AN INVESTIGATION OF LANGERHANS' CELL FUNCTION IN AGED SKIN

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Abstract

With increasing age, aspects of the innate and adaptive immune systems show functional decline. In the skin this is associated with an increased incidence of epidermal malignancies and infections, a decreased incidence of contact allergy, and the development of autoimmunity. The mechanisms underlying these clinical effects in aged skin are poorly understood. Langerhans’ cells (LCs), which are members of the wider family of dendritic cells (DCs), reside in the epidermis where they act as sentinels of the immune system by processing and presenting antigen and inducing T cell responses. Previous investigations have suggested that the number of epidermal LCs is reduced, and that the motility of LCs is impaired in aged skin.

A series of investigations was performed to characterise the mechanistic basis for the reduced frequency and restricted mobility of epidermal LCs in the skin of the elderly. Initially LC-like cells were cultured from circulating monocyte precursors and characterised using flow cytometry. The ability of precursors to differentiate into LC-like cells was not impaired in the aged; furthermore there were no age-associated differences in expression of markers of LC activation at baseline or upon stimulation. The phenotype of epidermal LCs was assessed using flow cytometric analysis of epidermal cell suspensions and did not appear altered in aged individuals. In addition, using the same techniques with dermal cell suspensions the dermal DC population was not altered with age. Langerhans’ cell migration from epidermal explants prepared from the skin of aged individuals was impaired but could be restored with exogenous interleukin (IL)-1β. There was no age-related reduction in the epidermal levels of IL-1β or caspase-1 (IL-1β converting enzyme which converts pro-IL-1β to the active form) or the expression of the IL-1 receptor I (IL-1RI), to account for this observation. However, the amount of IL-1 receptor antagonist was reduced in aged skin suggesting a change in the overall local cytokine balance. Based on previous reports that topical retinoic acid (RA) can increase cutaneous IL-1 production, a 4-day patch test assay was performed using 0.025% all-trans RA cream to explore whether this could restore LC migration in the aged. There was no effect on LC migration from epidermal explants prepared after treatment with RA in the aged.

These data demonstrate that changes in LC function in the elderly may not be associated with changes in systemic DC biology. Age related changes in the cutaneous microenvironment are likely to be more relevant.
Declaration

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Dedication
This thesis is dedicated to my husband, Ian, and son, James, who are always there for me.

