2014).

Cotransfer of allogeneic MataHari T cells led to the loss of CD11b$^+$ and CD103$^+$ cDC from the dermis. The long-term loss of these cells suggested that DC precursors were

either not recruited to the skin, did not differentiate into cDC, or did not survive in the GVHD environment. Interestingly, DN cDC were sensitive to irradiation and were reduced in both transplanted groups. This highlighted the undefined nature of this population and could point towards a different precursor. Future experiments are required to delineate the cause of cDC loss from the dermis during GVHD, possibly by lineage tracing of CDP-derived cells using *Clec9a*-reporter mice (Schraml et al., 2013).

The loss of cDC populations following GVHD is likely to result in functional consequences, given that dermal cDC are essential in patrolling the skin barrier environment, taking up antigen and migrating to the LN where they prime lymphocyte responses (Worbs et al., 2017). These responses can be immunostimulatory (e.g. in response to pathogen) or immunoregulatory (e.g. in response to self-antigen or harmless foreign antigen). Chow et al. described a division of labour where moDC predominantly promote Th polarisation and cDC predominantly promote Th proliferation and act as regulators. (Chow et al., 2016). Thus, after GVHD, T cell priming in the LN may be reduced whereas T cell polarisation in the dermis may be increased, leading to dysregulated immune responses. CD11b$^+$ dermal DC are important drivers of Th2-mediated immunity after skin immunisation, whereas CD103$^+$ DC are critical for cross-presentation of antigen to initiate anti-viral and anti-fungal immune responses (Stoecklinger et al., 2011, Brewig et al., 2009, Bedoui et al., 2009, Igyarto et al., 2011, Seneschal et al., 2014). Future work could employ various pathogen challenge models (e.g. *Leishmania*, *Candida*) to assess the consequences of loss of dermal cDC after allo-BMT.

Epidermis

Killing of LC by MataHari T cells initiated the recruitment of BM-derived cells which repopulated the epidermis in our model. BMT, irrespective of T cells, led to a reduction in number of LC, suggesting resident cells were lost after irradiation and were slow to recover. Irradiation may cause long-term damage to the extrinsic environment that could prevent the differentiation of LC. Alternatively, given the quiescent phenotype of mature LC (Merad et al., 2002, Vishwanath et al., 2006), the delay in repopulation could simply reflect the slow turnover of LC. LC turnover has been elegantly studied by Ghigo et al., who used a multicolour fate mapping mouse model to study repopulation and proliferation under homeostasis and inflammation (Ghigo et al., 2013). We propose using a similar model and analysing LC repopulation through a combination of confocal microscopy and EdU incorporation by flow cytometry. This would allow us to study recipient and donor LC division and determine whether turnover was impaired in our model. In any case, loss of LC following BMT could have a range of functional consequences, given these cells perform many functions within the skin. In the clinic, it

is unclear whether patients are more susceptible to cutaneous infections as these patients are treated with steroids and prophylactic antibiotics. However, there is evidence to suggest that viral and fungal infections affecting the skin are common in the late recovery period (from day 100) following HSCT (Park et al., 2006). In the following chapter, we began to test LC function following BMT.

Screening the cytokine profile of the epidermis indicated active recruitment of T cells through the CXCL10-CXCR3 axis, shown previously to play an important role in the pathogenesis of cutaneous acute GVHD and vitiligo (Rashighi et al., 2014, Piper et al., 2007). T cells could also be recruited to the epidermis through the CCL21-CCR7 axis; however, we postulate that CCL21 expression in the epidermis could impair LC and DC emigration as this mechanism is reliant on a positive CCL21 gradient from skin to lymphatics. Thorough assessment of LC and DC migration (e.g. in response to a stimulus or direct *ex vivo* emigration) is required.

The proteomic data further indicated that EMT regulators (HGF, Pentraxin 3) were active in GVHD epidermis. HGF is the exclusive ligand of the transmembrane tyrosine kinase receptor Met which is expressed by LC and other APC subsets (Hieronymus et al., 2015, Hubel and Hieronymus, 2015). The HGF-Met signalling pathway has been shown to be indispensable in skin wound healing (Chmielowiec et al., 2007, Xu et al., 2011, Singh et al., 2012, Miura et al., 2017) and is also a key mechanism of LC emigration from skin (Kurz et al., 2002, Baek et al., 2012). Pentraxin 3 is a pattern recognition molecule that plays a non-redundant role in the orchestration of tissue repair and remodelling (Doni et al., 2015). It therefore seems apparent that a genetic program similar to EMT is switched on following GVHD-induced skin injury to activate repair (Hieronymus et al., 2015, Sagi and Hieronymus, 2018). Whether mechanisms of EMT (LC migration, tissue repair) and MET (LC precursor migration into the epidermis) can effectively occur simultaneously in the skin remains to be addressed.

The epidermal proteomic screen additionally suggested the recruitment of neutrophils to the GVHD epidermis. Although we did not detect an infiltration of neutrophils at 10 weeks post-transplant, we suggest assessing an earlier time point after BMT as neutrophil infiltration is an innate and early response. Neutrophil-derived CSF-1 has been reported to be required for LC repopulation (Wang and Colonna, 2014); we therefore propose a thorough assessment of neutrophil numbers and CSF-1 production between 1 and 3 weeks post-transplant.

The overexpression of osteoprotegerin (OPG) in GVHD epidermis was notable because OPG is a soluble decoy receptor for receptor activator of nuclear factor-κB ligand (RANKL). In the skin, signalling through the RANK-RANKL axis is crucial for the peripheral expansion of Treg (Loser et al., 2006), and therefore overexpression of OPG

could limit cutaneous Treg responses. We hypothesise that induction of cutaneous tolerance and immunosuppression following inflammation in GVHD mice is impaired. Indeed, levels of circulating Treg are inversely correlated with acute or chronic GVHD (Zorn et al., 2005, Rieger et al., 2006, Rezvani et al., 2006, Miura et al., 2004) and there is evidence that Treg suppressive function is impaired in acute GVHD (Ukena et al., 2011). In the next chapter, we tested the induction of cutaneous tolerance in our model.

Our transcriptomics approach has allowed us to infer information about the differentiation of myeloid cells during GVHD. Our data indicated that long-term LC, as described by Sere et al., were generated in our model, suggesting that environment was dominant over cellular origin in determining cell fate, as has been postulated for other tissue macrophage populations (Mass, 2018, Scott et al., 2018, Lavin et al., 2014).

Furthermore, genes essential for long-term LC were being switched on in LC precursors in the GVHD environment. Entry into the epidermis appeared to instruct LC precursors to model the tissue and become responsive to TGF-β. Although TGF-β is not required for LC development, our data suggested that precursors were signalling via TGF-βR1. Binding studies would be useful to address whether TGF-β, or another cytokine, was binding to this receptor. For instance, BMP7 could be signalling through TGF-βR1, mediated by the coreceptor endoglin that we found to be upregulated in our model (Barbara et al., 1999). We have begun to test whether TGFβR1 signalling is critical in LC differentiation by inhibiting TGF-βRI signalling using the potent and selective inhibitor of TGF-βRI, SB 431542. So far, however, experiments have not been successful, owing to the short half-life of this drug and lack of experiment optimisation.

LC precursors acquired a repair profile that could be linked to radioresistance. LC radioresistance is dependent upon rapid repair mechanisms post-irradiation, particularly through intrinsic expression of cyclin-dependent kinase inhibitor 1A (CDKN1A; Price et al., 2015). Price et al. suggested that monocyte-derived LC expressed lower levels of *Cdkn1a* and exhibited higher radiosensitivity compared to embryonic LC. However, the authors were identifying monocyte-derived LC as MHC II+CD11b+EpCAM- cells, or short-term LC, as described by Sere et al. In contrast, our data suggested that LC precursors were differentiating into long-term LC. It could be possible that a repair program is switched on concurrently with a proliferative program as mutations can accumulate in actively dividing cells.

While the overexpression of the keratinocyte markers *Krt14* and *Ly6d* by LC precursors could be viewed as contamination, recent research has suggested that LC are able to acquire keratinocyte markers to prevent autoimmunity (Kitashima et al., 2018), suggesting that precursors have acquired this function before differentiation into LC. This could indicate that LC precursors are programmed for immunological tolerance

before differentiation and this program could potentially be switched on by epidermal entry.

The study of LC turnover in humans following GVHD and other diseases is more complicated for the obvious reasons, and further work is required in this field. BMT in humans also results in replacement by donor cells, but even after nonmyeloablative treatment and in the absence of overt GVHD (Collin et al., 2006, Mielcarek et al., 2014). There is currently no evidence to suggest that repopulating human LC are monocyte-derived, although a current hypothesis in the field is that LC originate from both monocytes and CD1c$^+$ DC, potentially paralleling the two wave repopulation reported in mouse models (Collin and Milne, 2016). In contrast, activated monocytes have been described in psoriasis patients and are referred to as "epidermal DC" (Martini et al., 2017). This is of particular interest as the precursors we observe in our model could persist as activated monocyte-derived cells and contribute to pathology, without differentiating into LC.

In summary, we have identified lasting changes to the cutaneous immune environment after BMT and have utilised transcriptional analyses of cells to infer information about myeloid cell differentiation following injury. This work depended heavily on the use of relative gene expression and therefore protein expression needs to be validated. It is also important to identify whether these changes were biologically significant; therefore, in the following chapter, we tested the functional implications of these changes following GVHD.

Chapter 4 Testing cutaneous immune functions following GVHD

The skin is a tightly regulated environment in order to maintain a default setting of immunological tolerance with the ability to induce protective immunity. Cutaneous GVHD significantly disrupts the balance of myeloid cells in the skin: in the epidermis, LC are replaced by monocyte-derived cells which do not recover to pre-transplant numbers, while in the dermis, ILC2, γδ T cells, CD11b$^+$ DC and DN DC are significantly reduced and the myeloid compartment is dominated by an ongoing influx of monocytes.

We hypothesised that the significant disruption to the cellular populations of the skin would alter the functions of LC and DC in priming protective immune responses and cutaneous tolerance.

Therefore, in this chapter, we aimed to test:

1. the induction of tolerance against hapten-induced contact hypersensitivity (CHS);
2. the humoral immune response following epicutaneous immunisation with a protein antigen; and
3. the protective immune response against the fungal pathogen *Candida albicans* in a skin infection model.

CHS mouse models are used to study the pathophysiology of allergic contact dermatitis, one of the most common skin diseases affecting 15-20% of the population (Peiser et al., 2012). CHS to epicutaneously-applied hapten is an established assay that has been used for decades to additionally investigate cutaneous adaptive immune responses. In this assay, mice are sensitised by hapten application to shaved abdominal skin and are then challenged 5 days later by application of the same hapten onto the ear. The induction of CHS critically depends on the activation of hapten-specific naïve T cells in skin draining LN during sensitisation, which then proliferate and differentiate into cytotoxic T cells that mediate a transient ear swelling reaction at the time of challenge (Akiba et al., 2002). Hapten-specific CD4$^+$ T cells are also generated which regulate the cytotoxic response. The ear swelling that develops quantifies the degree of inflammation and measures the extent of hapten-specific cytotoxic T cell responses (Grabbe and Schwarz, 1998). Prior application of a modified hapten leads to activation of Treg and deletion of cytotoxic T cells, suppressing the ear swelling response and leading to induction of tolerance to the sensitising hapten.

Comprehensive analyses of the roles of DC and LC in CHS, using independently generated transgenic mouse lines from which Langerin+ cells were inducibly deleted *in vivo*, revealed that LC were required for efficient induction of CHS at low hapten doses, however at higher hapten doses dermal DC were sufficient, indicating functional redundancy of the different skin APC subsets in the elicitation of CHS (Bursch et al., 2007, Noordegraaf et al., 2010, Romani et al., 2010, Clausen and Kel, 2010, Honda et al., 2010, Kaplan et al., 2008).

Intriguingly, human Langerin-DTA BAC transgenic mice, which constitutively lack LC throughout life, mounted enhanced ear swelling responses (Kaplan et al., 2005), suggesting a regulatory role for LC in CHS. Indeed, LC have been demonstrated to mediate tolerance in both Lang-DTR and Lang-DTA models through the induction of regulatory T cells (Gomez de Aguero et al., 2012, Strandt et al., 2017, Kautz-Neu et al., 2011, Yoshiki et al., 2009), IL-10 secretion (Igyarto et al., 2009, Yoshiki et al., 2009), incomplete maturation (Shklovskaya et al., 2011) and the deletion or anergy of effector T cells (Gomez de Aguero et al., 2012).

Skin APC are also important in the induction of humoral immunity. After gene gun immunisation, CD103+ dermal DC were required for optimal antibody switching to IgG2a/c and IgG2b isotypes, whereas LC facilitated generation of IgG1 (Nagao et al., 2009). Further studies reported that LC extend their dendrites through the tight junctions to the stratum corneum and could present acquired antigen in the LN to induce IgG1 responses in a model of staphylococcal scalded skin syndrome (Kubo et al., 2009, Ouchi et al., 2011). LC were later revealed to be critical for the development of Tfh cells and germinal centres (Zimara et al., 2014, Yao et al., 2015, Levin et al., 2017). Levin et al. reported that depletion of LC resulted in a partial loss of the Tfh cell and germinal centre B cell responses, whereas these responses were completely abolished when all skin LC/DC were depleted (Levin et al., 2017). This suggested that another skin migratory DC subset were involved in the induction of humoral immune responses and would explain the previously reported role of CD103+ dermal DC for optimal antibody formation (Nagao et al., 2009).

The localisation of skin APC at the skin barrier makes them the first APC to encounter foreign antigens and are thus key players in cutaneous pathogen defence. Several studies have suggested that skin APC subsets promote distinct immune responses: CD103+ dermal DC, and not LC were required for CD8+ T cell-mediated immune responses to skin scarification with vaccinia virus (Seneschal et al., 2014), whereas in a model of *Candida albicans* skin infection, LC were necessary and sufficient for a Th17 response while CD103+ dermal DC were required for the generation of a Th1 response (Igyarto et al., 2011). It was later revealed that the LC-mediated response to *C. albicans*

was dependent on dectin-1 engagement of the yeast form of *C. albicans* and subsequent production of IL-6 by LC (Kashem et al., 2015).

Given the concerted roles of LC and dermal DC in the protective immune response towards *C. albicans*, and our observations that LC and dermal DC were disrupted following GVHD, we utilised the *C. albicans* skin infection model established by Igyarto et al. (Igyarto et al., 2011) to preliminarily investigate potential changes to the immune response following GVHD.

We hypothesised that, following GVHD:

1. mice cannot establish hapten-mediated tolerance;
2. mice have a defective humoral immune response to epicutaneous immunisation with protein antigen;
3. mice have a defective response to cutaneous challenge with *Candida albicans*;
4. defective responses are a result of loss of priming of cutaneous APC;
5. defective responses are a result of upregulated dermal IL-6.

Therefore, we aimed to:

1. assess cutaneous function using the above-mentioned tests;
2. assess priming of immune responses by migratory skin APC; and
3. identify a potential mechanism of loss of function.

4.1. Loss of hapten-induced tolerance after GVHD

Given the critical role of LC in DNTB-mediated cutaneous tolerance (Gomez de Aguero et al., 2012), and our observation that recovery of the LC network following GVHD was incomplete, we utilised a CHS model to investigate potential changes to the induction of cutaneous tolerance following GVHD (Fig. 4.1A). Briefly, mice were sensitised on the abdomen with the chemical 2,4-dinitrofluorobenzene (DNFB) and 5 days later challenged by topical application of the same hapten to ear skin. The ear swelling response was measured as a direct correlate of the CHS response. Application of 2,4-dinitro-1-thiocyanobenzene (DNTB) - a related hapten with weaker sensitising properties that shares the dinitrophenyl-self peptide antigenic motif with DNFB - 7 days before sensitisation elicited tolerance to DNFB, measured by a dampened ear swelling response. Thus, we used this model to test the generation of tolerance in mice that had received BMT, with or without T cells, 6-10 weeks earlier. Non-sensitised mice challenged with DNFB were used as a non-specific inflammation control. Our data demonstrated that while DNFB-sensitised mice in untransplanted and BMT control groups developed a robust CHS response peaking at 48-72 hours after challenge, pre-painting of these mice with DNTB significantly reduced ear swelling in accordance with the reported tolerising property of DNTB (Fig. 4.1B). Mice that had received BMT with T cells (and therefore suffered from GVHD) showed non-specific inflammation upon challenge with DNFB alone. These mice further developed an enhanced CHS response with delayed onset and resolution, peaking at 96 hours after challenge. Strikingly, pre-treatment of these mice with DNTB did not induce tolerance, as demonstrated by the enhanced ear swelling responses compared to controls. Taken together, our data demonstrated that mice that had previously developed GVHD had long-term loss of cutaneous tolerance with altered kinetics of CHS and greater non-specific inflammation.
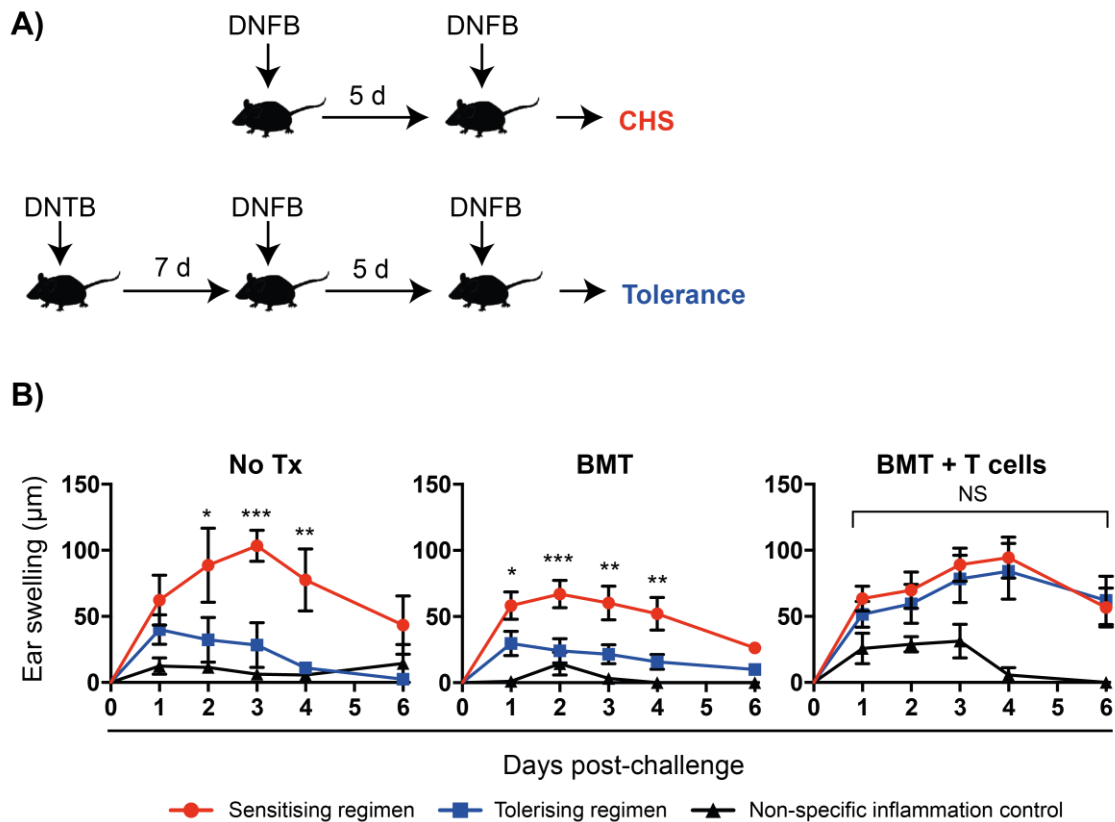
Figure 4.1 Cutaneous tolerance is lost in mice after GVHD

(A) Model of CHS. Mice were sensitised by epicutaneous painting of 0.5% DNFB on the abdomen at day -5. At day 0, mice were ear challenged with 0.2% DNFB and the ear swelling response measured over time. Mice were tolerised by epicutaneous painting of 0.1% DNTB on the back at day -12, prior to sensitisation and ear challenge. **(B)** The mean ear swelling response ($\mu m \pm$ SEM), relative to the control ear, was determined at various timepoints after DNFB ear challenge. Mice painted with vehicle at day -5 and challenged with DNFB were used as controls for non-specific inflammation. N = 3-12. Data from two independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P<0.0001$; NS = Not significant. No Tx = untransplanted mice. Statistics calculated using a 2 way repeated measures ANOVA with Tukey's multiple comparisons test.

4.2.   Characterising LN T cell populations in our CHS model

The observation that tolerance was not induced following GVHD led to the hypothesis that priming of T cells during tolerisation and/or sensitisation was impaired. To address this, the tolerising or sensitising regimen was employed 6 weeks after BMT with or without T cells, and draining LN were harvested 5 days following sensitisation. Figure 4.2 shows the flow cytometry gating strategy used to identify various lymphocyte populations within the LN. When comparing the tolerising regimen to the sensitising regimen, we could not detect a reduction in frequency of CD8+ T cells for any group, as would be predicted with tolerance induction (Fig. 4.2B). We suggest that this is because we are assessing the total CD8+ T cell population rather than hapten-specific IFN-γ-producing CD8+ T cells, potentially masking any small changes occurring to this population. Nevertheless, when the tolerising regimen was employed, we observed a

reduced frequency of CD8$^+$ T cells in the LN of the T cell group compared to controls, possibly due to egress of primed T cells out of the LN. Additionally, there was an increased frequency of LN CD4$^+$CD25$^+$FoxP3$^+$ Treg that were predominantly ICOS$^+$, shown in a previous study to be required for DNTB-mediated tolerance induction (Fig. 4.2B, C; Gomez de Aguero et al., 2012).

Following tolerisation and sensitisation, CD8$^+$ T cell, CD4$^+$ Tconv and CD4$^+$CD25$^+$FoxP3$^+$ Treg populations from the LN of the T cell group all exhibited increased CD44 expression compared to controls (Fig. 4.2D). CD44 is a marker for lymphocyte activation (Baaten et al., 2010) and therefore indicated a more activated phenotype of lymphocytes in the LN of mice following GVHD. This was similarly seen following sensitisation without prior DNTB application, supporting the concept that CD8$^+$ T cells are primed and rapidly enter the circulation after sensitisation. No differences were detected in CD62L expression levels (Fig. 4.2E). Together, these data suggested that naïve T cell populations were primed in the LN of mice following GVHD and displayed an activated phenotype.

A) Singlets, lymphocytes — CD8⁺ T cells; CD4⁺ — CD4⁺ Treg, CD4⁺ Tconv

B) CD8⁺ T cells; CD4⁺ Tconv; CD4⁺CD25⁺FoxP3⁺ Treg; CD4⁺CD25⁺FoxP3⁺ICOS⁺ Treg

Tolerising regimen; Sensitising regimen

C) BMT + T cells; BMT

D) CD8⁺ T cells; CD4⁺ Tconv; CD4⁺CD25⁺FoxP3⁺ Treg

E) CD8⁺ T cells; CD4⁺ Tconv; CD4⁺CD25⁺FoxP3⁺ Treg
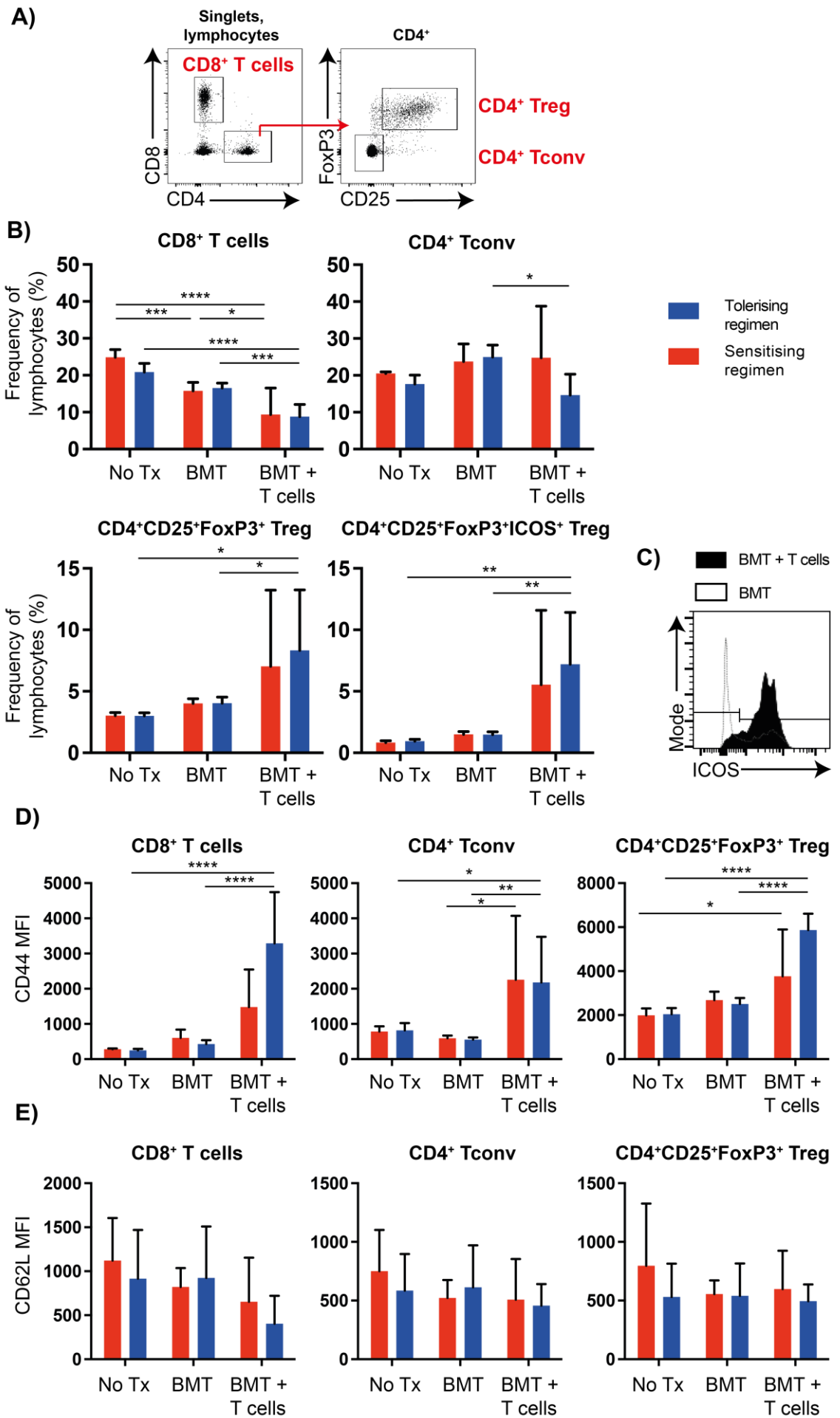
Figure 4.2 Lymphocytes are primed for tolerance induction in the LN of mice following GVHD

Skin draining LN were harvested and pooled 5 days after skin sensitisation, with prior DNTB application (tolerising regimen) or without prior DNTB application (sensitising regimen) and assessed by flow cytometry. **(A)** Gating strategy to identify CD8^{+} T cells, CD4^{+} conventional T cells (Tconv) and CD4^{+} regulatory T cells (Treg). **(B)** Frequency of various lymphocyte populations as a percentage of total lymphocytes (mean ± SD). **(C)** Histogram of ICOS expression on CD4^{+}CD25^{+}FoxP3^{+} Treg from tolerised mice receiving BMT or BMT + T cells. Y-axis has been normalised to mode. **(D)** Median fluorescence intensity of CD44 and **(E)** CD62L expression by various lymphocyte populations (mean ± SD). N = 5-8. Data from two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001. Statistics calculated using a 2 way ANOVA with Sidak's multiple comparisons test.

4.3.   Characterising changes to skin lymphocytes following ear challenge

Our previous experiments suggested that the breakdown in tolerance after GVHD could not be attributed to a reduction in the priming of Tconv or Treg in skin-draining LN; we therefore hypothesised that tolerance was lost due to a breakdown in cellular mechanisms *in situ* in the skin. Thus, at 6-10 weeks following BMT with or without T cells, we employed the tolerising regimen and assessed lymphocyte populations in ear skin 7 days after challenge (Fig. 4.4, 4.5).

Characterising lymphocyte populations separately in the epidermis and dermis was challenging because Dispase II incubation severely affects CD8 antibody staining (Autengruber et al., 2012). We therefore designed a flow cytometry panel, shown in Table 4.1, to identify CD8^{+} T cells by the phenotype CD3^{+}CD4^{-} γδ TCR^{-}CD64^{-}NK1.1^{-}CD11b^{-}, along with many other immune cells (Fig. 4.3).

Table 4.1 Flow cytometry staining panel to characterise lymphocyte populations within the skin

Marker	Conjugated fluorochrome	Antibody clone
CD4	BUV-737	GK1.5
Fixable viability dye	e450	-
CD45.1	BV605	A20
CD25	BV786	PC61
vβ8.3 TCR	FITC	1B3.3
CD45.2	PerCP-Cy5.5	104
γδ TCR	PE	eBioGL3
CD64	PE	x54-5.7.1
NK1.1	PE	PK136
CD11b	PE	M1/70
CD69	PE-Cy7	H1.2F3
FoxP3	APC	FJK-165
CD103	APC-R700	M290
CD3	APC-Cy7	145-2C11
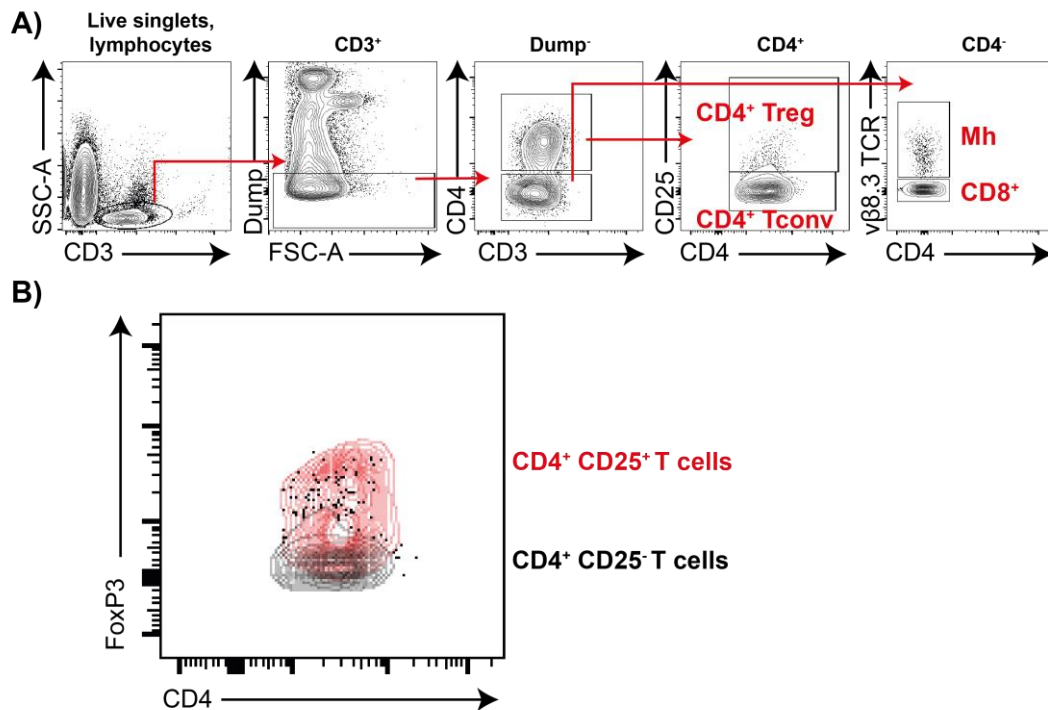
Figure 4.3 Flow cytometry gating strategy to characterise lymphocyte populations within the skin

(A) Dump channel includes $\gamma\delta$ TCR, CD64, NK1.1 and CD11b. This strategy allows for the identification of CD4+ regulatory T cells (CD4+ Treg), CD4+ conventional T cells (Tconv), CD8+ conventional T cells (CD8+) and CD8+ MataHari T cells (Mh). Mh T cells were a useful internal control as they did not expand in response to DNFB. **(B)** FoxP3 expression of CD4+CD25+ Treg and CD4+CD25- Tconv.

We first characterised infiltrating lymphocyte populations into the dermis following challenge. The challenged ear and unchallenged ear were analysed separately from each mouse and used as an internal control for non-specific T cell infiltration. Untransplanted and BMT controls had similar numbers of CD8+ conventional T cells (Tconv), CD4+ Tconv and CD4+ Treg entering the dermis following challenge (Fig. 4.4A). In contrast, there were fewer CD8+ Tconv and more CD4+ Tconv detected in challenged dermis of the BMT+T group. The number of CD4+ Treg was increased in challenged dermis of the BMT+T group compared to BMT controls, but no differences were detected when compared to untransplanted controls.

We assessed each lymphocyte population for expression of CD103 and CD69 as markers of tissue residency. Expression was unaffected by challenge as similar expression was detected in the challenged and unchallenged ear, within each group. However, CD103 expression by CD8+ T conv and CD4+ Tconv was downregulated in the BMT+T group (Fig. 4.4B), while CD69 expression by CD4+ Tconv and CD4+ Treg was upregulated in the BMT+T group (Fig. 4.4C).

Together, these data suggested that after GVHD, all three subsets of lymphocytes were primed in the LN and recruited to the dermis in response to ear challenge. Lymphocytes expressed markers of tissue retention which were unaltered by challenge.
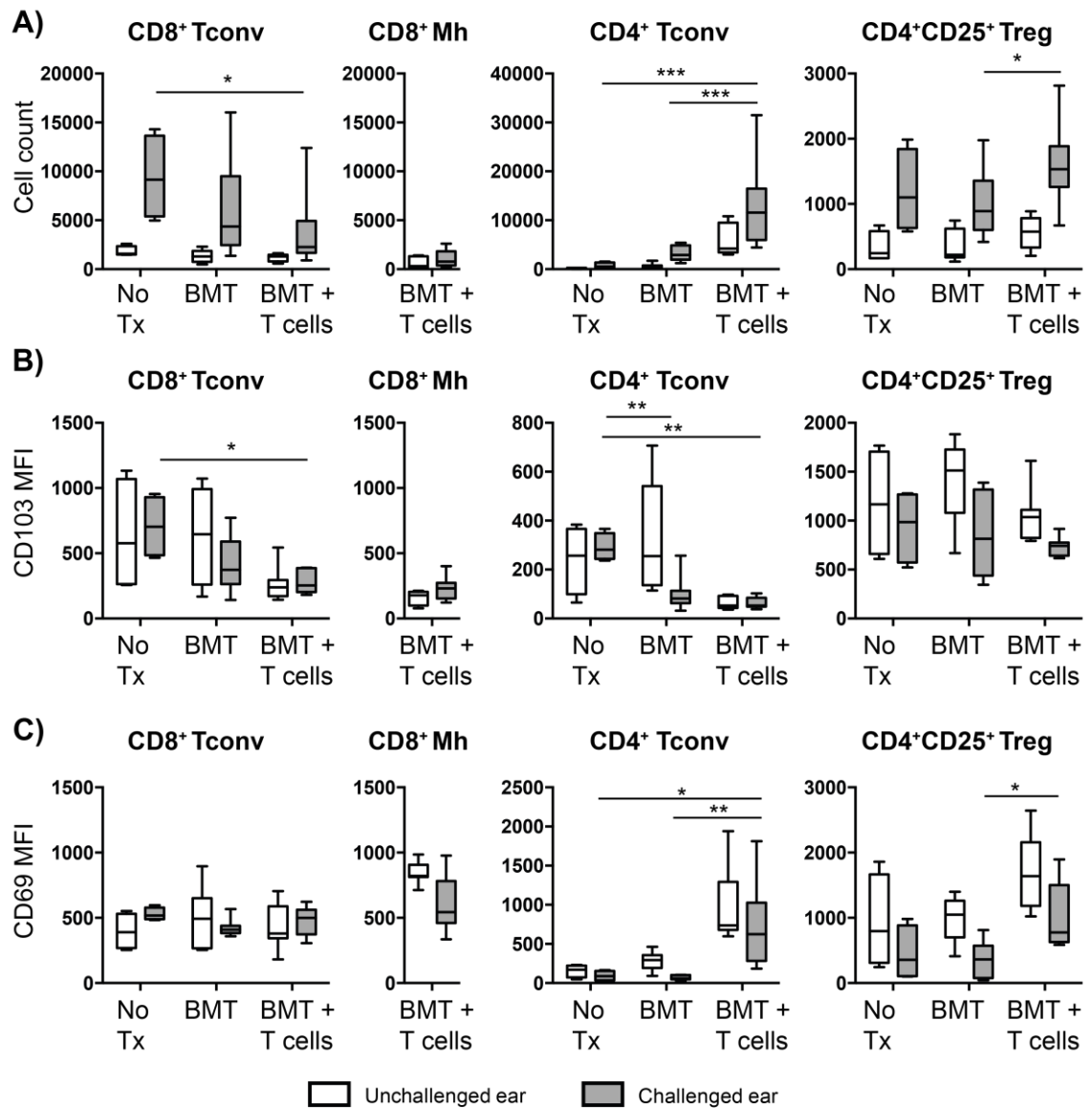
Figure 4.4 Greater lymphocyte infiltration into the dermis of ear-challenged mice following GVHD

Mice were painted with DNTB at day -12, sensitised with DNFB at day -5 and challenged on one ear with DNFB at day 0. At day +7, both ears were harvested separately, and dermal lymphocyte populations were assessed by flow cytometry for absolute number **(A)** and median fluorescence intensity of CD103 expression **(B)** and CD69 expression **(C)** (mean ± SD). N= 4-8, data from two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001. Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test.

The elicitation response of the CHS reaction occurs in the dermis of the skin. However, we observed accumulation of T cells in the epidermis following challenge. There was a reduction in the number of CD8^{+} T cells and an increase in the number of CD4^{+} Tconv in the epidermis of the BMT+T group compared to controls, but no difference in the size of the Treg compartment (Fig. 4.5A). We also assessed the lymphocyte populations for expression of CD103 and CD69 as an indication of residency. As for the dermis, expression was unaffected by challenge as similar expression was detected in the challenged and unchallenged ear, within each group. CD103 expression by CD4^{+}

Tconv was downregulated in the BMT+T group (Fig. 4.5B). Additionally, CD69 expression by CD4$^+$ Tconv was upregulated in the BMT+T group (Fig. 4.5C). Together, these data suggested that lymphocyte infiltration into the epidermis after GVHD was altered, skewing away from a CD8$^+$ T cell influx and toward a CD4$^+$ Tconv influx. These infiltrating CD4$^+$ T cells had higher CD69 expression, suggesting either prolonged tissue residency, or alternatively activation following APC engagement in the skin. We propose that these epidermal lymphocyte changes are likely to be a result of, and not causative in, breakdown in tolerance following GVHD, as CHS is largely constricted to the dermal compartment.
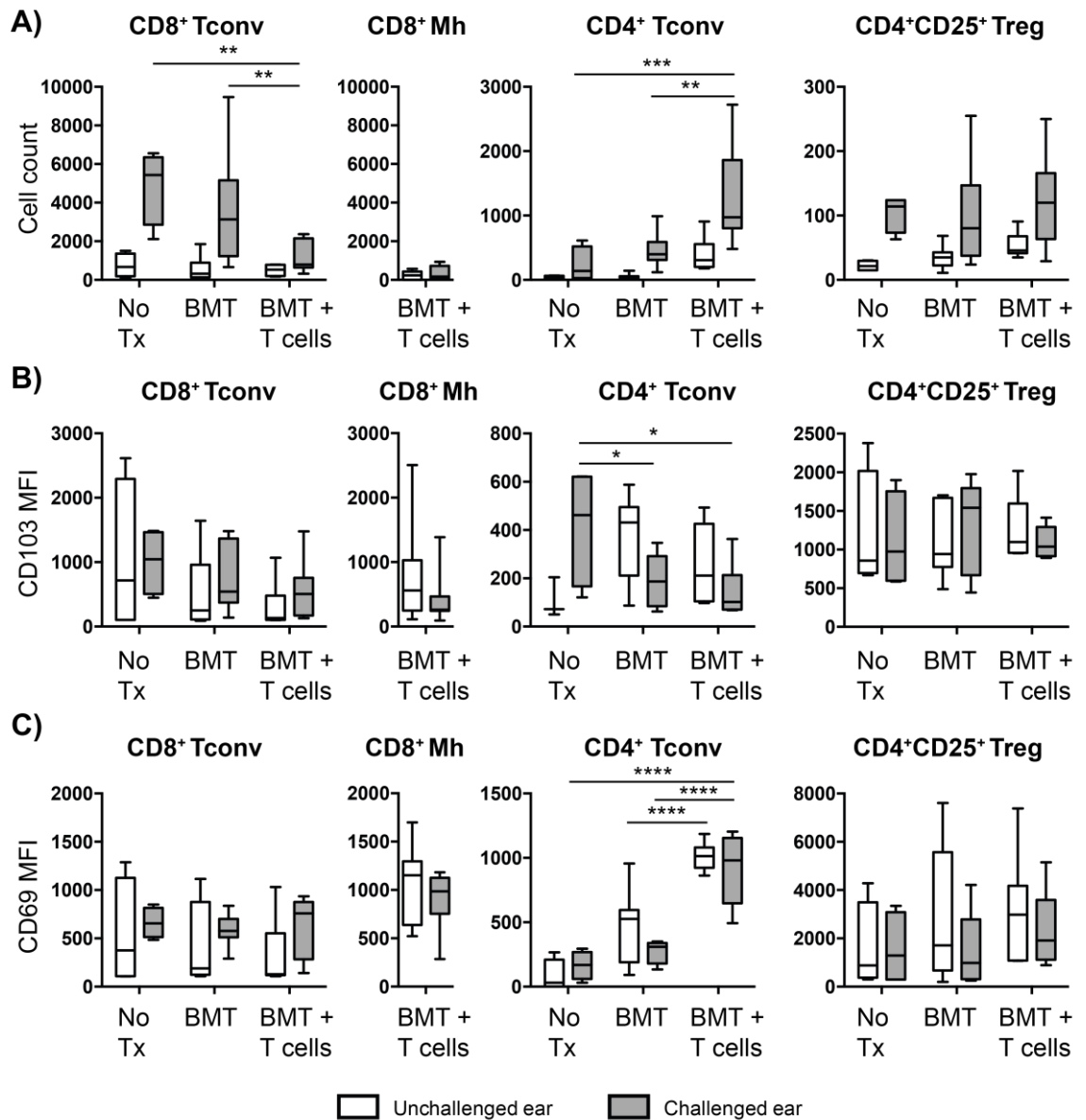
Figure 4.5 Lymphocyte infiltration into the epidermis following ear challenge is altered in mice following GVHD

Mice were painted with DNTB at day -12, sensitised with DNFB at day -5 and challenged on one ear with DNFB at day 0. At day +7, both ears were harvested separately, and epidermal lymphocyte populations were assessed by flow cytometry for absolute number **(A)** and median fluorescence intensity of CD103 expression **(B)** and CD69 expression **(C)** (mean ± SD). N = 4-8, data from two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001. Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test.

4.4.   Dermal Treg are potentially not suppressive after GVHD

Our data demonstrated the accumulation of CD4+CD25+FoxP3+ Treg in the dermis of GVHD mice after induction of tolerance. This observation generated two non-exclusive hypotheses: first, that loss of tolerance was due to the presence of dysfunctional Treg in the skin of mice that had previously suffered from GVHD; second, that CD8+ effector T

cells were resistant to suppression in these mice. Our original tolerance experiment had demonstrated a delay in the resolution of the CHS response after GVHD. Therefore, we tested the first hypothesis and analysed the suppressive function of dermal Treg in our model. Treg were isolated from the ears of tolerised and sensitised BMT or BMT + T mice, 6 days following challenge, and tested in an *in vitro* suppression assay. Previous studies have shown that Treg can effectively suppress proliferative responses of $CD25^-$ responder T cells stimulated with anti-CD3 and splenic APC *in vitro* (Collison and Vignali, 2011). Therefore, we sorted $CD4^+CD25^+$ Treg from the skin of transplanted mice, and $CD4^+CD25^+$ Treg, $CD4^+CD25^-CD127^+$ responder T cells and $CD19^+$ B cells from the LN of naïve mice. We cultured Treg from each group with varying ratios of responder T cells in the presence of anti-CD3 and B cells for 72 hours. In agreement with the literature, we demonstrated suppressive function of Treg sorted from the LN of untransplanted controls (Fig. 4.6A, B). Treg sorted from the skin of BMT controls were also suppressive, most notably at a Treg:Tresponder ratio of 1:2, although not to the same extent as LN Treg from untransplanted controls ($29.2\pm10.4\%$ c.f. $47.4\pm10.4\%$ suppression). Strikingly, Treg sorted from the skin of the T cell group did not suppress responder T cells at any ratio. A purity stain for FoxP3 expression of the sorted cells revealed that 96.1% of Treg sorted from the LN of naïve mice expressed FoxP3, in contrast to only 68.6% of Treg sorted from the skin of BMT + T recipients (Fig. 4.6C). It could therefore be possible that this population is contaminated with $CD25^+$ activated effector T cells. Future experiments are planned to repeat the suppression assay and adjust Treg:Tresponder ratios to compensate for lower FoxP3 purity, and to use Foxp3-EGFP reporter mice to isolate $FoxP3^+$ Treg.