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### List of Abbreviations

- **AAD**: aminoactinomycin
- **AF**: alexafluor
- **ANOVA**: analysis of variance
- **APC**: allophycocyanin
- **BSA**: bovine serum albumin
- **CCL**: cc chemokine ligand
- **CCR**: cc chemokine receptor
- **CD**: cluster of differentiation
- **CHS**: contact hypersensitivity
- **CSF-1R**: colony stimulating factor 1 receptor
- **Ct**: cycle threshold
- **CXCL**: cx chemokine ligand
- **CXCR**: cx chemokine receptor
- **DAPI**: 4',6-diamidino-2-phenylindole
- **DC**: dendritic cell
- **DC-SIGN**: dendritic cell specific ICAM-3 grabbing nonintegrin
- **DNA**: deoxyribonucleic acid
- **DT**: diphtheria toxin
- **ECM**: extracellular matrix
- **EDTA**: ethylenediaminetetraacetic acid
- **ELISA**: enzyme linked immunosorbent assay
- **EpCAM**: epithelial cellular adhesion molecule
- **EU**: endotoxin unit
- **FACS**: fluorescence activated cell sorting
- **FcR**: Fc receptor
- **FCS**: foetal calf serum
- **FITC**: fluorescein isothiocyanate
- **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase
- **G-CSF**: granulocyte colony stimulating factor
- **GM-CSF**: granulocyte macrophage colony stimulating factor
- **HIV**: human immunodeficiency virus
- **HLA**: human leukocyte antigen
- **Hpf**: high powered field
- **HPRT**: Hypoxanthine-guanine phosphoribosyltransferase
- **HRP**: horseradish peroxidase
- **ICAM**: intercellular adhesion molecule
- **ICE**: interleukin converting enzyme
- **IFN**: interferon
- **Ig**: immunoglobulin
- **IL**: interleukin
- **IL-1RI**: interleukin-1 receptor I
- **IL-1RII**: interleukin-1 receptor II
- **IL-1Ra**: interleukin-1 receptor antagonist
- **IRF-8**: interferon regulatory factor 8
- **LC**: Langerhans’ cell
- **LPS**: lipopolysaccharide
- **MACS**: magnetic activated cell sorting
- **MFI**: mean fluorescence intensity
- **MHC**: major histocompatibility complex
- **Min**: minutes
MIP macrophage inflammatory protein
MMP matrix metalloproteinases
MMR macrophage mannose receptor
MoDC monocyte derived DC
MoLC monocyte derived LC
mRNA messenger ribonucleic acid
NALP-1 NACHT leucine-rich-repeat protein 1
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PCR polymerase chain reaction
pDC plasmacytoid dendritic cell
PE phycoerythrin
PFA paraformaldehyde
PGD2 prostaglandin D2
PI propidium iodide
PMA phorbol 13-myristate 12-acetate
RA retinoic acid
RANKL receptor activator of nuclear factor kappa-B ligand
RAR retinoic acid receptor
RT room temperature
RT-PCR real time PCR
RXR retinoid x receptor
SDS sodium dodecyl sulphate
Sec seconds
SLS sodium lauryl sulphate
T0 time zero
T24 time 24 hours
TBS tris buffered saline
TBST tris buffered saline with Tween
TGF-β1 transforming growth factor beta-1
TLR toll-like receptor
TNF-α tumour necrosis factor-alpha
TNF-R tumour necrosis factor receptor
Treg T regulatory cell
1. Introduction

1.1 Introduction to Skin Ageing

Chronological or intrinsic ageing of the skin, can be identified clinically by the presence of fine wrinkles, loss of elasticity and dryness (Ghadially et al., 1995; Koehler et al., 2009; McGrath et al., 2012). Microscopic changes in skin biopsies include a thinning of the epidermis, flattening of the dermo-epidermal junction, and a reduction in elastic fibres and types I and III collagen (El-Domyati et al., 2002; Lavker, 1979). A reduction in collagen fibres has also been observed in aged skin in vivo using confocal microscopy (Wurm et al., 2012). There is an age-related reduction in epidermal and dermal glycosaminoglycans, including hyaluronic acid, which in addition to changes in elastin and collagen, may contribute further to the clinical signs of wrinkling and reduced elasticity (Gheretich et al., 1994; Oh et al., 2011). In terms of cutaneous vasculature, although there appears to be no reduction in cutaneous blood vessels in intrinsically aged skin, the vessels appear reduced in size (Chung et al., 2002).

Loss of collagen is thought to be related to a combination of a reduction in dermal fibroblasts and an increased expression of matrix metalloproteinases (MMPs) in aged skin (Varani et al., 2000). Reduced levels of tissue inhibitors of MMPs have also been identified in intrinsically aged skin, which may enhance collagen degradation (Ashcroft et al., 1997a; Ashcroft et al., 1997b). Interestingly, in aged skin there is a reduced activation of the extracellular-signal-related mitogen-activated protein kinase pathway which results in reduced cyclin D2 expression, an important regulator of cell cycle progression (Chung et al., 2000). In contrast, the stress activated mitogen-activated protein kinase pathway is up-regulated in old compared with young skin, resulting in increased cutaneous levels of c-Jun mRNA and protein; a component of the transcription factor activator protein-1, which regulates transcription of MMPs (Chung et al., 2000; Fisher et al., 2009). Intrinsic skin ageing is also associated with signs of increased oxidative stress, and fibroblasts cultured in MMP-1 degraded collagen lattices produce higher levels of oxidants and have higher concentrations of oxidised proteins (Fisher et al., 2009).