Highly suppressive Treg have previously been shown to express low levels of the IL-7 receptor alpha chain (CD127; Liu et al., 2006). Our data suggested that $CD25^+$ cells from BMT + T mice expressed higher levels of CD127, an observation that may be consistent with loss of suppressive function (Fig. 4.6D). High expression of CD127 has previously been reported for murine skin memory Treg and activated Treg in models of skin inflammation (Gratz et al., 2013, Simonetta et al., 2010).

A)

	0:1	1:16	1:8	1:4	1:2
No Tx	87.9	74.3	75.0	67.9	33.7
BMT	75.6	90.7	87.7	68.4	57.5
BMT + T	82.6	92	92.2	96.3	93.8

CTV

Treg:Tresponder   0:1   1:16   1:8   1:4   1:2

B)

C)

No Tx — 96.1   BMT + T — 68.6

FoxP3 / CD25

D)

Pre-sort Live CD4⁺ T cells | Post-sort CD25⁺ Treg

No Tx — 97.1   BMT — 94   BMT + T — 94.9

CD127 / CD25
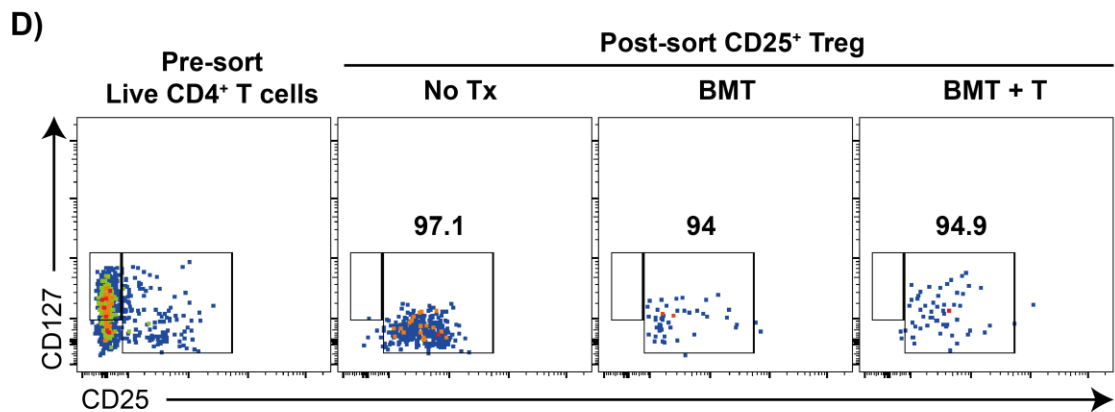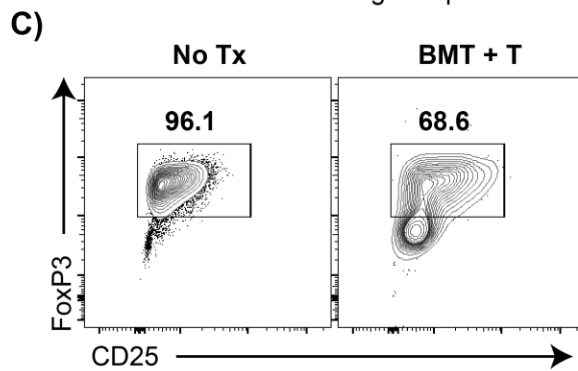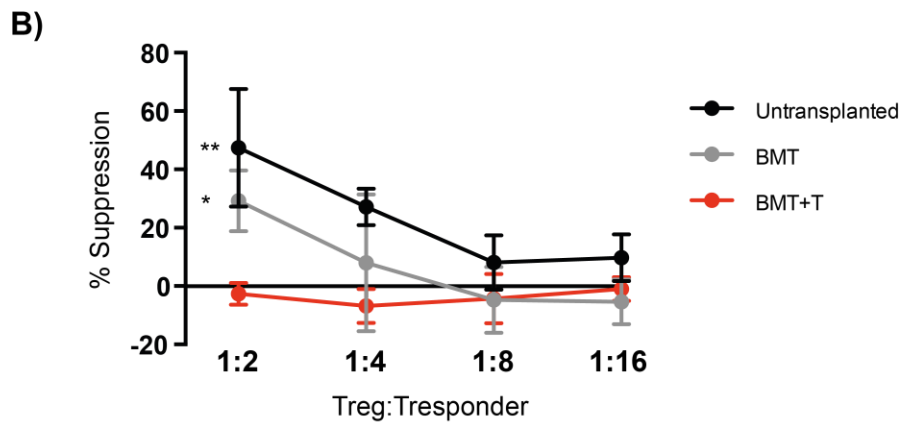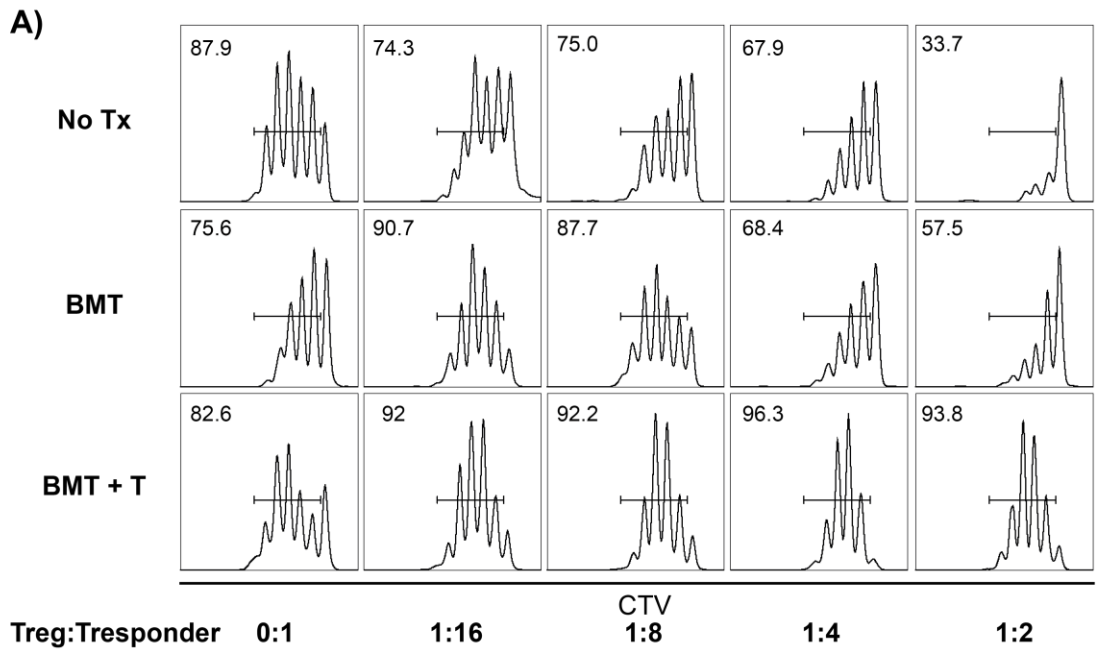
Figure 4.6 Treg sorted from the skin of mice following GVHD are not suppressive

Mice were painted with DNTB at day -12, sensitised with DNFB at day -5 and challenged on both ears with DNFB at day 0. At day +7, both ears were harvested and five mice per group were pooled. Whole skin was processed and Treg were sorted by FACS, based on CD4$^+$CD25$^+$ expression. Naïve responder T cells (Tresponder) were sorted by FACS from the LN of naïve mice, based on CD4$^+$CD25$^-$CD127$^+$ expression, and subsequently CTV-labelled. Treg were cultured at various ratios with Tresponder cells in the presence of anti-CD3 and splenic B cells (sorted by FACS based on CD19$^+$ expression) for 72 hours. **(A)** Proliferation of Tresponder cells after 72 hours in culture with Treg was tracked using CTV dye dilution and assessed by flow cytometry. The number in each panel indicates the percentage of proliferating responders. **(B)** Percent suppression was determined (mean ± SD). **(C)** FoxP3 purity of sorted Treg populations from naïve LN (left panel) and the skin of GVHD mice (right panel). **(D)** Dot plots of pre-sort and post-sort Treg with prior gating on live, singlet CD4$^+$ cells. Numbers indicate the purity of the sorted populations. Data from two independent experiments, each experiment pooled from N = 5 per group. *P < 0.05; **P < 0.01. Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test.

4.5.   Monocyte-derived IL-6 is upregulated in the dermis after GVHD

Our data suggested that loss of tolerance in the skin following GVHD may be due to the dysfunction of Treg within the dermal environment. Previous studies have reported that inflammation can block Treg function (Korn et al., 2009, Bettelli et al., 2006). Therefore, we returned to our proteomic screen and focused on inflammatory cytokines that were potentially upregulated in the dermis after GVHD (Chapter 3, Fig. 3.7). Of particular interest to us was IL-6, as previous studies have shown that IL-6 induced the generation of Th17 cells from naïve T cells and inhibited TGF-β-induced generation of Treg (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). Furthermore, TLR-induced IL-6 production by DC inhibited the suppressive function of Treg (Pasare and Medzhitov, 2003).

Therefore, we measured IL-6 production in the dermis of mice 3 and 6 weeks after BMT by flow cytometry. Validating our proteomic screen, we detected an increased frequency of IL-6-producing CCR2$^+$ cells (including monocytes and moDC), CCR2$^-$ macrophages and migratory LC (based on CD24$^+$CD11b$^+$ phenotype) in the dermis of GVHD mice, compared to controls (Fig. 4.7A, B, C). This was significantly upregulated at 3 weeks after allo-BMT within the CCR2$^+$ population but had not reached significance for the other populations. IL-6 was sill increased in the dermis of mice 6 weeks after allo-BMT, suggesting ongoing inflammation.

These data suggested a possible model in which IL-6 production by dermal myeloid cells leads to dysregulation of Treg function in the dermis and a loss of tolerance to contact sensitisers. To attempt to validate the role of IL-6, we transplanted mice with IL-6-deficient BM and tested skin Treg suppressive function as described above. We hypothesised that the absence of IL-6 production by donor-derived cells would prevent loss of Treg function. On the contrary, we did not observe a restoration of suppressive function in these mice. However, this does not disprove our hypothesis as this

experiment included a number of caveats, including the possibility of reduced GVHD owing to lack of IL-6 production (Hill, 2009). An alternative experiment with fewer caveats would block IL-6 using anti-IL-6 antibodies during the time period where tolerance is induced.

A)

Singlets, Lineage⁻ → CD11b⁺ CD24^low → Ly6C^low-to-hi CD64l^ow-to-hi → CCR2⁺ / CCR2⁻

B)

	No Tx	BMT 3 wks post-tx	BMT + T cells 3 wks post-tx	BMT + T cells 6 wks post-tx	
CCR2⁺ monocytes	0.009	0.006	0.042	0.046	
CCR2⁻ macrophages	0.009	0.002	0.023	0.011	
Migrating LC	0.032	0.022	0.270	0.028	

IL-6 (y-axis) / CCR2 (x-axis)

C)

CCR2⁺ monocytes — CCR2⁻ macrophages — Migrating LC

% IL-6⁺ cells

Conditions: No Tx, BMT (3 wk), BMT + T cells (3 wk), BMT + T cells (6 wk)

Figure 4.7 IL-6 is upregulated in the dermis of GVHD mice

(**A**) Flow cytometry gating strategy to identify migrating LC (mLC), CCR2⁺ monocytes and moDC (CCR2⁺) and CCR2⁻ macrophages (CCR2⁻). Lineage exclusion gate included NK1.1, Ly6G, CD3 and CD19. **(B)** Representative contour plots of direct *ex vivo* IL-6 production by CCR2⁺ monocytes/moDC (top panel),

CCR2[-] macrophages (middle panel) and migrating LC (bottom panel) from the dermis of mice 3 or 6 weeks post-transplant. Numbers indicate the frequency of IL-6^{+} cells from total singlet cells. **(C)** The frequencies of IL-6^{+} CCR2^{+} monocytes/moDC, IL-6^{+} CCR2^{-} cells and IL-6^{+} migrating LC are shown as a percentage of the lymphocyte gate (mean ± SD). N = 3-10. Data from two independent experiments. *$P < 0.05$. Statistics calculated using a one way ANOVA with Tukey's multiple comparisons test.

4.6. Humoral immune responses to epicutaneous immunisation are dysregulated after BMT

Previous experiments tested T cell activation and function. Given the roles of skin APC in eliciting humoral immunity, and our observations that LC and dermal DC were disrupted following GVHD, we also utilised an epicutaneous immunisation model to investigate potential changes to the induction of humoral immunity following GVHD. In this model, the stratum corneum is permeabilised by tape-stripping while leaving the epidermis intact, followed by application of protein antigen to elicit a potent systemic response. Previous studies have shown that the primary immune responses generated were strongly Th2-biased and involved antigen presentation by LC (Strid et al., 2004). The authors detected high levels of specific IgE and IgG1 with no Ig2a. This model facilitated the examination of antibody responses mediated by skin APC following GVHD.

LC and CD103^{+} dermal DC are required for the induction of Tfh cells, germinal centre formation and generation of antigen-specific antibody responses (Nagao et al., 2009, Ouchi et al., 2011, Zimara et al., 2014, Levin et al., 2017, Yao et al., 2015). We hypothesised that alteration to LC and DC subsets following GVHD would impact upon humoral immunity. To investigate this, we utilised the experimental model as outlined in Figure 4.8A. 6 weeks after BMT with or without T cells, the stratum corneum was damaged by 8 repetitions of tape stripping of both ears, which has been shown to permeabilise the outer layer, while leaving the epidermis intact. Topical application of highly purified OVA antigen diluted in PBS after tape stripping induced a dominant Th1 response in untransplanted mice, associated with production of antigen-specific IgG2c in the absence of antigen-specific IgG1 or IgE (Fig. 4.8B). No OVA-specific IgG response was elicited in transplanted mice. A strong non-specific IgE response was detected in the T cell group, supporting published data demonstrating that IgE production was dysregulated in GVHD (Claman and Spiegelberg, 1990, Amedei et al., 2014, Heyd et al., 1988). These data suggested that epicutaneous elicitation of immune responses was dysregulated following BMT. Further work is needed to define the APC populations responsible for priming humoral responses in transplanted mice and identify any functional consequences. We could utilise a pathogen challenge model such as experimental staphylococcal scaled skin syndrome employed by Ouchi et al., allowing

us to vary the delivery of antigen to either the epidermis or the dermis to test whether LC or dermal DC were priming responses (Ouchi et al., 2011).
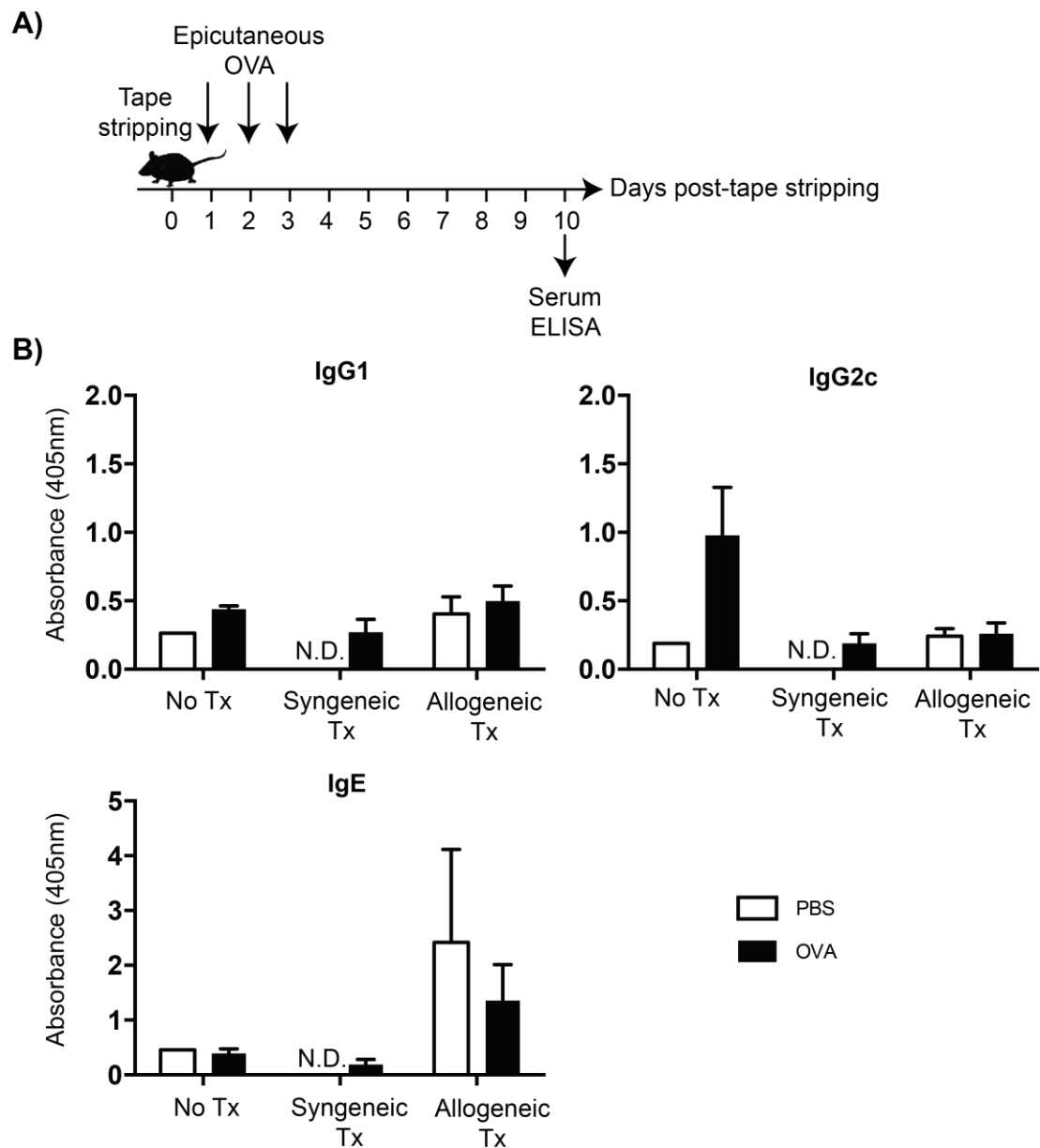
Figure 4.8 Humoral immune responses are dysregulated following GVHD

(A) At ten weeks after transplantation, sellotape was applied to and removed from the ears of mice a total of eight repetitions. Highly purified OVA protein or PBS was deposited on the ears on the following three days. Serum was harvested ten days post-tape stripping and assessed by ELISA for relative immunoglobulin levels. **(B)** OVA-specific IgG1, IgG2c and IgE in serum diluted tenfold in PBS was measured by ELISA and expressed as mean absorbance ± SD. N = 1-4. Data from two independent experiments. N.D. = Not determined.

4.7.   Defining the PRR profile of LC after GVHD

During GVHD, recipient LC are lost from the epidermis and replaced by donor-derived cells. In the clinic, patients who are recovering from GVHD can suffer from ongoing skin infections (Ferrara et al., 2009). Previous experiments tested the elicitation of contact hypersensitivity (often used as a model for atopic dermatitis) and humoral immunity. In order to complement this data with a pathogen challenge model, we aimed to profile pattern recognition receptor (PRR) expression by LC in order to select a suitable model. To test this, first we analysed the relative mRNA expression by RNA sequencing of toll-like receptor (TLR) genes, inflammasome-related genes and C-type lectin receptor (CLR) genes by LC from untransplanted mice and donor LC from mice 10 weeks after BMT with T cells (Fig. 4.9A). We detected broad upregulation of PRR by donor LC in comparison to untransplanted LC, including *Tlr3*, *Tlr4* and *Tlr12*, the inflammasome components *Lrrfip1*, *Ddx58*, *Nlrp1a*, and the C-type lectin receptors *Cd207* (Langerin), *Clec5a*, *Dectin2* and *Cd205* (DEC205).

Second, we transplanted irradiated male (allogeneic) and female (syngeneic) recipients with BM and T cells and purified total LC from the epidermis at 10 weeks after BMT by FACS, based on the gating strategy shown in Figure 4.9B. LC were additionally sorted from untransplanted control mice. RNA was extracted, and reverse transcription (RT)-PCR utilised to assess expression of TLR (*Tlr1-7*, *Tlr9*) and CLR (*Dectin1* and *Dectin2*), relative to *Gapdh*. We detected expression of *Tlr2*, *Tlr4* and *Tlr9* by LC from untransplanted mice, in agreement with a previous report (Mitsui et al., 2004). Contrary to this report however, we additionally detected expression of *Tlr6* and *Tlr7*. Expression levels were low, and variable between experiments, for all TLR (relative expression to *Gapdh* < 0.1). *Dectin1* and *Dectin2* were also expressed by LC from untransplanted mice (0.097±0.010 and 0.154±0.024 arbitrary units, respectively), in agreement with the literature (Yokota et al., 2001, Ariizumi et al., 2000). After transplantation, we detected slight changes in the PRR profile of LC, but no changes were significantly different. These data differed from the RNA sequencing analysis in which we observed broad upregulation of PRR. We propose that this was due to exaggeration of small changes in absolute values by relative comparative heatmaps. However, the sensitivity of RT-PCR is lower than RNAseq, and it is therefore possible that small changes to PRR expression have not been detected by RT-PCR, especially with low sample numbers. Further experiments are required to resolve these conflicting data, ideally assessing expression of PRR at the protein level. Additionally, PRR agonists could be used with isolated primary cells in culture to assess activation and indirectly characterise PRR expression. The C-type lectin Langerin (CD207) is a defining protein for mature LC and has previously been shown to be reduced on monocyte-derived "short-term" LC (Sere et al., 2012). However, our RNA sequencing data suggested that it was highly expressed by

donor monocyte-derived LC in our model. Therefore, we compared the Langerin protein expression by LC at 3 weeks post-transplant. At this timepoint, both recipient and donor LC are present in the same epidermal environment. Langerin protein expression by donor LC was similar to resident cells from untreated mice (Fig. 4.9D). Langerin was, however, upregulated on recipient LC in both transplanted groups. This could suggest activation of these cells following irradiation and/or transplantation.

In sum, while the data were variable, the increased expression of Dectin-1 and Dectin-2 seemed to be consistent between the RNA sequencing data and one sample of the RT-PCR analyses. Detection of Dectins by LC and dermal DC in the skin is required for the activation of anti-fungal immunity. Therefore, we decided to challenge our GVHD mice with the fungal pathogen *Candida albicans*.
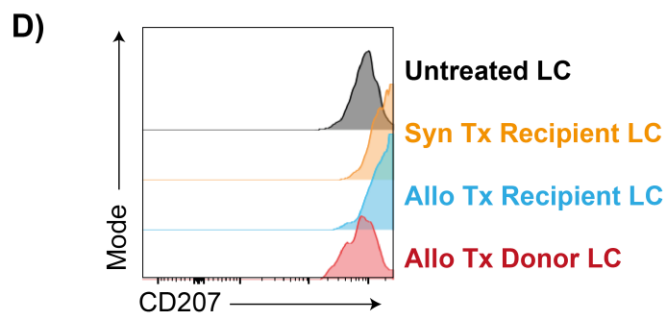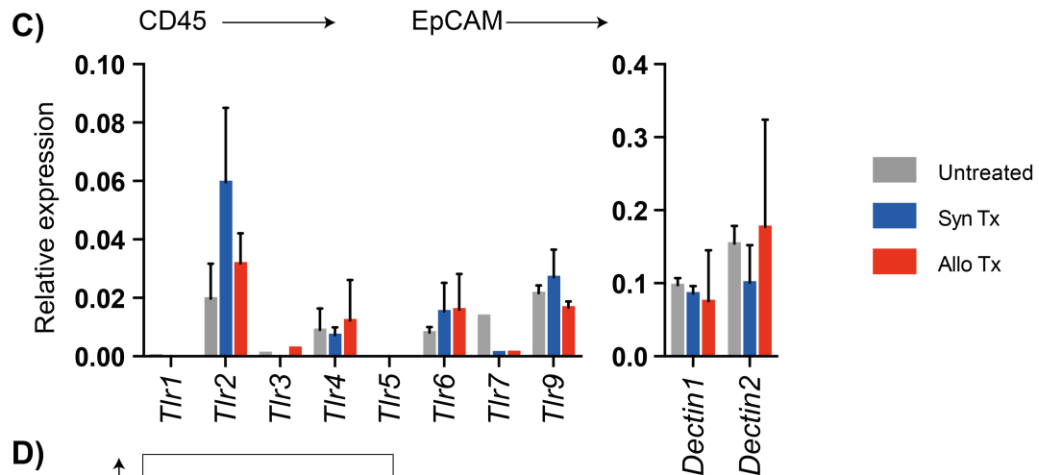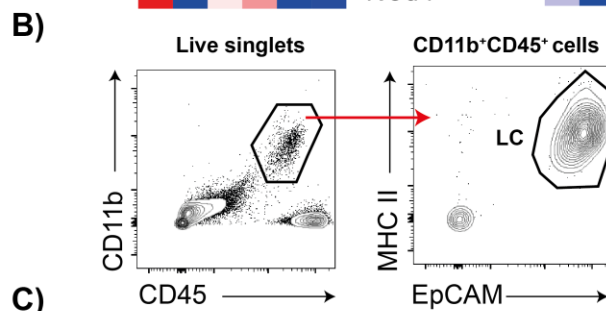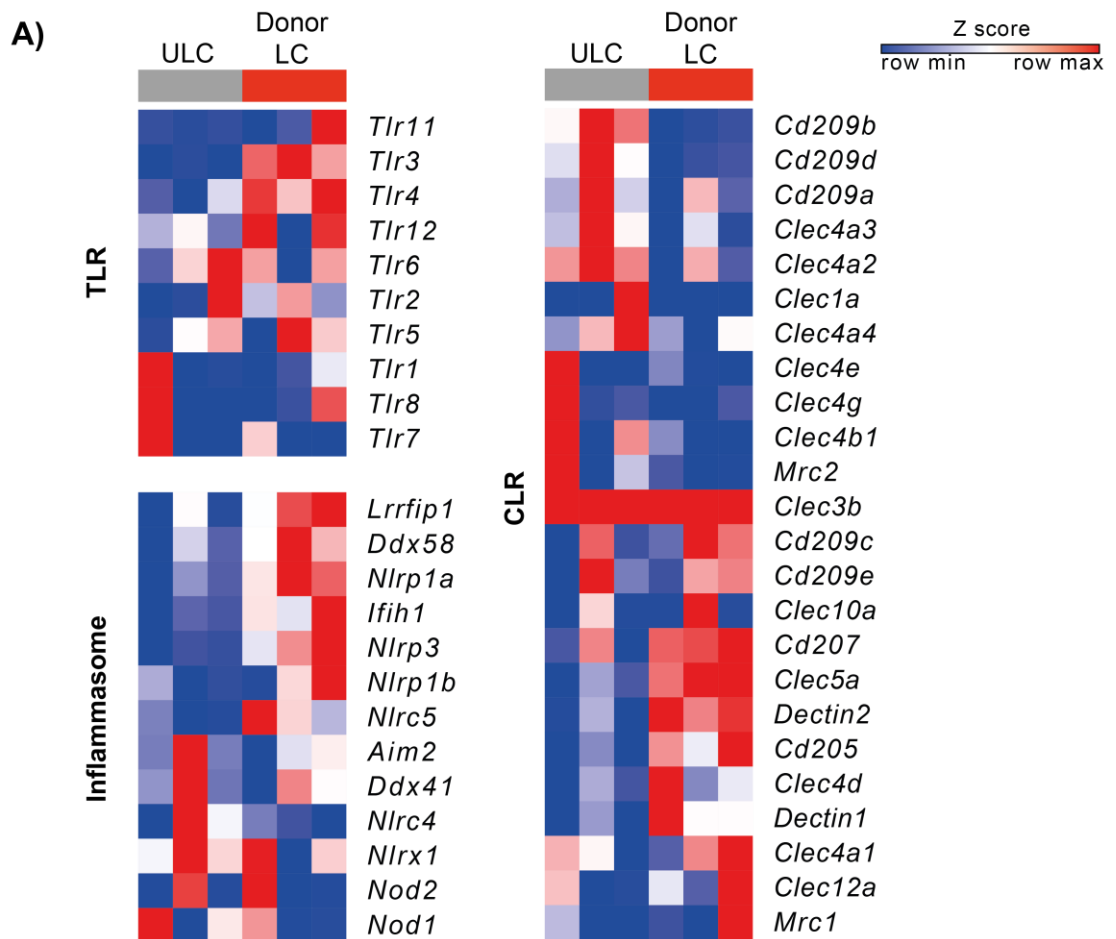
Figure 4.9 Assessing PRR profile of LC

(A) Heat maps of the relative expression of panels of toll-like receptor (TLR) genes, inflammasome-associated genes and C-type lectin receptor (CLR) genes by donor LC at 10 weeks post-transplant and age-matched untreated LC (ULC). **(B)** Gating strategy for sorting LC for subsequent RT-PCR analysis. **(C)** Relative expression of TLR and CLR by LC from naïve, syngeneic transplanted and allogeneic transplanted mice, determined by RT-PCR (mean ± SD). **(D)** CD207 (Langerin) protein expression by LC from untreated mice, recipient LC from syngeneic transplant recipients and recipient and donor LC from allogeneic transplant recipients at 3 weeks post-transplant (normalised to mode). N = 1-3, data from two independent experiments.

4.8. After GVHD, mice are more susceptible to cutaneous *Candida albicans* infection

The protective immune response to *C. albicans* skin infection requires a concerted response of LC and CD103$^+$ dermal DC (Igyarto et al., 2011). The authors of this study utilised a self-limiting infection that could be induced in mice (that are not natural hosts of *C. albicans*) by removal of the stratum corneum and epicutaneous application of *C. albicans*. The authors demonstrated that the infection was sub-lethal and cleared within 7 days. This required direct presentation of antigen by LC for the generation of a Th17 response and cross-presentation of antigen by CD103$^+$ dermal DC for the generation of a Th1 response. Kashem et al. later reported that Th17 responses required Dectin-1 ligation by LC and provided protection against cutaneous infection while Th1 responses provided protection against systemic infection (Kashem et al., 2015). We hypothesised that alteration of LC and DC subsets following GVHD would affect susceptibility to *C. albicans* infection. One the one hand, the presence of donor LC with enhanced levels of Dectin-1 could lead to the more rapid activation of anti-fungal T cell responses. On the other, loss of dermal DC in irradiated mice may impair early innate responses to the challenge. To test this, we employed the epicutaneous challenge model established by Igyarto et al. (Igyarto et al., 2011) and outlined in Figure 4.10A. Initial work using this protocol required significant optimisation as the mice became infected, but fungal burden calculations were not successful. Standard techniques in the lab of dissociating skin tissue (chopping and vortexing; tissue dissociation using a GentleMACS) were not adequate to release the yeast from the tissue, either because the yeast remained embedded in the tissue or died as a result of processing. In our hands, only tissue homogenisation using a manual homogeniser was successful in releasing the yeast.

Following experimental optimisation, we employed the model using transplanted mice. 6 weeks following BMT, we removed the stratum corneum of the back skin using 15 strokes of 220 grit sandpaper and challenged the same area with 2 x 10^8 *C. albicans*. At various timepoints after challenge, the infected area was cleansed with povidone-iodine and fungal burden of the skin was calculated by homogenisation of tissue followed by culture on YPD plates. Infection was evident in these mice by red, scabbed skin (Fig.

4.10B). Our data demonstrated an increased fungal burden at day 2 in the skin of mice following GVHD, compared to controls (Fig. 4.10C). Furthermore, resolution of infection was delayed, such that the fungal burden remained increased at day 4. This, however, did not reach statistical significance due to the small sample size, but strongly implied that mice were more susceptible to *C. albicans* infection following BMT.

We attempted to assess the T cell response in these mice by harvesting the LN and restimulating with phorbol 12-myristate 13-acetate (PMA) and Ionomycin or the Dectin-1/2 agonist Furfurman. These experiments, however, were unsuccessful. We suggest that future experiments to determine the cellular response require TCR transgenic mice or detection of antigen-specific T cells by tetramer staining. Further experiments are also required with a BMT control group to control for the effect of irradiation.
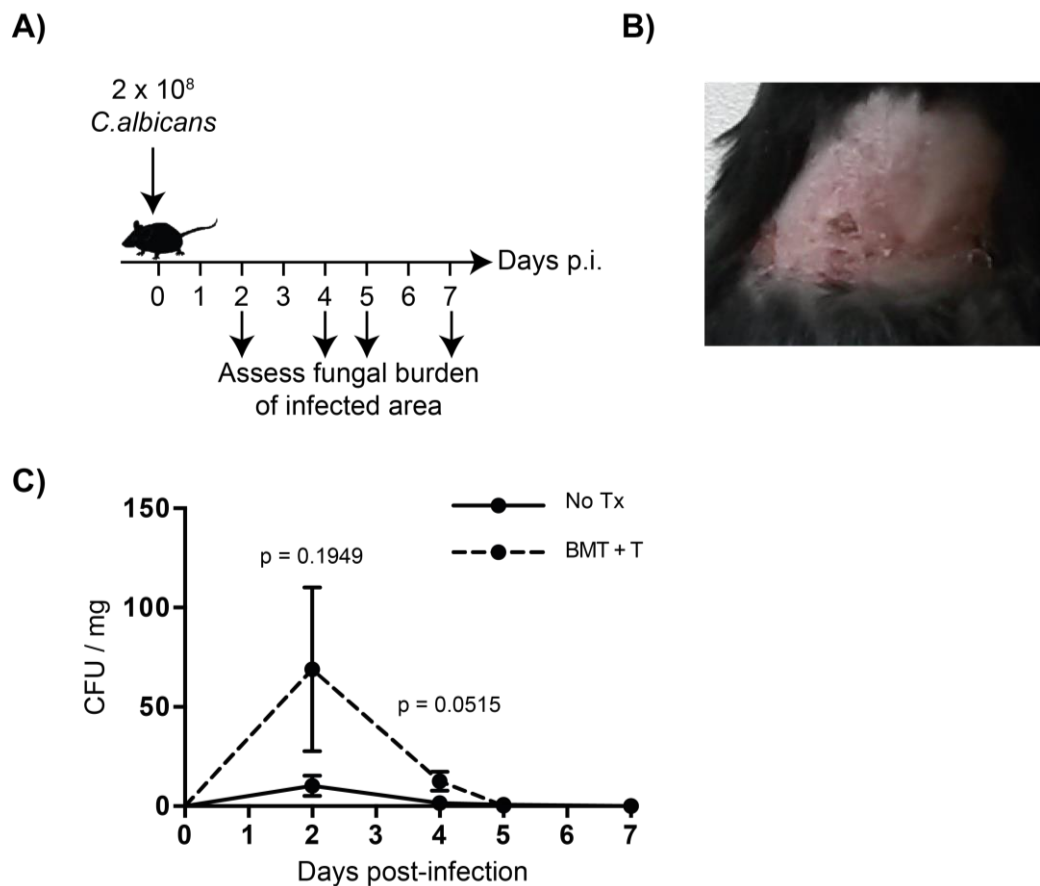
Figure 4.10 Following GVHD, mice are more susceptible to *C. albicans* infectious challenge

(A) Experimental outline. At 6 weeks after transplantation, a 2.0cm^2 area of back skin was depilated and the stratum corneum partially removed using fifteen strokes of 220 grit sandpaper. 2 x 10^8 blastoconidia of the ATCC10231 standardised strain of *C. albicans* was applied to the area and fungal burden of the infected area (mean ± SD) was determined at days 2,4,5 and 7 post-infection. (B) An image of evident skin infection on day 4 post-infection in an untransplanted mouse. (C) *C. albicans* CFU is expressed as colonies per mg of tissue. N = 2-5 per timepoint, data from two independent experiments. p values are indicated on the graph. Statistics calculated using multiple student t-tests.

4.9.   Summary

In this chapter, we tested the impact of skin pathology after GVHD on cutaneous immunity. The main body of work in this chapter demonstrated a breakdown in tolerance to topical haptens in mice that had previously suffered from GVHD. We have linked this to a loss of suppression by dermal Treg. Further, we have demonstrated that, while increased frequencies of monocytes are recruited into the skin of irradiated mice irrespective of T cell transfer, in the context of GVHD these cells are activated to produce IL-6, with the potential to suppress Treg function.

<u>Hapten-induced tolerance</u>

Our data revealed that GVHD mice were more susceptible to inflammation. One possible explanation is that pro-inflammatory cytokines, such as IL-1α and IL-6, were upregulated in the dermis of GVHD mice and contributed to sensitisation. A single application of DNFB to the ear has been reported to upregulate IL-1β and IL-6, which, by stimulating migration of skin APC to LN, has a key role in sensitisation (Bonneville et al., 2007). Indeed, in preliminary experiments we assessed induction of tolerance in GVHD mice at 3 weeks post-BMT, at a timepoint where mice displayed symptoms of active GVHD; experiments were discontinued owing to the high degree of non-specific inflammation in these mice (data not shown). This would indicate that the skin environment during and following GVHD is more prone to skin contact irritation.

We were unable to induce tolerance in mice that had previously developed GVHD, and these mice additionally displayed a delayed resolution of ear swelling. Analysis of skin APC migration was inconclusive, owing to difficulties with autofluorescence and baseline TRITC staining. These experiments were technically difficult, as it was tricky to distinguish between autofluorescent cells and Langerin$^+$ cells, as the Langerin staining was dim (data not shown). However, we could exclude a defect in T cell priming within the LN of these mice. CD4$^+$ and CD8$^+$ T cells were clearly primed and upregulated CD44. The expression of ICOS by Treg was an indication of their suppressive capacity, and suggested that the breakdown in tolerance was not occurring in the LN. Unfortunately, we were unable to directly test LN Treg function because the LN in these mice were very small. We cannot exclude the possibility that T cells in mice following GVHD are intrinsically less functional; however, CD8$^+$ T cells in these mice are clearly functioning in the CHS response. An additional control group of untreated mice is required in each group to assess LN lymphocyte populations without tolerisation or sensitisation. We can then determine whether endogenous T cells in mice following GVHD are intrinsically different.

We next tested whether primed Treg infiltrated the challenge site. Our data demonstrated that Treg effectively migrated to skin of mice following GVHD, albeit in a more non-specific manner. Given these data, we generated two hypotheses: firstly, that CD8$^+$ T cells causing the CHS response were resistant to suppression by Treg; and secondly, that Treg were dysfunctional.

We propose that Treg isolated from the skin of mice following GVHD are not suppressive and have identified CCR2$^+$ cell-derived IL-6 as a potential mediator of loss of Treg function. We have previously shown that the frequency of CCR2$^+$ cells was increased in the dermis of mice after BMT, in the presence or absence or T cells. What then, was stimulating IL-6 production in GVHD mice, compared to BMT controls? We suggest that damage induced during the pathophysiology of GVHD stimulates influxing CCR2$^+$ cells, through DAMP signalling, to produce IL-6. Increased levels of IL-6 in the dermis of mice following GVHD could potentially be inhibiting the suppressive function of Treg, resulting in a loss of cutaneous tolerance.

IL-6 has been well characterised to promote Th17 cell generation and inhibit Treg generation (Korn et al., 2009, Bettelli et al., 2006). Furthermore, *in vitro* studies revealed the ability of IL-6 to abrogate Treg-mediated suppression (Pasare and Medzhitov, 2003). Far less is known about the direct effect of IL-6 on mature Treg in peripheral tissues. One study has shown that increased levels of IL-6 in lesional psoriatic skin enable cutaneous T cell escape from Treg suppression (Goodman et al., 2009). We propose that IL-6 was directly acting on peripheral Treg and preventing suppression of effector cells. However, we cannot exclude the possibility that effector T cells were additionally resistant to suppression by Treg. Indeed, IL-6 has additionally been shown to render effector T cells refractive to suppression (Pasare and Medzhitov, 2003). We can test this by isolating effector T cells from the ears of challenged mice in our model and performing an *in vitro* Treg suppression assay using Treg from the LN of naïve mice. This could be challenging however, as suppression assays typically use CD4$^+$ T cells as responders, whereas effector T cells in our model are CD8$^+$ T cells, which are notoriously harder to suppress *in vitro*. However, there is precedent in the field for Treg suppression of CD8$^+$ T cells *in vitro* (Hasenkrug and Myers, 2011, Zhang et al., 2014).

CD25$^+$ T cells isolated from the skin did not express FoxP3 to the same extent as those isolated from the LN, and as a result fewer FoxP3$^+$ Treg were present in the cocultures of BMT+T samples. It could be argued that we were isolating CD25$^+$ activated effector T cells. Alternatively, FoxP3 could be downregulated or degraded in GVHD skin. Previous studies have shown that FoxP3$^+$ Treg are prone to shift toward a Th17-like effector phenotype in the presence of TGF-β and IL-6 by promoting FoxP3 degradation (Zhu et al., 2010, Bettelli et al., 2006, Gao et al., 2012b, Gao et al., 2015). Indeed, we

detected an increased CD4$^+$ Tconv population in the epidermis and dermis of challenged mice following GVHD. Further experiments are required to characterise this population and identify if these cells were bona fide Tconv or were originally Treg that were converted in the periphery. The use of *FoxP3*-Timer of cell kinetics and activity (Tocky) mice would be useful for these experiments, as real time *Foxp3* expression could be followed and we would be able to identify if FoxP3 expression was downregulated in the skin (Bending et al., 2018). We propose that a skewed ratio of effector T cells to Treg is responsible for the breakdown in local immune control within the skin and could have implications for all cutaneous immune responses. In one respect, polarisation of effector T cells towards a Th17 phenotype could affect protective immune responses towards pathogens; in another, lack of Treg suppression within the skin could lead to ongoing inflammation after insult and adverse reaction to harmless antigens, including commensal organisms and self-antigens.

Topical challenge with Ovalbumin

We could not elicit IgG responses to topical OVA in transplanted mice irrespective of the presence of MataHari T cells. Given the reported role for CD103$^+$ dermal DC in the induction of IgG responses (Nagao et al., 2009), one possibility is that loss of DN cDC from the dermis of irradiated mice could account for this defect. However, our experiments provided no clear evidence that GVHD specifically leads to an altered IgG response. Further experiments are required to test helper T cell priming following epicutaneous immunisation. Additionally, a better approach may be the use of infectious challenge model to test humoral immune responses towards a physiological pathogen, for example Staphylococcus aureus, as described by Ouchi et al. (Ouchi et al., 2011).

We need to consider why we were generating a Th1 response to epicutaneous immunisation in untransplanted mice, in contrast to the Th2 response that has been described in the literature. One possibility is that this was due to our use of Th1-skewed B6 mice in comparison to the use of Th2-skewed BALB/c mice by Strid et al. (Strid et al., 2004). However, this study also tested responses in B6 mice and reported a Th2 response in this strain. Our only suggestion is that the different housing of mice led to different microbiota, which has been previously reported to influence immune responses (Ericsson et al., 2014, Franklin and Ericsson, 2017).

Responses to *C. albicans*

Screening of PRR on LC highlighted Dectin-1 and Dectin-2 as potentially altered candidates following GVHD. We tested this by challenging mice with the fungal pathogen *C. albicans* and revealed that mice following GVHD were more susceptible to infection. Further experiments are required to determine the cause of this difference.

One possible explanation is an altered balance of LC and dermal DC following BMT. We have detected loss of DN cDC after BMT which are critical for the Th1 response to *C. albicans* that acts in concert with the LC-mediated Th17 response. Another explanation for increased susceptibility of mice following BMT is loss of IL-17 production, reported to be essential in the *C. albicans* protective immune response (reviewed in Sparber and LeibundGut-Landmann, 2015, Conti and Gaffen, 2015). This could potentially be accounted for by loss of dermal γδ T cells following BMT, reported previously to express IL-17 and control *C. albicans* infection (Conti et al., 2014). If loss of dermal DC or dermal γδ T cells was causative in the impaired protective immune response, we would hypothesise that BMT control mice would also be more susceptible to infection with *C. albicans*, as these mice would similarly lack dermal DN cDC and γδ T cells. This control is lacking in our experiments and needs to be tested. Further experiments are additionally required to determine whether the defect lies within the innate or adaptive immune response. We could test the contribution of various cytokines (e.g. IL-6, IL-17) by intra-dermally injecting blocking antibodies or recombinant proteins during the course of infection. In parallel, we could deplete the various populations to identify the source of the defect.

In summary, we have identified a breakdown in local immune responses in the skin following GVHD, affecting both cellular and humoral responses. Our data could implicate IL-6 as one potential mediator of this breakdown, and points to anti-IL-6 therapy as an attractive target for restoring tolerogenic responses in the skin.

Chapter 5 Modelling LC differentiation *in vitro*

The work shown in the previous chapters has described the plasticity in monocyte-derived cells that are recruited to the dermis and epidermis during, and after, acute GVHD. Here, we sought to further define the signals that control differentiation of monocytes into replacement LC in the epidermis in this setting.

Embryonic-derived precursors seed the epidermis at birth to establish an LC network that self-renews throughout life without input from the circulation (Hoeffel et al., 2012, Merad et al., 2002). Tissue macrophages are never physiologically depleted in large numbers and as such, most studies have focused on the development and seeding of the LC niche at birth. However, man and medicine have caused situations where LC are substantially depleted and repopulated by a blood-borne precursor. We are now beginning to understand that development of these ontogenetically distinct LC has different cytokine dependency and potentially a unique repopulation mechanism.

The embryonic LC niche is seeded by a single wave of $CX_3CR1^+CD45^+Langerin^-$ precursors, which enter the skin and differentiate into $CX_3CR1^{low}CD45^+Langerin^+MHC$ II^+ LC, before undergoing a burst of proliferation to fill the niche (Chorro et al., 2009). By comparison, the relative importance of recruitment or proliferation of cells to repopulate the epidermis after loss of the original LC network is less well defined. Guilliams and Scott proposed a model whereby repopulation of resident tissue macrophages occurs if the niche is accessible (i.e. no barrier preventing precursor entrance) and available (i.e. presence of unoccupied niche within the tissue from e.g. loss of cells or growth of tissue), and finally that the most competitive progenitor would engraft (Guilliams and Scott, 2017). LC niche availability alone is not sufficient for recruitment of blood-borne precursors, demonstrated by the lack of replacement from blood-borne precursors when LC were substantially depleted by x-irradiation or topically administered acetone and oil (Merad et al., 2002). This could potentially be explained by the lack of niche accessibility; the epidermis is a physical barrier to circulating BM-derived monocytes that explains their strict embryonic origin. It is possible that inflammation reduces the integrity of the epidermal barrier, causing the skin to become "leaky" and allow access to circulating BM-derived monocytes. Indeed, recruitment of BM-derived LC precursors following UV exposure correlated with UV dose and exposure, suggesting that the degree of inflammation may control repopulation of the epidermis (Merad et al., 2002). Recruitment of donor LC, not expansion of recipient LC, facilitated recovery of the LC population to pre-treatment numbers in this study. Parallels can be drawn to the mucosal LC system, where maintenance of the LC population is dependent on recruitment and

proliferation of BM-derived precursors, and not maintenance of existing cells (Capucha et al., 2018, Capucha et al., 2015).

Niche accessibility and availability is evident in our model of acute GVHD, as we see replacement of LC. However, unlike replacement following UV irradiation, LC numbers do not recover to pre-treatment numbers. Are LC niches being destroyed during GVHD pathophysiology, or are BM-derived LC precursors intrinsically less functional in the GVHD environment? Or is there a further factor, such as a recruitment signal, that Guilliams and Scott did not account for in their model?

Work in our lab, including the transcriptional analyses described in chapter 3, defined the differentiation of epidermal myeloid cells into repopulating LC after killing of the original LC population by allogeneic MataHari T cells. This occurred via an intermediary CD11b$^+$MHC II$^+$EpCAM$^+$Langerin$^-$ population, which displayed a unique transcriptional profile. Our data suggested that LC precursors switched on the expression of a proliferative program upon entry into the epidermis. The aim of the work described in this chapter was to begin to define the environmental signals that control epidermal myeloid cell differentiation after allo-BMT.

The generation of human LC-like cells *in vitro* has been used for several decades as a means to model function and further understand their development *in vivo*. The addition of TGF-β to cultures of CD34$^+$ haematopoietic stem cells with GM-CSF, TNF-α, SCF and FLT-3L suppressed myelomonocytic cell differentiation in favour of LC-like cells (Strobl et al., 1996, Strobl et al., 1997). Monocytic precursor cells derived from CD34$^+$ cells also developed into LC-like cells in a TGF-β1-dependent manner (Jaksits et al., 1999, Jurkin et al., 2017). Consistent with these findings, *ex vivo* human CD14$^+$ peripheral blood monocytes differentiated into LC-like cells in the presence of GM-CSF and TGF-β, supplemented with IL-4 (Geissmann et al., 1998) or Delta-1 (Hoshino et al., 2005). CD14$^+$ dermal DC isolated *ex vivo* were also able to differentiate into immature LC-like cells in culture with TGF-β alone, or into mature LC-like cells with the addition of GM-CSF and IL-4 (Larregina et al., 2001). With the adjusted understanding in the field that LC are generated from macrophage precursors, it seems unlikely in this scenario that dermal DC would re-differentiate into LC; instead, LC may be generated from CD14$^+$ monocytic cells that could have been contaminating their cultures. More recently, culture systems have been developed to generate LC-like cells from CD1c$^+$ blood DC, in the presence of GM-CSF and TGF-β (Milne et al., 2015), or TGF-β and thymic stromal lymphopoietin (TSLP; Martinez-Cingolani et al., 2014). BMP7 was initially defined as an instructive factor for the generation of LC-like cells from CD34$^+$ HSC in the presence of GM-CSF and FLT-3L (Yasmin et al., 2013), or from CD1c$^+$ blood DC in the presence of GM-CSF (Milne et al., 2015). In both of these studies, LC-like cells could be generated

in the absence of TGF-β, potentially identifying a redundancy between these two cytokines.

Fewer *in vitro* systems have been developed for murine models. Becker et al developed a system wherein murine BM cells were cultured in the presence of GM-CSF, CSF-1 and TGF-β to generate LC-like cells with the phenotype CD11c$^+$MHC II$^+$EpCAM$^+$ (Becker et al., 2011). This system was optimised by Chopin et al., who described the generation of LC-like cells from murine BM cells after a 3 day culture in the presence of GM-CSF and TGF-β (Chopin et al., 2013). The generated LC-like cells had the phenotype CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$, with low Langerin protein expression but high mRNA expression, as previously described (Becker et al., 2011). The transcriptional profile of LC-like cells was consistent with primary cells, expressing *Runx3*, *Irf4*, *Irf8* and *Id2*, and lacking *Batf3*, *Klf4* and *Xcr1*. LC-like cells were also generated from murine BM cells after a 5 day culture in the presence of TGF-β, GM-CSF, SCF, FLT-3L and TNF-α (Capucha et al., 2018); interestingly, BMP7 could not replace TGF-β in this model, in contrast to human systems.

Understanding how different factors synergise to generate LC-like cells from different precursors *in vitro* may be instructive for LC development and homeostasis *in vivo*.

In this chapter, we hypothesised that:

1. LC differentiation *in vitro* mimics monocyte-derived LC differentiation in our GVHD model;
2. BMLC have different growth factor requirements to LC in the steady state;
3. BMLC differentiate via a precursor that proliferates before differentiation *in vitro*;
4. donor LC differentiate via a precursor that proliferates before differentiation *in vivo*.

Therefore, we aimed to:

1. establish culture conditions to directly compare the differentiation of myeloid cells into macrophages, DC or LC;
2. define growth factor requirements for the generation of LC-like cells in culture;
3. validate the proliferation of LC precursors *in vitro*; and
4. validate the proliferation of LC precursors *in vivo*.

5.1.   Establishing a 6 day culture for generation of BMLC

BM cells are routinely cultured for 3-5 days to generate LC-like cells (Chopin et al., 2013, Capucha et al., 2018), or 6-8 days to generate DC or macrophages (Helft et al., 2015, Inaba et al., 1992, Wang et al., 2016, Trouplin et al., 2013). The shorter culture time for generation of LC-like cells could support the hypothesis that LC are directly generated from a pre-committed cell such as monocytes in the BM, as opposed to a more pluripotent MDP or CDP. In our experiments, we aimed to directly compare the generation of DC, macrophages and LC. To this end, we adapted an established 3 day culture model to generate LC-like cells (herein referred to as BMLC) to a 6 day model, to effectively compare the differentiation of BMLC, BM-derived DC (BMDC) and BM-derived macrophages (BMMac).

In initial experiments, we directly compared the generation of LC-like cells in 4 or 6 day cultures. $CD11c^+MHC\ II^+$ cells were expanded after 6 days in culture with GM-CSF. By gating on $EpCAM^-DEC205^+$ cells, we could identify BMDC and BMMac based on the expression of MHC II and CD11b, as described previously (Fig. 5.1A; Helft et al., 2015). Addition of TGF-β to the culture system led to the emergence of $EpCAM^+DEC205^+$ cells within the $CD11c^+MHC\ II^+$ population. These cells had lower expression of CD11b than BMDC and BMMac and have previously been described as BMLC. However, we could further divide this population based on the expression of MHC II: we classified MHC II^{hi} cells as BMLC and $MHCII^{low}$ cells as immature BMLC (im-BMLC). BMLC and im-BMLC had similar expression of CD11b, CD11c and EpCAM when harvested after 4 or 6 days in culture (Fig. 5.1B).

We next compared the number of cells generated after 4 or 6 days in culture, revealing a reduction in cell number by day 6 due to loss of cells at the $EpCAM^+DEC205^+$ stage. This suggested that BMLC were developing rapidly from differentiated cells in the presence of TGF-β, rather than from rare progenitors. However, these cells did not survive well in culture.
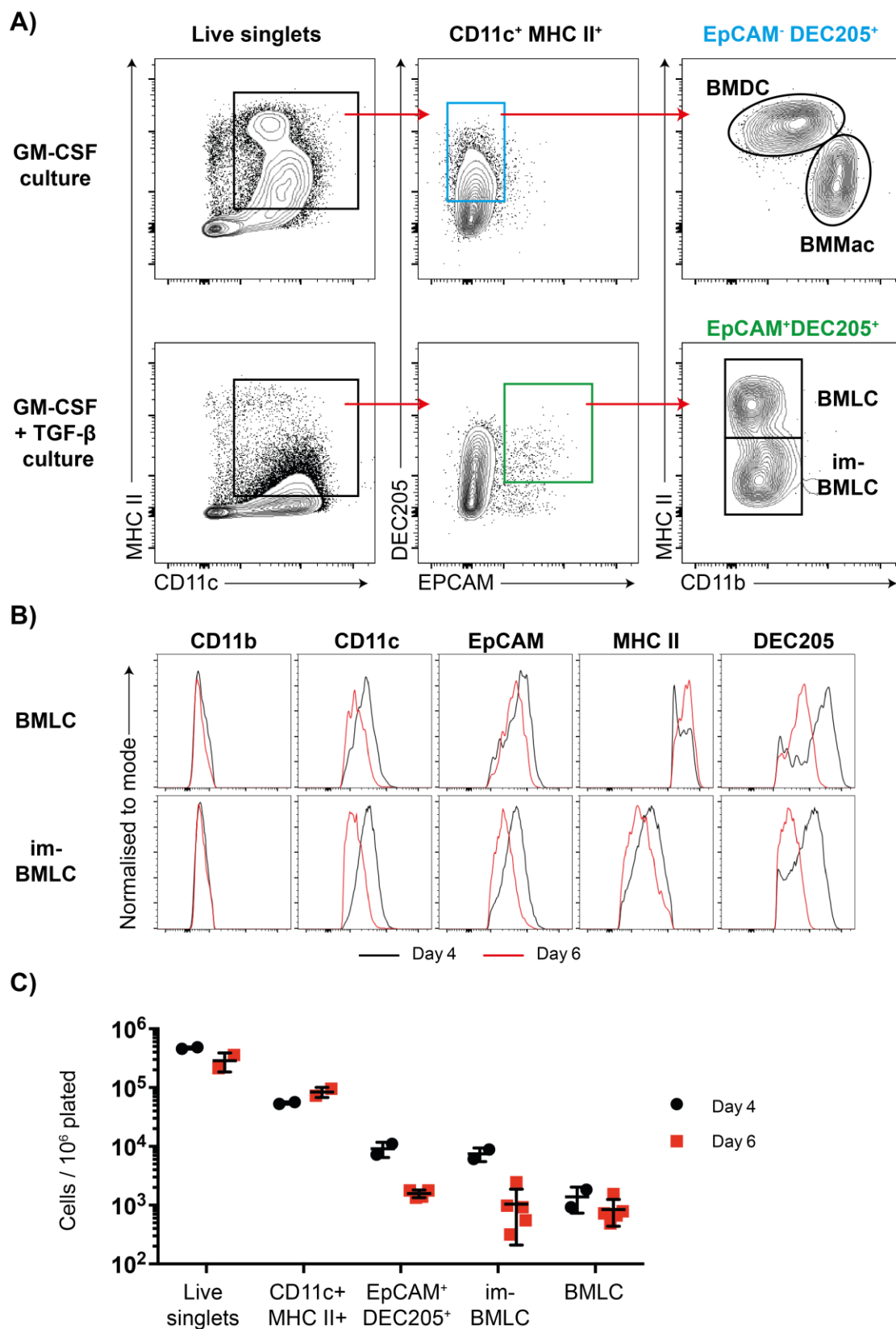
Figure 5.1 BMLC are generated after 4 or 6 days in culture

WT BM cells were cultured for 4 or 6 days in the presence of GM-CSF or GM-CSF and TGF-β. **(A)** Representative contour plots showing the gating strategy used to identify BMDC and BMMac from GM-CSF cultures, and BMLC and im-BMLC from GM-CSF+TGF-β cultures. **(B)** Representative histograms of BMLC and im-BMLC phenotype at day 4 versus day 6. **(C)** Cell counts of live singlet cells, CD11c$^+$MHC II$^+$ cells, CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells, im-BMLC and BMLC harvested on day 4 versus day 6 (mean ± SD). N = 2-4, data from two independent experiments.

126

5.2. Phenotyping BMLC after 6 days in culture

Having established a 6 day culture system, we next compared the phenotype of BMLC, BMDC and BMMac. Thus, we harvested cells after a 6 day BM culture with GM-CSF alone (generating BMDC and BMMac) or GM-CSF and TGF-β (generating BMLC) and assessed phenotype by flow cytometry (Fig. 5.2).

BMLC and im-BMLC had similar expression of CD11b, CD11c, EpCAM, CD24 and CD64, and differed in expression of maturation/activation markers, with BMLC displaying a more mature phenotype (increased expression of MHC II, DEC205, CD40, CD70, CD80 and CD86). BMDC and BMLC expressed similar levels of DEC205, MHC II, CD24 and CD64, and had a similar activation profile. BMLC expressed slightly lower levels of CD11b and CD11c compared to BMDC, and vastly upregulated EpCAM.

In line with previous reports (Helft et al., 2015), BMMac expressed higher levels of CD11b, and CD64 compared to the other populations. However, in our hands, BMMac expressed higher levels of CD11c. This was not reported by Helft et al., but it is known that tissue and tumour macrophages express CD11c *in vivo* (e.g. Hotblack et al., 2018). Together, our data suggested that, aside from EpCAM expression, BMLC and BMDC had a similar phenotype. The costimulatory molecule expression profile of BMLC and im-BMLC fitted with our division of CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells into the two sub-populations. This observation was consistent with primary LC, that express high levels of MHC II within the TGF-β-rich epidermis, but was surprising, considering that TGF-β is considered a suppressive factor. Therefore, we questioned whether we were really generating LC-like cells, or a population of EpCAM$^+$ BMDC, in our TGF-β cultures. To test this, we assessed lineage of cells generated in these cultures.
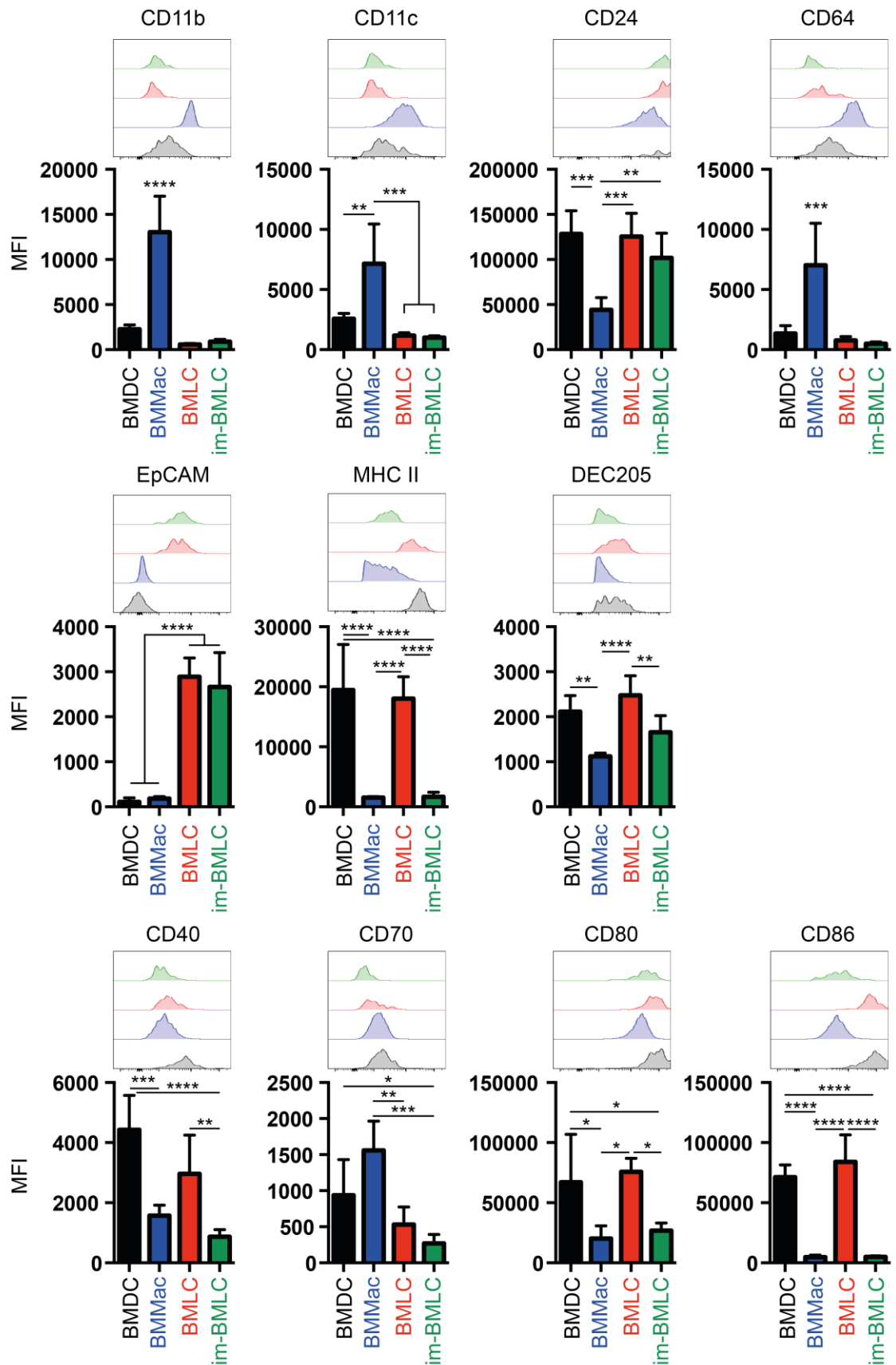
Figure 5.2 Phenotype of cells generated from GM-CSF and GM-CSF+TGF-β cultures

Representative histograms and summary bar charts of median fluorescence intensity (MFI) (mean ± SD) of phenotyping markers on BMDC and BMMac from 6 day GM-CSF cultures, and BMLC and im-BMLC from 6 day GM-CSF+TGF-β cultures. Histogram y axis was normalised to mode. N = 5, data from 3 independent

experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistics calculated using a one way ANOVA with Tukey's multiple comparisons test.

5.3.   Assessing the ancestry of cells generated in culture

It has previously been shown that expression of lineage-defining growth factor receptors by differentiated DC and macrophages provides evidence of their ancestry in culture (Helft et al., 2015). BMMac come from CSF1R$^+$ cells, reflecting a monocytic origin, whereas BMDC come from both CSF1R$^+$ cells and FLT3$^+$ cells, reflecting a monocytic or pre-DC origin for moDC and cDC, respectively. To investigate whether monocytes were seeding LC-like cells in our cultures, we analysed expression of these proteins (Fig. 5.3). Preliminary analyses revealed that BMDC were composed of cells with CSF1R$^+$FLT3L$^-$, CSF1R$^-$FLT3$^+$ and CSF1R$^-$FLT3$^-$ phenotypes, in line with previous observations, and identifying moDC and cDC in these cultures (Helft et al., 2015). BMMac were almost entirely CSF1R$^+$FLT3$^-$, reflecting their monocytic origin. By comparison, neither LC population resembled BMDC or BMMac, however FLT3$^+$ cells were not detected, arguing strongly that BMLC were not derived from pre-DC in these cultures. Only im-BMLC showed evidence of a monocytic ancestry and the "mature" BMLC population did not express CSF1R. This was unexpected as primary LC express CSF1R. This could potentially reflect downregulation of CSF1R on differentiation or signalling, as has been shown for CSF1R expression by Ly6C$^+$CD11c$^+$ cells in GM-CSF culture (Rogers et al., 2017), or the mature cells could be expanding from the CSF1R$^-$ cell population. Together, our data implied that BMLC were derived from monocytes in these cultures, reflecting the repopulation of LC *in vivo*.
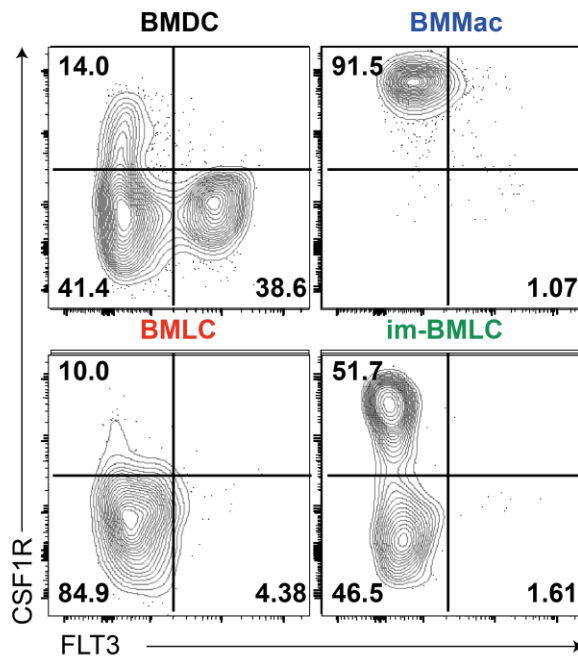
Figure 5.3 CSF1R and FLT3 expression of cells generated from GM-CSF and GM-CSF+TGF-β cultures

Representative contour plots of CSF1R and FLT3 expression. Numbers indicate the frequency of the gated population. N = 1, data from one independent experiment.

5.4. Continuous TGF-β is required for BMLC differentiation

TGF-β1 signalling is not required for seeding of the epidermis at birth by LC but is an absolute prerequisite for maintenance of LC within the niche (Xu et al., 2012, Kel et al., 2010, Zahner et al., 2011, Li et al., 2016, Kaplan et al., 2007, Borkowski et al., 1996). We therefore hypothesised that TGF-β was not required for the initial generation of BMLC and was only required later in the culture. In addition, we questioned whether addition of TGF-β later in the culture would allow expansion of increased numbers of LC from their precursors. To test this, we set up BM cultures in the presence of GM-CSF for 6 days, GM-CSF and TGF-β for 6 days (D1-6), or GM-CSF for the first three days followed by GM-CSF and TGF-β for the final 3 days (D3-6).

Unexpectedly, addition of TGF-β for the duration of the cultures increased the live cell yield (Fig. 5.4B). No difference was detected in the number of CD11c⁺MHC II⁺ cells, suggesting that cells outside this gate were expanding in response to TGF-β. The increase in cell yield could potentially be attributed to an expansion of MHC II^{low} monocytes and macrophages, outside of the CD11c⁺MHC II⁺ gate.

Contrary to our hypothesis, BMLC and im-BMLC required TGF-β for the full 6 days of culture, with very low number of cells generated when TGF-β was absent or added at a later timepoint. This was not the case for BMDC and BMMac. BMDC numbers were sequentially suppressed by addition of TGF-β for the last 3 days or all 6 days of culture,

as has previously been reported (Felker et al., 2010). However, BMMac showed an inverse response, and increased in numbers with prolonged exposure to TGF-β. Previously, TGF-β has been shown to enhance the GM-CSF stimulated proliferation of macrophages (Sere et al., 2012, Celada and Maki, 1992).

Together, these data demonstrated that TGF-β was essential throughout the *in vitro* differentiation process of BMLC. This indicated that BMLC precursors were a limited pool that were not continually being generated from the BM and were either being diverted to another differentiation pathway or were undergoing apoptosis. Furthermore, these data suggested that, in response to TGF-β, BMLC behaved more similarly to BMMac than BMDC, consistent with a monocytic origin of BMLC.
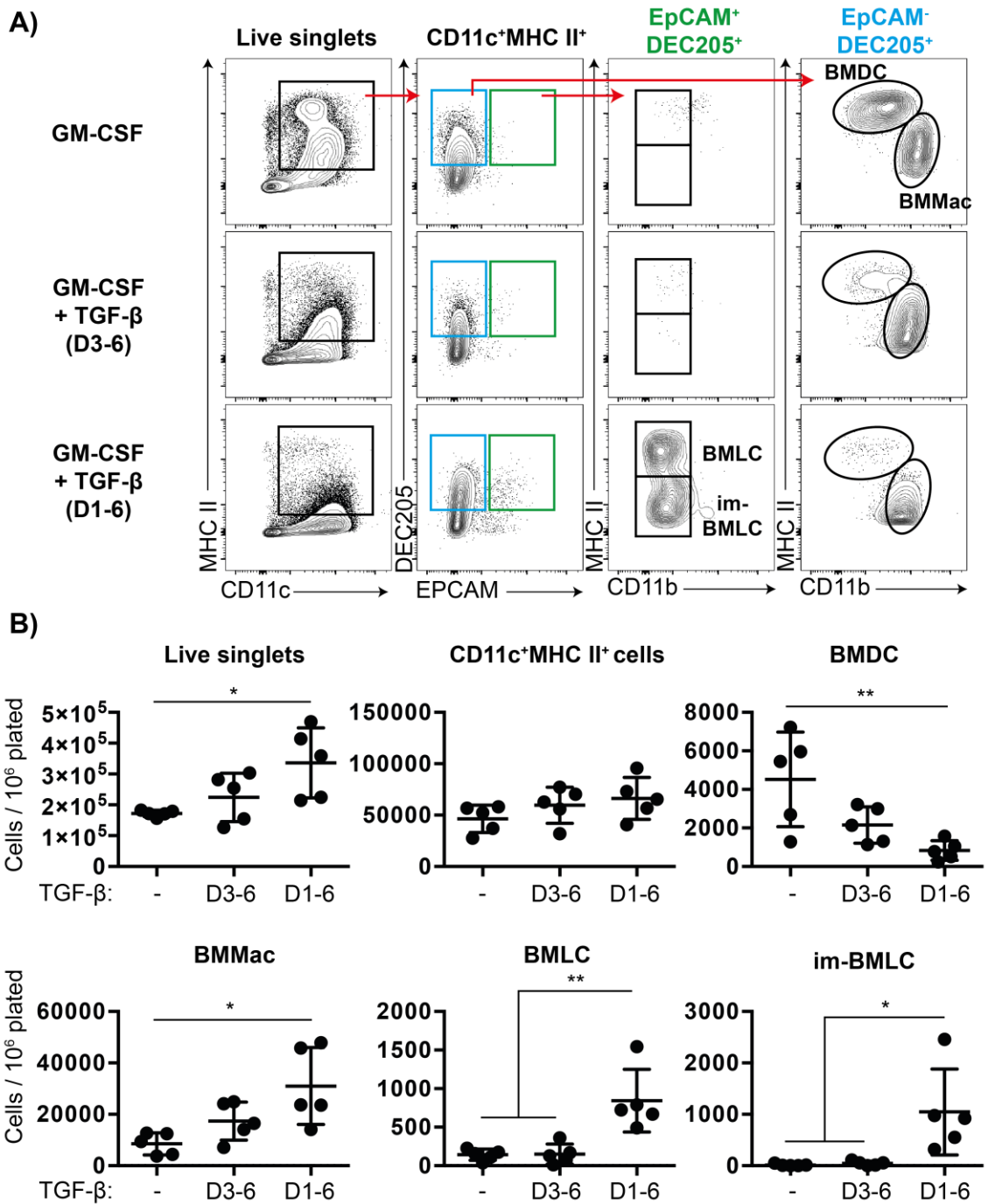
Figure 5.4 BMLC require constant TGF-β

WT BM cells were cultured in the presence of GM-CSF for 6 days (GM-CSF), GM-CSF for the first 3 days and GM-CSF + TGF-β for the final 3 days (D3-6) or GM-CSF + TGF-β for 6 days (D1-6). **(A)** Representative contour plots of the cells generated under the different culture conditions and harvested on day 6. **(B)** Cell counts of the various subsets generated under the different culture conditions and harvested on day 6 (mean ± SD). N = 5, data from two independent experiments. *P < 0.05; **P < 0.01. Statistics calculated using a one way ANOVA with Tukey's multiple comparisons test.

5.5.   IL-34 increases the viability of im-BMLC

The requirement of GM-CSF and TGF-β for the generation of LC-like cells in culture has been established, however fewer reports document the effects of other growth factors involved in LC differentiation or homeostasis. We tested whether addition of BMP7, IL-

34 or CSF-1 would lead to enhanced accumulation or survival of LC-like cells. Although we demonstrated that mature BMLC did not express CSF1R, we hypothesised that precursor cells to BMLC signalled through this receptor. Therefore, we supplemented BMLC cultures with these cytokines, alone or in combination, and assessed cell counts, viability and proliferation by flow cytometry. Our data demonstrated that significantly more CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells were present in all cultures supplemented with IL-34 (Fig. 5.5A). This increase was not detected when cultures were supplemented only with BMP7 or CSF-1. This difference was not due to increased proliferation of these cells (determined by Ki67 nuclear staining); however, viability of CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells was increased in cultures supplemented with IL-34 (Fig. 5.5B).

More detailed analysis indicated that the expansion in the EpCAM$^+$DEC205$^+$ population was due to an expansion of im-BMLC, in accordance with their expression of CSF1R (Fig. 5.5C, D). The lack of coincident expansion of the BMLC population was therefore surprising, suggesting that im-BMLC were not successfully maturing into BMLC, or alternatively were not BMLC precursors.

To investigate why im-BMLC survived better in the presence of IL-34, we sorted EpCAM$^+$DEC205$^+$ cells after 6 days in culture with GM-CSF and TGF-β, with or without IL-34, and assessed relative expression of key transcription factors and receptors involved in LC differentiation by RT-PCR (Fig. 5.5E). CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells expressed *Id2*, *Runx3*, *Tgfbr1* and *Bmp1ra,* like their primary counterparts. Of the four transcripts tested, only *Id2* was significantly upregulated when IL-34 was added to the culture. These data suggested that IL-34 caused a downstream effect of increased ID2 and increased viability of CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells.
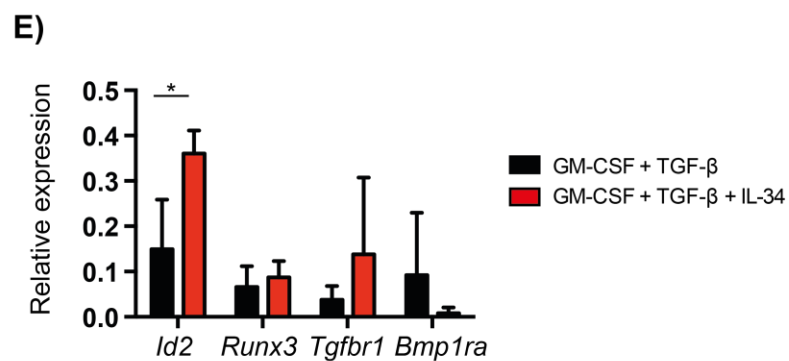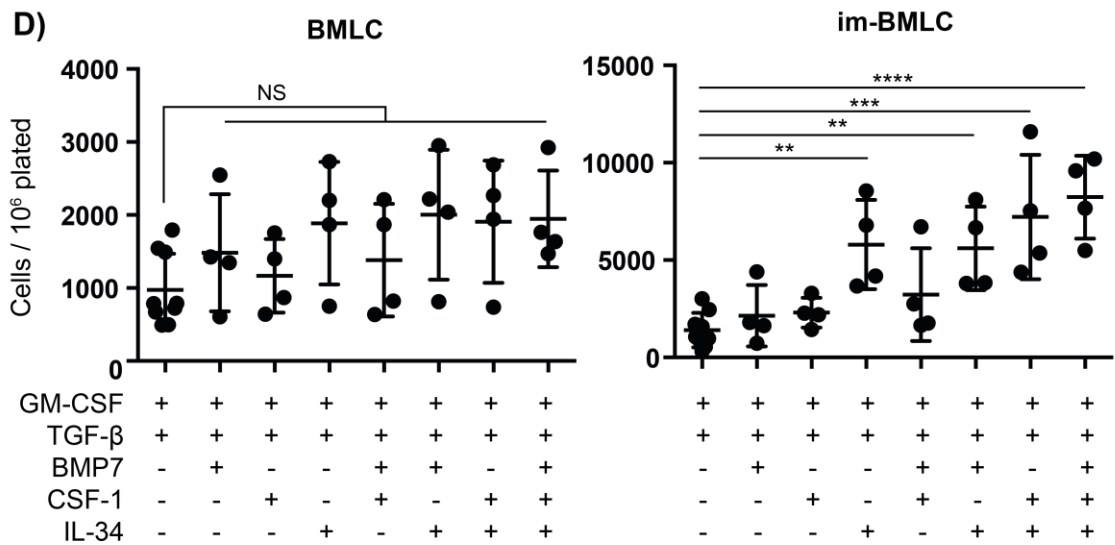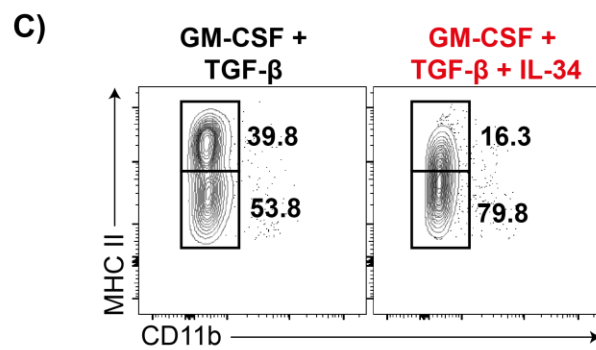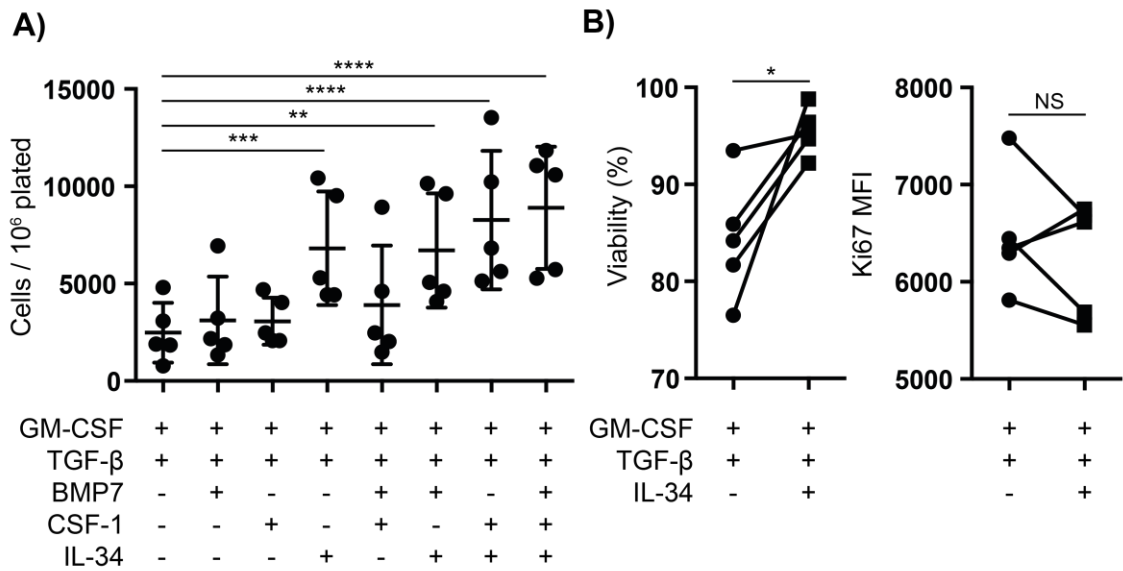
Figure 5.5 IL-34 increases the viability and *Id2* expression of im-BMLC

BMLC cultures were supplemented for 6 days with BMP7, CSF-1 and IL-34 in different permutations and harvested on day 6. **(A)** Cells counts of CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells harvested on day 6 under the different culture conditions (mean ± SD). Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test. **(B)** Viability (left panel) and Ki67 median fluorescence intensity (MFI; right panel) of CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells harvested on day 6 from GM-CSF + TGF-β and GM-CSF + TGF-β + IL-34 cultures (mean ± SD). Statistics calculated using a Mann-Whitney U test. **(C)** Representative contour plots of MHC II and CD11b expression by CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells harvested on day 6 from GM-CSF + TGF-β and GM-CSF + TGF-β + IL-34 cultures. The numbers indicate the frequency of the gated population. **(D)** Cells counts of BMLC (left panel) and im-BMLC (right panel) harvested on day 6 under the different culture conditions (mean ± SD). N = 5, data from four independent experiments. Statistics calculated using a 2 way ANOVA with Tukey's multiple comparisons test. **(E)** Gene expression relative to *Gapdh* (mean ± SD) of transcription factors by sorted CD11c$^+$MHC II$^+$EpCAM$^+$DEC205$^+$ cells generated by GM-CSF + TGF-β and GM-CSF + TGF-β + IL-34 cultures and harvested on day 6. Statistics calculated using multiple student's t-tests. N = 4, data from two independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; NS = not significant.

To complement this data, we aimed to determine levels of IL-34 in the skin at the time point when our potential precursors were differentiating into LC. We harvested back skin epidermis at 3 weeks after BMT, floated it on RPMI (2% FCS) for 16 hours and assayed the supernatant for IL-34 by ELISA. Unfortunately, we could not detect IL-34 using this method. We are of the opinion that the supernatant volume was too large, thus diluting the cytokine to levels below the sensitivity of the detection method. Further experiments are planned to detect levels of IL-34 by immunohistochemistry.

5.6. Proliferation of LC precursors leads to LC accumulation *in vitro* and *in vivo*

The transcriptional profiling detailed in chapter 3 had highlighted the activation of proliferative gene programmes in monocyte-derived LC precursors as they entered the skin. Having established differentiation of LC-like cells in culture, we tested the hypothesis that accumulation of monocyte-derived LC required proliferation of precursor cells. GM-CSF + TGF-β BM cultures were pulsed with 10μM EdU on day 2 or day 5, for 24 hours before washing the cells. We then harvested cells on day 6 and analysed incorporation of EdU by BMLC and imLC by flow cytometry. Both BMLC and im-BMLC incorporated EdU when it was given on day 2 of culture (BMLC 43.8±12.7%; im-BMLC 35.6±23.9%), but not on day 5 (Fig. 5.6A). These data suggested that precursor cells proliferated before differentiating into EpCAM$^+$DEC205$^+$ LC, and proliferation was absent in differentiated BMLC, which require a minimum of 3 days in culture for their generation (Becker et al., 2011, Chopin et al., 2013).

Following these experiments, we tested whether we could detect proliferation in our putative LC precursor population after a short pulse of EdU *in vivo*. 3 weeks after allo-BMT, we injected 100 μg EdU i.p. and harvested blood and ear skin 4 hours later. Uptake

of EdU by blood monocytes, LC precursors (population [pop] 1 and 2) and LC (pop 3) was analysed by flow cytometry.

Figure 5.6B outlines the gating strategy used to identify the myeloid cell populations found in the epidermis at this time point. Blood monocytes, which do not proliferate once they leave BM, (Patel et al., 2017) were negative for EdU. Likewise, mature LC, which divide very slowly in the epidermis (Chorro et al., 2009), also did not incorporate EdU in the 4 hour pulse. By comparison, both precursor populations displayed high levels of EdU incorporation. Together, these data supported the hypothesis that proliferation of precursors leads to the accumulation of LC in the skin after allo-BMT.
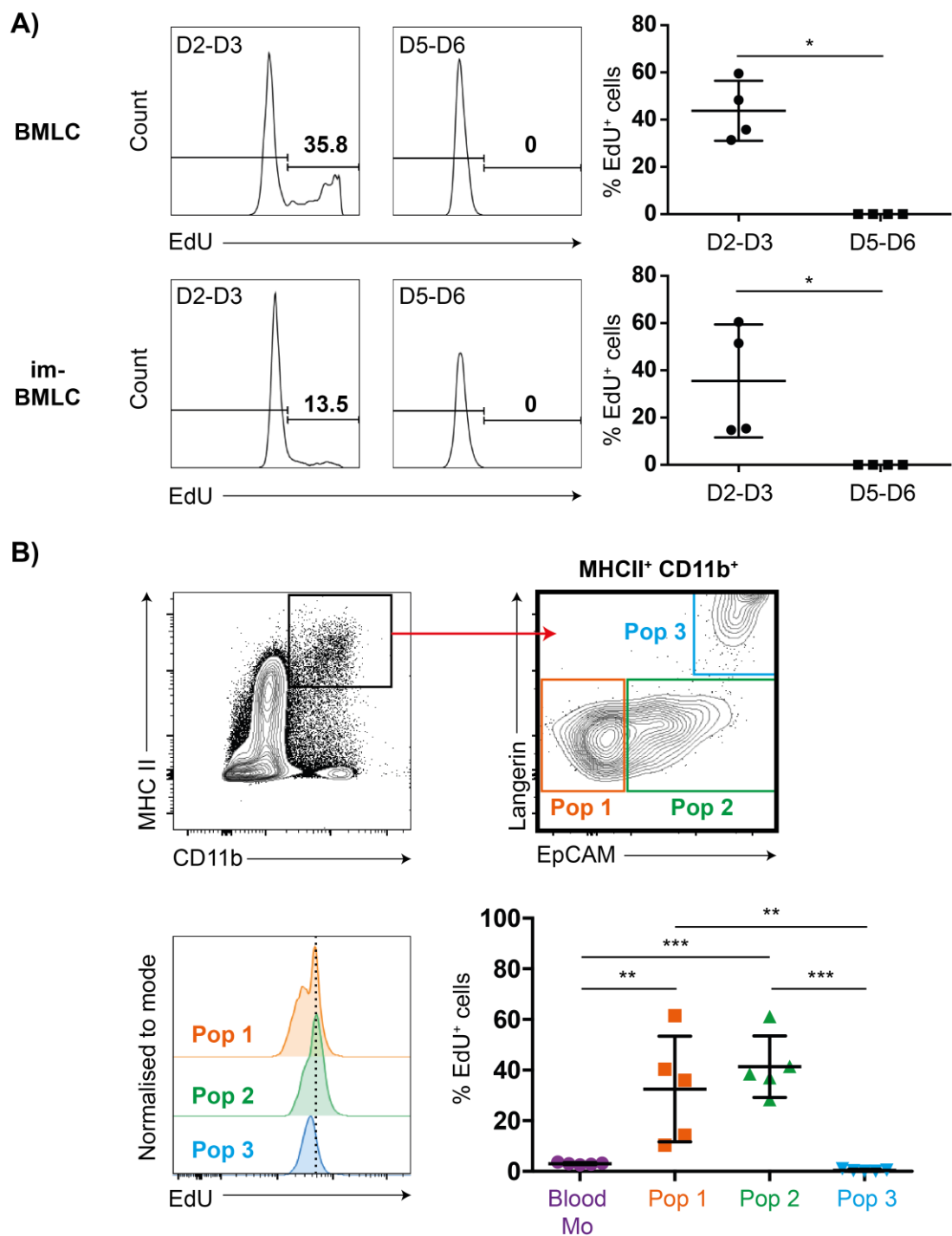
Figure 5.6 LC precursors proliferate in the epidermis

(A) BMLC cultures were pulsed with EdU for 24 h at day 2 (D2-D3) or at day 5 (D5-D6) and harvested on day 6.   Representative histograms (left panel) with numbers indicating frequency of the gated population,.and summary data (mean ± SD; right panel) of percentage of EdU$^+$ cells within the BMLC population (top panel) and im-BMLC population (bottom panel). EdU gates were set using an EdU negative control. N = 4, data from two independent experiments. Statistics calculated using a Mann-Whitney U test. **(B)** Allo-BMT mice at 3 weeks post-transplant were injected i.p. with EdU and harvested 4 hours later. EdU incorporation into epidermal populations was assessed. Blood monocytes (CD11b$^+$Ly6C$^+$CSF1R$^+$ cells were used as a control. The top panel shows the gating strategy used to identify populations 1, 2 and 3 from the epidermis. The bottom panel shows the representative histograms and summary data (mean ± SD) of percentage of EdU$^+$ cells within the gated populations. EdU gates were set using an EdU negative control. One experiment shown, N = 5, representative of two independent experiments (N = 8 total).   Statistics calculated using a one way ANOVA with Tukey's multiple comparisons test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

5.7.   Summary

In this chapter, we aimed to optimise the generation of BMLC in *in vitro* cultures in order to further dissect the extrinsic signals that control the generation of monocyte-derived replacement LC. Inflamed skin following GVHD is a complex environment and blockade of cytokine signalling or deletion of cytokines in genetically engineered mice can often lead to compensatory effects that mask physiological processes. Therefore, we used a reductionist approach to identify the role of specific factors.

Evidence for a monocytic precursor for LC

The rapid generation of LC-like cells in these cultures provided strong evidence for a monocytic precursor for LC. Furthermore, expression of CSF1R by im-BMLC was consistent with a monocyte ancestry, in accordance with reports that mucosal LC are derived partially from monocytes (Capucha et al., 2018). To further define this, *Cx3cr1-*Cre reporter mice could be utilised to trace the lineage of the different cells. This reporter strain fate maps tissue macrophages and monocytes (Yona et al., 2013) and LC precursors have been shown to express CX_3CR1 *in vivo* (Bennett and Ferrer, unpublished).

While we can be confident that BMLC were developing from a committed myeloid progenitor, we need to consider the possibility that our BMLC population is contaminated with pre-DC-derived cells; further experiments are required to assess expression of DC-associated transcription factors (e.g. *Batf3*, *Xcr1*).

It is surprising that GM-CSF is required in all systems to generate LC-like cells *in vitro,* when it is not required for *in vivo* LC development. LC precursors extravasate from the blood and migrate through the dermis before reaching the LC niche in the epidermis. The signals that the precursors receive in this migration step and the subsequent effects are currently unexplored. GM-CSF could potentially be providing the same signal in culture that LC precursors receive in the dermis. Reports have shown GM-CSF to be upregulated in injured skin and accelerate wound healing (Mann et al., 2001, Braunstein et al., 1994, Jyung et al., 1994). Indeed, we have shown upregulation of GM-CSF in the epidermis and dermis of GVHD mice (Fig. 3.7). Another possibility is that BMLC are generated via a pre-DC precursor that requires GM-CSF signalling. This would mirror mucosal LC differentiation and explain the requirement for TGF-β during BMLC development (Capucha et al., 2018). In this regard, the environment would be the dominant signal in LC development, allowing pre-DC precursors to differentiate into LC in certain conditions (e.g. in the mucosa), and monocytes to differentiate into LC in others (e.g. during skin inflammation). An interesting experiment would sort labelled LC precursors from the mucosal epithelium and transfer them into recipients with skin injury;

generation of skin LC derived from these precursors would provide evidence that environment is dominant over precursor origin.

Role of TGF-β and IL-34

Our experiments demonstrated that the development of BMLC from precursors was dependent on exposure to TGF-β throughout the culture. Possible explanations for this include apoptosis of TGF-β-sensitive cells in the absence of TGF-β, or more likely, commitment of the precursors to another differentiation pathway. Indeed, BMDC were expanded in GM-CSF cultures compared to TGF-β-supplemented cultures. Together, this data suggested that BMLC precursors were present early in the culture but were either absent or unresponsive to TGF-β signalling later in the culture.

The role of TGF-β in our culture system needs to be further tested. BMLC precursors were clearly responsive to TGF-β, even though TGF-β is not required for LC differentiation *in vivo*. We can test TGF-β dependency through the specific deletion of its receptor in myeloid cells, by crossing myeloid cell lineage Cre reporter mice with TGF-βR floxed mice. Initially we could use *Langerin-Cre* mice, which would specifically delete TGF-βR signalling in *Langerin*-expressing cells. However, Langerin protein was not expressed by BMLC, although they expressed *Langerin* mRNA (Chopin et al., 2013). This conditional deletion system would therefore not be suitable if TGF-β signalling was required before Langerin expression. Another option would be the use of *LysM*-Cre mice, which would specifically delete TGF-βR signalling in macrophages and neutrophils and allow us to identify whether BMLC were generated from macrophage precursors. A preferred option would be the use of *Cx3cr1*-Cre mice, which would specifically delete TGF-βR signalling in monocytes and conclusively demonstrate whether TGF-β signalling by monocyte-derived precursors was critical for BMLC differentiation. It is important to note that TGF-β can signal through TGF-βRI, II and III, and the use of floxed mice would only delete one receptor. If redundancy existed in precursor TGF-β signalling, then these experiments would not be suitable. In this regard, we could attempt to block TGF-β signalling using a combination of TGF-βR antagonists (SB-431542 blocks TGFβRI (Inman et al., 2002); LY2109761 blocks TGFβRI/II (Sawyer et al., 2003)).

As CSF-1, BMP7 and IL-34 are all involved in LC development and/or maintenance *in vivo*, we tested the effects of these cytokines on BMLC generation. IL-34 was the only cytokine to enhance BMLC numbers, due to an increased viability within MHC II^{low} im-BMLC. Responsiveness to IL-34 by im-BMLC fitted with their expression of CSF1R. By contrast, CSF-1, which also competes for CSF1R, did not enhance LC numbers in these cultures. This was unexpected because, while IL-34 has been shown to be essential for LC maintenance *in vivo,* CSF-1 is more important for initial repopulation of LC by recruited cells (Wang et al., 2016). Assessment of transcription factors revealed that

addition of IL-34 to the cultures increased the expression of *Id2* by EpCAM$^+$DEC205$^+$ cells. The generation of the persistent LC network is ID2-dependent, and outside of the skin, ID2 expression has been shown to promote survival of glioblastoma cells during metabolic stress by regulating mitochondrial function (Zhang et al., 2017), and control the survival of hepatic NKT cells (Monticelli et al., 2009). Given these data, we propose that IL-34 induces *Id2* expression in BMLC precursors, to increase their survival and differentiation into long-term LC. In these cultures, however, the factor driving the maturation of MHC II^{low} im-BMLC appeared to be missing. It would be interesting to test whether stimulation with a TLR agonist, for example, would lead to increased numbers of MHC II^{hi} BMLC under these conditions.

Repopulation of the LC network after injury has been reported to be independent from ID2 and IL-34. While we have demonstrated upregulation of *Id2* in response to IL-34 in our cultures, we have not shown dependency on ID2 for BMLC generation. To confirm lack of dependency, we could culture BM from *Id2*-deficient mice with GM-CSF and TGF-β and assess BMLC generation.

Aside from cytokine dependence for development, BMLC drew many parallels with primary cells; they were generated in the absence of IL-34, while addition of IL-34 enhanced survival. The question remains of why did CSF-1 have no effect on BMLC generation in our cultures? One suggestion is that CSF-1 is actually a recruitment signal for LC precursors, and is thus irrelevant in the culture setting. Wang et al demonstrated that CSF-1 expression in the skin peaked one week after UV-induced skin injury, coincident with inflammation, whereas IL-34 expression decreased (Wang et al., 2016). The two wave model of LC repopulation described by Sere et al. could potentially be explained by the first wave of Gr-1^{hi} monocytes signalling via CSF-1 and not receiving survival signals from IL-34. These LC do not differentiate into persistent LC, retain a monocytic signature and subsequently die. When skin inflammation has been regulated, and IL-34 coincidently is increased, precursors recruited to the skin by CSF-1 receive the survival signals required to differentiate into long-term LC. Moreover, in our model of GVHD, CSF-1 may not be upregulated to the same extent as in UV injury (possibly by lack of epidermal neutrophil infiltration), and therefore precursor recruitment and LC repopulation would be limited. Additionally, this would explain why we only see the recruitment of one wave of LC precursors to the epidermis, and that transcriptionally, these precursors have begun to differentiate into long-term persistent LC.

To test this hypothesis, future experiments are required to characterise neutrophil infiltration and assess CSF-1 and IL-34 protein expression in GVHD skin. An early time point, for example one week after BMT, would coincide with precursor recruitment to the

epidermis and would be a suitable starting place. This will determine whether CSF-1 and/or IL-34 is limiting in our model.

<u>Proliferation of precursors prior to differentiation into quiescent LC</u>

Finally, we have demonstrated that BMLC precursors proliferated before differentiating into mature BMLC, a finding that was mirrored *in vivo* and in accordance with the proliferative program highlighted by our transcriptional analyses of LC precursors. These data supported our hypothesis that proliferation, and not recruitment, of LC precursors was required for LC repopulation.

During LC differentiation, monocyte-derived LC precursors switch on a proliferative program that must subsequently be switched off for final differentiation into mature LC. While the specific signals for these switches during LC differentiation have not yet been elucidated, there is robust evidence for the role of notch signalling in the maintenance of epidermal cellular differentiation and proliferation (Artavanis-Tsakonas et al., 1999, Moriyama et al., 2008, Nickoloff et al., 2002, Okuyama et al., 2004, Nguyen et al., 2006, Wang et al., 2008, Yamamoto et al., 2003, Blanpain et al., 2006). Recently, repression of KLF-4 by epithelial notch-signalling has been shown to be required for monocyte-derived LC commitment (Jurkin et al., 2017).

Self-renewal in macrophages involves downregulation of c-Maf and MafB that relieves the repression of enhancers of self-renewal genes (Soucie et al., 2016). MafB/c-Maf deficiency was shown to dissociate cell cycle exit from terminal differentiation (Aziz et al., 2009), and these transcription factors have been shown to be essential mediators of epidermal progenitor differentiation by binding to epidermal differentiation transcription factor genes, including *Klf4* (Lopez-Pajares et al., 2015). A working model for LC differentiation would include downregulation of c-Maf and MafB – as monocytes have high expression of MafB (Sieweke et al., 1996) – to allow LC precursor proliferation. These transcription factors would later be upregulated to suppress proliferation and promote differentiation. Upstream regulators of c-Maf and MafB include the long non-coding RNAs (lncRNA) ANCR and TINCR (Lopez-Pajares et al., 2015), the transcription factor C/EBPδ (Borrelli et al., 2010) and the IL-10/STAT3 signalling pathway (Gemelli et al., 2014). However, the environmental signals that cause the switches in these transcriptional programs are not yet known; further experiments are required to define this mechanism.

In summary, our data strongly supported a monocytic origin for repopulating LC that can become long-term LC due to IL-34-dependent upregulation of *Id2*. We have defined proliferation of LC precursors as a vital, and unreported, step in LC development. Our data highlights the benefits of utilising *in vitro* culture models to study the differentiation

and survival of LC in a reductionist environment; however, these findings need to be further tested *in vivo*.

Chapter 6 Discussion

While myeloid cell differentiation in the steady state is reasonably well characterised, gaps in our understanding remain for myeloid cell differentiation during pathology. It is widely recognised that inflammation leads to altered recruitment of myeloid cells, however it is far less appreciated that pathology may lead to dysregulated differentiation, or even that dysregulation could potentiate disease. This project therefore aimed to characterise how pathology in GVHD alters myeloid cell populations and cutaneous immune function. To achieve this, we utilised a combinatorial experimental approach, assessing myeloid cell differentiation and function in an *in vivo* murine model of acute GVHD, complemented by a reductionist *in vitro* model of myeloid cell differentiation.

The pathology caused in the skin during GVHD has two critical effects. Firstly, the cutaneous environment is significantly altered which can impact upon function of resident and recruited cells. Secondly, allogeneic T cells force the replacement of LC by an ontogenetically distinct progenitor.

This project has further characterised the cellular and environmental changes to the skin as a result of GVHD. We identified ongoing myeloid infiltration into the dermis following BMT that was independent of T cells. Proteomic assessment of the skin compartments during GVHD revealed that the epidermis was not an overtly inflammatory environment and permitted the differentiation of monocyte-derived LC that were transcriptionally similar to their embryonic-derived counterparts. In contrast, screening of the dermis during GVHD indicated a pro-inflammatory environment, and IL-6 production by monocyte-derived cells was identified as a potential mediator of long-term breakdown in immune function.

Modelling LC differentiation from BM cells *in vitro* confirmed that this process was remarkably inefficient. We could infer that the growth factor requirements for LC differentiation *in vitro* were distinct from those *in vivo*, highlighting the importance of the cellular microenvironment in the development of immune cells. In particular, our data demonstrated that the differentiation of epidermal LC was distinct to mucosal LC as proliferation, and not recruitment, of LC precursors was evident in the skin.

Characterising the changes to the cutaneous immune environment following BMT

This project demonstrated that irradiation and BMT caused long lasting changes to the dermal immune compartment. This is so often ignored in experimental settings when these significant changes could seriously alter interpretation of experiments involving BMT. Recently, studies have begun to highlight the impact of BMT on recipients. BMT

has been shown to significantly alter cardiac macrophage phenotype and response to myocardial infarction (Protti et al., 2016). Syngeneic BMT in mice was also shown to cause a hyper-activated phenotype of CD11b$^+$ cDC in lungs with impaired migration, resulting in pathogenic Th responses to gammaherpesvirus infection (Zhou et al., 2016). A greater understanding of the effects of BMT on different tissues is required; this will help us to understand and treat complications in patients after BMT.

Our data revealed that LC repopulation was inefficient as there were fewer LC in the epidermis after BMT compared to steady state. Our functional analyses in chapter 4 revealed altered cutaneous immune functions after GVHD, however our experiments did not go so far as to determine a causative link between loss of LC and loss of function. Further functional assessment is required to determine if LC loss alters cutaneous immune function.

We hypothesise that LC function *in situ* may be impacted more significantly than functions that require migration of LC to the LN and T cell priming. These functions have in the past been clouded by the assumption that LC function primarily as migratory cells. However, evidence is accumulating to suggest a critical role for LC locally within the skin, in line with their macrophage origin (West and Bennett, 2017). Numerous studies have demonstrated that interactions between DC and T cells within peripheral tissues are critical for lymphoid function and survival (Bennett and Chakraverty, 2012, Honda et al., 2014), and our lab has demonstrated that LC directly license epidermal CD8$^+$ effector function in a murine model of GVHD (Bennett et al., 2011).

Further, LC may function in the local control of TRM. In the vaginal mucosa, related tissue macrophages produce CCL5, which is required for the recruitment and maintenance of clusters of protective CD4$^+$ TRM (Iijima and Iwasaki, 2014). Although the formation of CD8$^+$ TRM is not impaired in the absence of LC (Mohammed et al., 2016), LC have been reported to interact directly with CD4$^+$ TRM in human skin (Seneschal et al., 2012) and TRM in murine skin (Zaid et al., 2014). The concept of LC eliciting rapid localised immunity is, therefore, very attractive (West and Bennett, 2017). In the context of autoimmunity, psoriatic human skin TRM produce IL-17 (Cheuk et al., 2014), but healthy human skin TRM could not be polarised by IL-6, IL-1β and IL-23 to produce IL-17 *in vitro* (Cheuk et al., 2017), suggesting a requirement for cognate TCR-mediated interactions *in situ*. Indeed, in the vaginal lamina propria, cognate antigen presentation by CD301b$^+$ DC drove CD8$^+$ TRM-mediated protection after HSV-2 infection (Shin et al., 2016), and we therefore anticipate a similar role for LC in the skin. Evidence also suggests that LC directly interact with peripheral Treg (Seneschal et al., 2012), as has been suggested for resident tissue macrophages of the gut and lung (Denning et al., 2007, Soroosh et al., 2013, Coleman et al., 2013).

Future experiments are required to assess resident functions of LC following BMT. For example, loss of local Treg control could lead to chronic inflammation, infection or even autoimmunity. In the clinic, understanding the underlying cause of these complications could direct therapy development.

Testing cutaneous immune functions following GVHD

This project has demonstrated that monocytes are recruited to the dermis during GVHD and are persistent and pathogenic in the inflammatory environment. We demonstrated that mice following GVHD have a breakdown in cutaneous tolerance. Accumulating evidence supports the notion that Treg cells harbour plasticity by sensing microenvironmental factors (Gao et al., 2012a, Koenen et al., 2008, Miyao et al., 2012, Oldenhove et al., 2009, Wohlfert and Belkaid, 2010). In some cases, this is beneficial to the host, reprogramming the cells towards an effector phenotype and permitting immune activation adequate to fight infection. However, our data indicated that Treg reprogramming in a chronic inflammatory environment led to a breakdown in immune function.

Treg reprogramming has been shown to cause the onset of various autoimmune and chronic inflammatory diseases (Dominguez-Villar et al., 2011, Kimura and Kishimoto, 2010, Miyara et al., 2011). IL-6 has been described as an important factor for determining Th17/Treg balance (Kimura and Kishimoto, 2010), and anti-IL-6 blockade effectively suppressed autoimmune disease in mice (Fujimoto et al., 2008, Hohki et al., 2010, Haruta et al., 2011, Serada et al., 2008). It could therefore be possible that IL-6 mediated dysregulated Treg reprogramming during acute GVHD is a causative factor in the onset of the autoimmune resembling chronic GVHD. While several studies have investigated IL-6 blockade as a therapy for acute GVHD (Chen et al., 2009, Noguchi et al., 2011, Tawara et al., 2011), few have studied the causative link between the acute and chronic stages of the disease. One study observed increased IL-6 levels during disease progression of sclerodermatous chronic GVHD, however IL-6 blockade was only effective in attenuating disease severity when administered prior to onset of chronic GVHD (Le Huu et al., 2012). This indicated that IL-6 may indeed be a causative link between acute and chronic GVHD. Prophylactic IL-6 blockade has only been investigated in one clinical trial for acute GVHD, with encouraging results (Kennedy et al., 2014). Future studies are therefore warranted to assess anti-IL-6 therapy in recovering acute GVHD patients as a prophylaxis for chronic GVHD.

Modelling LC differentiation *in vitro*

This project further demonstrated that monocytes were recruited to the epidermis and appeared to generate long-term LC that were transcriptionally similar to steady state LC.

This indicated that, even in the GVHD setting, environment was dominant over ontogeny of cells for LC repopulation, and was in line with studies in other tissues revealing that adult monocytes can generate tissue-resident macrophages with a very similar transcriptional profile (van de Laar et al., 2016, Scott et al., 2016b, Gibbings et al., 2015).

It may be the case that all of the precursors of replacement LC have not yet been fully defined, but we are certain that monocytes are at least a major population that have been reported to either partially (Sere et al., 2012) or fully (Ginhoux et al., 2006, Nagao et al., 2012) acquire the phenotype of steady state LC. Most commonly, LC repopulation has been studied in models where the LC network is depleted by UV radiation. Studies using this inflammatory stimulus have described full repopulation of the LC network in murine and human skin, returning to original steady state numbers (Merad et al., 2002, Achachi et al., 2015). In this project however, LC numbers did not recover to steady state levels following BMT. A difference between the models is that UV radiation does not fully deplete the LC network, and therefore recipient LC persist following UV exposure that can divide to contribute to LC repopulation. It is possible that experimental models of GVHD are creating a clinical scenario that the skin has not evolved mechanisms to deal with. We have not assessed T cell-mediated damage to the BM, nor to keratinocytes in the epidermis. Keratinocyte-derived TGF-β is critical for LC maintenance, and alteration of TGF-β production following damage would certainly affect LC survival. Indeed, reduction of TGF-β in the oral epithelium in a titanium dental implant model led to impaired differentiation of LC and a build-up of immature LC that phenotypically mirrored LC precursors in our model (Heyman et al., 2018).

The question remains: why do monocytes fully differentiate into LC in some models, and only partially differentiate in others? Sere et al. described a first wave of activated monocytes that only generate short-term LC, and Heyman et al. described a build-up of immature LC in a dental implant model (Sere et al., 2012, Heyman et al., 2018). Similarly, monocyte-derived inflammatory LC-like cells have been implicated in mediating inflammation in psoriasis (Singh et al., 2016, Martini et al., 2017). It could be possible that activated monocytes in these models are not receiving survival signals that would enable them to differentiate into LC and become long-lived cells. One study has revealed that autonomous TNF is critical for *in vivo* monocyte survival in the steady state and during inflammation (Wolf et al., 2017). Upregulated TNF-α is a hallmark of GVHD (Korngold et al., 2003) and thus the ongoing inflammatory environment could in fact be beneficial for survival of monocytes and explain why we do not detect short-term LC in our model. Likewise, upregulation of TNF-α promotes survival of infiltrating dermal monocytes and explains persistence of these cells in our model.

But what is the functional relevance of these recruited monocytes? The LC precursors that we detected in our model resembled monocyte-derived inflammatory LC in models of psoriasis (Singh et al., 2016). Our transcriptional analyses of the precursor cells indicated that these cells were poised for antigen presentation, and thus suggested the potential of these cells to be pathogenic. One possibility is that accumulation and persistence of LC precursors could cause pathology. Further work is required to investigate whether under pathological conditions, such as during psoriasis, LC precursors contribute to disease.

Understanding the differentiation and persistence of cells requires a thorough appreciation of the complicated interplay of cellular cytokine requirements. This is beginning to be elucidated for LC, however this area is still not fully defined. This project utilised a reductionist *in vitro* culture model to further understand LC differentiation from BM. TGF-β was absolutely required for LC differentiation *in vitro*, in line with previous studies (Strobl et al., 1996, Chopin et al., 2013, Jaksits et al., 1999, Jurkin et al., 2017, Geissmann et al., 1998, Hoshino et al., 2005, Larregina et al., 2001). However, several studies have identified a redundancy between TGF-β and BMP7 for differentiation of LC from human haematopoietic cells (Yasmin et al., 2013, Milne et al., 2015). In agreement with Capucha et al., we could not detect a role for BMP7 in murine LC differentiation *in vitro* (Capucha et al., 2018). BMP7 has been shown to be required for the transition to the epithelium by mucosal LC (Capucha et al., 2018), so potentially the requirement for this cytokine is bypassed in a culture scenario. This project has however highlighted IL-34 as a mediator of long-term LC survival through the upregulation of ID2. Further work is required to assess the applicability of this finding to the repopulation of LC *in vivo*, with the potential of reprogramming pathogenic monocytes in inflammatory diseases.

Limitations and future work

A critical limitation of this project is that we utilised a murine model of acute GVHD. An essential next step is corroborating our findings in the clinical context. Firstly, we would aim to directly compare the dermal cellular environment after BMT. This obviously has its difficulties as patients undergoing BMT have underlying disease. We would need to obtain samples from autologous or syngeneic BMT recipients to assess the effects of conditioning on the cellular composition of the dermis. Similarly, it would be useful to identify if pathogenic monocytes and dysfunctional Treg are present in human skin following GVHD. If possible, a direct comparison of murine and human RNAseq samples in the context of GVHD could identify similar transcriptomic signatures and validate our findings.

While clinical results are necessary, basic science is an excellent tool to accelerate our knowledge of skin immunology. Following on from our findings, we aim to disrupt IL-6

signalling in GVHD mice, possibly using anti-IL-6 monoclonal antibodies, to identify if tolerance is restored. This would demonstrate that IL-6 was a crucial mediator in the breakdown in tolerance. We additionally need to identify the signal in GVHD skin that is switching on the pathogenic signature in dermal monocytes. A first experiment would screen the skin environment in the early stages of GVHD for known DAMP, for example by western blot and qRT-PCR. We could also isolate dermal monocytes and assess PRR responsiveness *in vitro*.

Further work is required to fully characterise the putative LC precursors in our model. Identifying cytokine requirements for development and recruitment signals is essential. Experiments to block precursor TGF-βRI signalling are planned to identify if this receptor is important for development or proliferation. Additionally, we aim to isolate monocytes and track their development into damaged LC depleted skin, by labelling or utilising a fluorescent reporter strain (e.g. ubiquitous RFP). Identification of labelled monocytes in GVHD epidermis would validate our findings.

Summary

Overall, this work has highlighted the developmental plasticity of monocytes during acute GVHD. The inflammatory cutaneous environment facilitated LC replacement by monocytes in the epidermis but in parallel generated pathogenic monocytes in the dermis. Understanding the environmental cues underpinning these processes is crucial to the future directions of this project. Furthermore, delineating the developmental requirements for replacement LC could potentiate therapy design for chronic inflammatory diseases.

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Chapter 8 Appendix

The following journal article is included as an appendix:

WEST, H. C. & BENNETT, C. L. 2017. Redefining the Role of Langerhans Cells As Immune Regulators within the Skin. *Front Immunol,* 8**,** 1941.

Redefining the Role of Langerhans Cells As Immune Regulators within the Skin

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Langerhans cells (LC) are a unique population of tissue-resident macrophages that form a network of cells across the epidermis of the skin, but which have the ability to migrate from the epidermis to draining lymph nodes (LN). Their location at the skin barrier suggests a key role as immune sentinels. However, despite decades of research, the role of LC in skin immunity is unclear; ablation of LC results in neither fatal susceptibility to skin infection nor overt autoimmunity due to lack of immune regulation. Our understanding of immune processes has traditionally been centered on secondary lymphoid organs as sites of lymphocyte priming and differentiation, which is exemplified by LC, initially defined as a paradigm for tissue dendritic cells that migrate to draining LN on maturation. But, more recently, an awareness of the importance of the tissue environment in shaping effector immunity has emerged. In this mini-review, we discuss whether our lack of understanding of LC function stems from our lymph node-centric view of these cells, and question whether a focus on LC as immune regulators *in situ* in the skin may reveal clearer answers about their function in cutaneous immunology.

Keywords: Langerhans cells, skin, epidermis, macrophages, migration

INTRODUCTION

Langerhans cells (LC) are a unique population of mononuclear phagocytes that are seeded from common macrophage precursors in the skin epidermis before birth (1) (and reviewed in this topic). They are highly conserved across vertebrate species (2, 3) and this, with their location at the interface with the environment, suggests the strategic importance of LC as immune sentinels at the skin barrier surface.

Traditionally, immunologists have focused on secondary lymphoid organs as the center of T cell immunity, assuming that instructions given during the priming of naïve T cells by migratory and resident dendritic cells (DC) were sufficient for differentiation and effector function by T cells recruited to peripheral sites of tissue injury. However, the field is beginning to appreciate importance of the tissue environment in regulating effector and regulatory T cell function, and it is clear that antigen-presenting cell-T cell interactions play a key role in T cell survival and function outside lymphoid organs (4). Despite sharing an origin with other tissue-resident macrophages, differentiation of LC is associated with the acquisition of DC-like functions, namely the ability to migrate to skin-draining lymph nodes (LN) and interact with naïve T cells. Observation of this property in the 1980s has dominated the field, and as a result, studies to define LC function in the skin have focused largely on their role as DC-like cells in priming T cell immunity [reviewed by Romani et al. (5)]. However, to date, a consistent role for LC as primers of T cell immunity has not emerged.

In particular, there are few scenarios, if any, in which removal of LC ablates immunity to infection, or results in the development of severe autoimmunity in mice; and, despite the common observation that LC are sufficient to prime T cell immunity after the experimental provision of antigen and adjuvants that may favor LC activation and migration [e.g., Ref. (6)], few papers have explicitly identified a crucial role for migratory LC, and not dermal DC, under physiological conditions.

We suggest that shifting our focus to potential roles for LC *in situ* in the skin, a function more in keeping with their development as macrophages, will provide clearer answers about the importance of these unique cells in skin immunity. In this mini-review, we will consider the evidence for LC functions within the skin (**Figure 1**), and discuss whether our historic focus on LC as exemplars of migrating DC has skewed our understanding of their role in skin immunity.

ELUCIDATING LC FUNCTION *IN SITU* IN THE SKIN

Barrier Site Surveillance
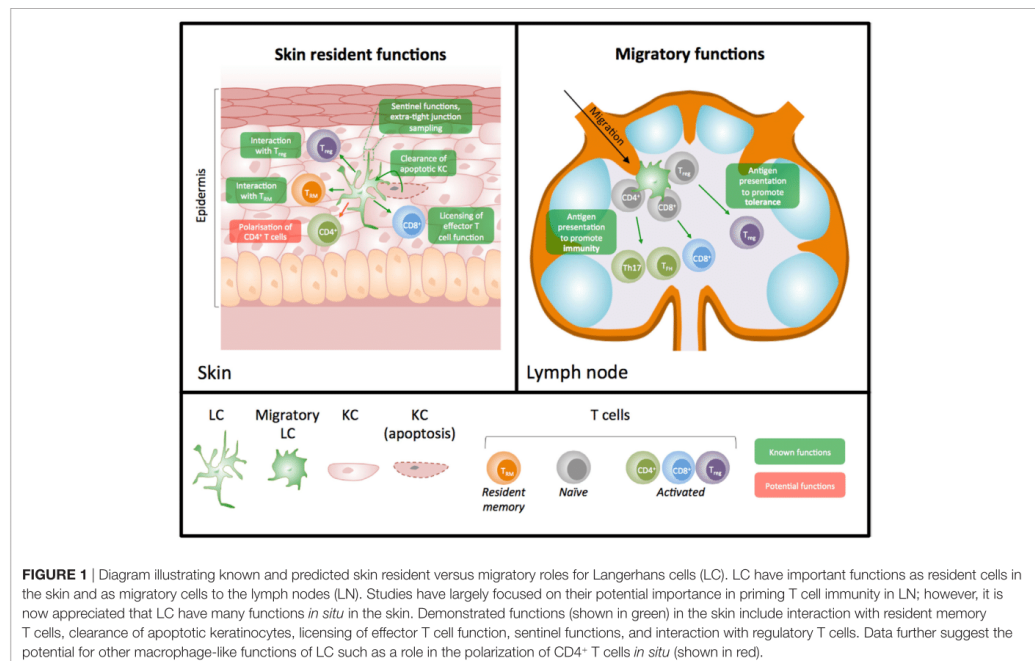#### Sensing the Local Environment
Mononuclear phagocytes have important functions within tissues, and barrier site non-migratory macrophages are essential for surveillance of the local environment. $CX_3CR_1^+$ intestinal macrophages form dendritic projections, termed transepithelial

dendrites, which penetrate the intestinal epithelium to sense commensal microbes and sample luminal antigens. Barrier integrity is maintained by the formation of tight junctions between the macrophages and epithelial cells (7, 8). In the central nervous system, microglia are highly dynamic in their resting state and rapidly extend and retract their processes (9, 10). This motility, termed synaptic pruning, allows them to make frequent and transient contact with synapses, actively engulfing synaptic material (11, 12), and is essential in nervous system development and maintenance (9, 13, 14).

Langerhans cells also constantly extend and retract dendrites between keratinocytes, in a behavior termed dendritic surveillance extension and retraction cycling habitude (15). Barrier integrity is maintained by the *de novo* formation of tight junctions between keratinocytes and LC, enabling LC to sample the extra-tight junction environment without loss of integrity (16). Thus, in this respect, LC can be seen to closely mimic non-migratory cells, leading to the question of how LC behavior compares to other resident macrophage populations once foreign material has been detected.

Local Interaction with Viruses
Langerhans cells are ideally positioned to respond to viruses that enter the body *via* the skin, namely human immunodeficiency virus (HIV), herpes viruses such as herpes simplex virus (HSV) or varicella zoster virus, and poxviruses (human papilloma

FIGURE 1 | Diagram illustrating known and predicted skin resident versus migratory roles for Langerhans cells (LC). LC have important functions as resident cells in the skin and as migratory cells to the lymph nodes (LN). Studies have largely focused on their potential importance in priming T cell immunity in LN; however, it is now appreciated that LC have many functions *in situ* in the skin. Demonstrated functions (shown in green) in the skin include interaction with resident memory T cells, clearance of apoptotic keratinocytes, licensing of effector T cell function, sentinel functions, and interaction with regulatory T cells. Data further suggest the potential for other macrophage-like functions of LC such as a role in the polarization of CD4$^+$ T cells *in situ* (shown in red).

181

virus, HPV), and this interaction has been extensively reviewed elsewhere [e.g., Ref. (17–19)]. LC express a number of pattern recognition receptors but do not efficiently internalize bacteria *in vitro*, leading to the suggestion that they preferentially prime antiviral immunity (20, 21). However, infection of LC by HSV or HPV leads to their destruction; priming of T cell immunity in this situation probably depends on transfer of viral antigen to other DC. In the case of HSV, non-infected epidermal LC may acquire antigen from apoptotic LC in the skin and migrate to draining LN. Here, transfer of antigen to cross-presenting DC subsets is required for the initiation of CD8$^+$ T cell immunity (22, 23). By comparison, while LC appear to be the major infected immune cell after HIV infection *via* the anogenital tract (24), there is evidence that they may restrict HIV replication or transmission (25, 26), and their protective role in HIV infection continues to be investigated (27). In this respect, LC resemble other tissue macrophages, which also restrict HIV and other viral infections (28, 29). Thus, LC may provide a repository of virally infected cells under some conditions, but the implications for this interaction for innate and adaptive immunity still remain unclear.

Innate Control of Skin Immune Homeostasis

A key role for macrophages as resident tissue cells is the maintenance of immune homeostasis. This occurs partly through their scavenger function, rapidly clearing debris from dying cells in the steady state and after infection. This uptake and recognition of apoptotic cells is an important mechanism for maintaining local immune tolerance to self-antigens (30, 31). TAM (TYRO3/ AXL/MER) family receptor tyrosine kinases are expressed by macrophages, DC and natural killer cells in the immune system. Engagement of the receptors by their ligands, growth-arrest-specific (GAS)6, and protein S, enhances uptake of apoptotic cells and suppresses inflammation (32). In the gut, MER is upregulated by macrophages in response to induction of apoptosis in intraepithelial cells, probably to control local immune suppression to self-antigens (31). LC employ a similar innate mechanism to control local tolerance in the skin: apoptotic keratinocytes accumulate in the skin of mice depleted of LC (33); and transforming growth factor (TGF)-β induced expression of AXL by LC enhances uptake of GAS6-expressing apoptotic keratinocytes, inhibiting of production of inflammatory cytokines (34).

Regulation of T Cell Function in the Skin

In addition to innate control of the immune environment, tissue-resident cells may also influence adaptive immunity by recruited regulatory and conventional T cells. The following section considers the evidence for LC mediating this function directly in the skin.

Enhancing the Accumulation and Function of Regulatory T Cells *In Situ*

Tissue macrophages play a key role in the suppression of local adaptive immunity, *via* direct and indirect interaction with CD4$^+$ regulatory T cells (T$_{reg}$). CX$_3$CR$_1$-expressing macrophages are an abundant population in the lamina propria of the small intestine,

where they regulate T$_{reg}$ differentiation *in situ* by production of IL-10, retinoid acid (RA) and TGF-β (35), and are critical for the local proliferation of T$_{reg}$ and induction of oral tolerance (36). Likewise, lung-resident tissue macrophages constitutively express TGF-β and RA to induce T$_{reg}$ within the lung tissue (37, 38), while IL-10 production by Kupffer cells in the liver, and microglia in the brain, has been implicated in the induction of T$_{reg}$-induced tolerance (39–42). In addition to promoting cytokine-mediated differentiation and function, macrophages directly interact with T$_{reg}$ *in situ*. In a model of experimental autoimmune encephalomyelitis, direct contacts between sialoadhesin$^+$ macrophages and T$_{reg}$ regulated local immune suppression to limit progression of disease (43), and in the liver, presentation of antigen and arrest of T$_{reg}$ by Kupffer cells led to local secretion of IL-10 (41).

Langerhans cells have been directly implicated in a number of T$_{reg}$-dependent models of immune suppression in the skin but, consistent with the LN-centric view of LC research, it is often unclear whether cellular interactions between LC and T$_{reg}$ are occurring in the LN, the skin, or both. For example, while use of bone marrow chimeras in which radio-resistant LC are selectively deficient for the gene *Cdkn1a* suggests a clear role for LC in the priming of CD4$^+$ T$_{reg}$ in LN after exposure to ionizing radiation (44), the precise location(s) at which LC are required has not been defined for the accumulation of T$_{reg}$ in the ear skin of *Leishmania*-infected mice (45), or expansion and function of activated ICOS$^+$ T$_{reg}$ in a murine model of skin sensitization and tolerance (46). However, cognate interaction between LC and CD4$^+$ T cells inhibits effector T cell responses after challenge with topical sensitizers (47), and resting LC from human skin selectively and specifically induce the activation and proliferation of skin resident T$_{reg}$ *in vitro* (48). Upregulation of receptor activator of nuclear factor κ B (RANK) ligand by apoptotic keratinocytes induces IL-10 production by LC (49), inferring the potential of LC to directly regulate effector T cell immunity *in situ*, after exposure to ultraviolet (UV)B radiation. This concept is supported by Loser et al. who demonstrated that UV-induced immunosuppression is mediated by RANK-RANKL signaling between LC and keratinocytes, resulting in an increased capacity of LC to induce IL-10-driven CD4$^+$ T$_{reg}$ proliferation (50). CD300a is a glycoprotein expressed by myeloid cells, which binds phosphatidylserine when exposed on the plasma membrane of apoptotic cells. Interaction between CD300a$^+$ LC and apoptotic epithelial cells in the skin restricts numbers of local T$_{reg}$, mimicking interactions between epithelial cells and CD11b$^+$CX$_3$CR$_1^+$ cells in the lungs and gut. This interaction was required for control of *S. typhimurium* in the gut, but promoted deleterious inflammatory responses including atopic dermatitis in the skin (51). Together, these data strongly support a tissue role for LC in controlling immune suppression by T$_{reg}$.

Elegant *in vivo* imaging studies by the Germain lab recently revealed close localization of migrating dermal DC with T$_{reg}$ clusters in mouse LN in the steady state (52), supporting a previous study that demonstrated a specific role for dermal RelB$^+$ DC in the maintenance of skin tolerance (53). Given the sessile behavior of LC in the steady state (54), these data support a dominant role for dermal DC in maintaining day-to-day tolerance to skin antigens. Therefore, the challenge now is to understand whether there are

contexts in which LC may take over this regulatory role in LN. A recent study attempted to address this question using a novel genetic model of inducible neo-antigen expression by LC, but not other Langerin$^+$ DC, in the steady state (LCre-GFPOVA mice) (55). In this model, presentation of endogenous ovalbumin leads to priming of CD8$^+$ T cells in LN and accumulation of cutaneous CD4$^+$ T$_{reg}$. It would be informative to test how the T$_{reg}$ are primed in this model and whether LC are required for their suppressive function in the skin.

Activation of Effector T Cell Immunity in the Skin

While it is accepted that the tissue-immune environment is important for the differentiation and function of T$_{reg}$, textbook immunology tells us that priming of naïve conventional T cells in LN provides all the requisite signals for differentiation into functional effector/memory T cells. However, numerous studies have now demonstrated the importance of interactions between myeloid cells and conventional T cells for T cell function and survival within peripheral tissues [reviewed in Ref. (4), see also Ref. (56)]. We have demonstrated a unique and novel role for LC in licensing effector function of CD8$^+$ T cells in the epidermis (57). In this section, we will consider the evidence to suggest that LC may also drive the function of other effector T cells *in situ*.

Licensing of CD8$^+$ T Cell Function by LC

Langerhans cells are highly radio-resistant and, as such, persist following conditioning of patients prior to allogeneic stem cell transplantation for blood cancers and other hematopoietic diseases. Entry of activated donor T cells into inflamed organs, including the skin, frequently leads to graft-versus-host disease (GVHD) in these patients (reviewed in this topic by Santos e Sousa and Chakraverty). LC are sufficient to prime the donor T cell response leading to GVHD (58), but are not required for systemic GVHD in the presence of conventional DC populations (59). To understand whether LC are important in cutaneous GVHD, we combined the Langerin-diphtheria toxin receptor model [in which LC are inducibly depleted upon injection of diphtheria toxin (60)] with a murine allogeneic model of GVHD (61). We demonstrated that LC were not required for priming of donor T cells, in agreement with the published literature (59). However, when we focused on the cellular interactions occurring in the skin, we observed that LC licensed the upregulation of epidermal effector CD8$^+$ T cell function, leading to GVHD at the site of inflammation (57). This research, therefore, revealed a novel role for LC outside LN.

LC-Dependent Control of CD4$^+$ T Cell Function

Polarized CD4$^+$ T cells that exit LN after priming maintain the plasticity to adapt to the tissue environment at their destination (62). This is particularly true for Th17 cells that may acquire a different functional fate depending on signals received from their surroundings. IL-23 production by monocytes and macrophages in inflamed tissues has been closely linked to the activation of IL-17-producing CD4$^+$ T cells [e.g., Ref. (63–65)], and Foucher et al. demonstrated that interaction between human macrophages and memory CD4$^+$ T cells *via* membrane-bound IL-1α is sufficient to polarize T cells toward Th1 or Th17-like phenotypes (66).

Moreover, "inflammatory DC" from patient synovial fluid or tumor ascites stimulate IL-17 production from autologous CD4$^+$ T cells, suggesting that myeloid cells may directly induce differentiation of Th17 cells within tissues (67).

Based on these macrophage data, it seems likely that LC perform a similar role in the skin, and it is clear that activated LC produce Th17-polarizing cytokines *ex vivo* (68–70). However, the ability of LC to induce differentiation and activation of epidermal Th17 cells *in situ* has not been directly investigated. LC are required for the priming of Th17 cells in response to topical *Candida albicans* infection (71, 72), and for the accumulation of both IL-17$^+$CD4$^+$ $\alpha\beta$ and $\gamma\delta$ T cells in the epidermis of mice with *Staphylococcus aureus* dysbiosis (73); however, neither study investigated whether LC are required *in situ* for the functional activity of cutaneous Th17 cells. Likewise, there are currently conflicting data on whether IL-23 production by LC is important for IL-17-dependent psoriasis-like disease (74, 75), and, to date, studies have focused on an Imiquimod-driven, $\alpha\beta$ T cell-independent, $\gamma\delta$ T cell-dependent model of disease. Thus, the potential importance of interactions between LC and epidermal CD4$^+$ T cells in psoriasis has not been established.

LC and Cutaneous-Resident Memory T Cells

Human skin contains billions of T cells, and the majority express markers associated with retention as resident memory cells (T$_{rm}$) (76). The concept of LC eliciting rapid localized immunity *via* activation of these cells is, therefore, very attractive. Precedent for interaction between tissue macrophages and T$_{rm}$ has been set in the vaginal mucosa, where macrophage-derived CCL5 is required for the recruitment and maintenance of clusters of CD4$^+$ T$_{rm}$ that protect against viral challenge (77). However, despite evidence that LC interact directly with CD4$^+$ memory T cells in human skin (48), and data demonstrating that LC interact with, and may control local migration of T$_{rm}$ within the epidermis (78), formation of CD8$^+$ T$_{rm}$ is not impaired in the absence of LC (79).

IL-17 production by human skin T$_{rm}$ is associated with psoriasis (80). However, a subsequent study from the same lab demonstrated that incubation with IL-6, IL-1β, and IL-23 were not sufficient *in vitro* to induce IL-17 production by CD8$^+$ T$_{rm}$ from healthy skin, suggesting a requirement for cognate T cell receptor-mediated interactions *in situ* (81). These data strongly infer a role for local interactions with LC, or other epidermal cells, in controlling T$_{rm}$ fate in the context of autoimmunity.

CONCLUDING REMARKS: WHY DO LC EVOLVE THE CAPACITY TO MIGRATE?

Despite the development of LC from a common macrophage precursor (1), transcriptional profiling demonstrates a gene expression profile that is more similar to migrating DC than other macrophage populations (3, 82), and LC share an antigen processing and presentation machinery with DC (83). Resident macrophage precursors that are recruited into different sites respond to tissue-specific signals and differentiate into a functional cell type that is adapted to that niche (84). For example, lung macrophages develop the capacity to phagocytose excessive

surfactant proteins, thereby preventing alveolar proteinosis (85). These observations lead us to anticipate the existence of environmental pressures in the epidermis leading to functional evolution of LC toward a DC-like cell. However, given the paucity of data showing a clear requirement for LC in priming immunity to infections, and the frequency of DC in the dermis, the unanswered question remains of why the acquisition of a DC-like function by LC is so important for protection of the organism?

Egress of LC from the keratinocyte network in the epidermis to LN is a carefully choreographed process. In the epidermis, inflammation and/or pathogen-derived signals block TGF-β-mediated retention by integrins (79, 86) and promote down-regulation of the cell adhesion protein E-cadherin (87), together allowing release of LC from surrounding keratinocytes. Activated LC then secrete metalloproteinases (MMP) 2 and 9, required to break though the basement membrane and enter the dermis (88). Once in the dermis, LC upregulate the chemokine receptor CCR7 that conducts entry into the dermal lymphatics (89, 90), mirroring the migratory pathway used by tissue DC to enter LN.

The requirement to extricate themselves from the keratinocyte network in the epidermis means that LC are slower to arrive in draining LN than dermal DC after antigenic challenge, suggesting that dermal DC may dominate in priming T cell responses to antigens that enter the dermis (54, 91). However, there are scenarios in which LC appear to take charge. Epicutaneous infection models with the pathogens *C. albicans* or *S. aureus* have demonstrated clear roles for LC in priming protective CD4$^+$ T cell responses (71, 72, 92), and ionizing radiation specifically activates LC to prime T$_{reg}$ in LN (44). Human, but not murine, LC express high levels of the invariant MHC-like molecule, CD1a. Recognition of CD1a on LC by recruited autoreactive T cells, or T cells specific for the poison ivy lipid urushiol, results in enhanced activation of IL-22- or IL-17-producing CD4$^+$ T cells, respectively (93, 94). These data support murine studies suggesting that LC prime CD4$^+$ T cells responses to some topically applied sensitizers that do not reach the dermis (95, 96), potentially demonstrating a requirement for migratory epidermal cells in the rapid initiation of T cell responses to topical sensitizers or irritants.

Langerhans cells have also emerged as key players in the activation of CD4$^+$ T follicular helper cells (T$_{fh}$), which are required for germinal center formation and antibody affinity maturation in response to infection or vaccination (97). Here, ablation of LC results in clear defects in the formation of germinal centers and production of mature antibodies (92, 98). Importantly, this requirement for LC persists when antigen is delivered into the dermis and, therefore, should theoretically be preferentially acquired by dermal DC (97, 99). By contrast, an alternative study demonstrated that loss of LC led to increased production of autoantibodies in a murine model of lupus dermatitis (100), implying that, under steady state conditions, LC may also regulate activation of T$_{fh}$, either directly or indirectly *via* T$_{reg}$. However, dermal DC also prime T$_{fh}$ (101), indicating that the need to generate antibody responses to skin antigens is not a sufficient functional pressure to explain the acquisition of migratory function by LC.

In conclusion we argue that, while significant progress has been made in our understanding of the key role LC play in cutaneous immunity, we now need to shift our focus from LN to the skin. Precise definition of the sites of interaction between LC and T cells, or other cells, is needed to determine the selective pressures that drive the relative tissue-resident or migratory DC-like functions of these unique cells.

AUTHOR CONTRIBUTIONS

HW and CB formulated opinions and concepts for the mini-review and wrote it together.

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185

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186

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187