Over recent years genomic and proteomic studies of intrinsic skin ageing have been performed to identify candidate genes and proteins involved in the ageing process. Gene expression profiling studies have shown a reduced expression of genes
associated with skin barrier function (for example, fatty acid synthase and several keratins), collagen, and anti-oxidant defences. In accordance with other studies, gene expression profiling of aged skin has demonstrated an increased expression of genes associated with oxidative stress (McGrath et al., 2012; Oender et al., 2008). One study using proteomic analysis of young and old foreskin samples corroborated the findings of earlier reports, for example in terms of demonstrating a reduction in type I collagen in aged skin; however a number of other candidate proteins potentially contributing to the ageing process were identified (Laimer et al., 2010). Ageing was associated with a reduced level of phosphatidyl-ethanolamine binding protein, which has been shown to promote differentiation of keratinocytes and suppress cell proliferation, and an increased level of carbonic anhydrase which may be a result of increased energy metabolism and intracellular carbon dioxide production (Laimer et al., 2010; Yamazaki et al., 2004).

Age-related changes also occur in the cutaneous immune response with a variety of clinical consequences including: increased prevalence of epidermal cancers and skin infection, an impairment of allergic contact hypersensitivity (CHS), and increased frequency of autoimmunity, for example bullous pemphigoid (Balato et al., 2008; Brewster et al., 2007; Hope-Simpson, 1965; Parker et al., 2008; Piaserico et al., 2004; Tosti et al., 1998; Wohrl et al., 2003).

1.2 Skin Immune System: the Innate Immune Response

The skin is an immunologically active organ capable of mounting immune responses to external challenges such as infectious microorganisms. The innate immune response provides a rapid onset first line of defence against pathogens. Immune cells (neutrophils, natural killer cells, macrophages) perform cytotoxic or phagocytic functions, whilst production of antimicrobial peptides and inflammatory mediators by a variety of cells in the skin further augments the host defence (Meyer et al., 2007). Keratinocytes are important mediators of the cutaneous innate immune response; in addition to expression of pattern recognition receptors, they produce antimicrobial peptides, such as β-defensins which have been shown both to have a bactericidal effect on Staphylococcus aureus, and to reduce viral load following infection with varicella zoster virus (Baker et al., 2003; Crack et al., 2012; Kisich et al., 2007; Lebre et al., 2007). Following injury keratinocytes produce chemokines which are involved
in the attraction of innate and adaptive immune cells including neutrophils and dendritic cells (DCs) (Kennedy-Crispin et al., 2012; Roupe et al., 2010), and keratinocyte derived cytokines can also affect the polarisation of the adaptive immune response (Lebre et al., 2003).

1.3 Skin Immune System: the Adaptive Immune Response

The adaptive immune response, mediated principally by T and B lymphocytes, is of slower onset but is specific, has memory, and has the ability to distinguish between self and non-self. The potency of the innate response influences the adaptive response, the latter being driven by DCs, which are professional antigen presenting cells. Dendritic cells are able to capture and process antigen, upregulate markers of maturation, and migrate to lymphoid organs to initiate the T cell response. Immature DCs also play a role in maintenance of self-tolerance (Meyer et al., 2007). In the steady state they sample the environment and induce T cell tolerance (Garbi et al., 2010; Hawiger et al., 2001). The functional importance of DCs has been highlighted recently by Ohnmacht et al who found that constitutive depletion of DCs from mice led to fatal autoimmunity within weeks (Ohnmacht et al., 2009). Dendritic cell ablation also provoked a reduced T lymphocyte response, reduced elimination of parasites, and an increase in the proportion of macrophages and neutrophils. Recently identified human DC deficiency syndromes, such as interferon regulatory factor 8 (IRF 8) deficiency, also suggest that DCs are indispensable for an efficient immune response. For example, in the most severe form of IRF 8 deficiency there is a failure of DC development which leads to death in infancy unless stem cell transplantation is performed (Collin et al., 2011).

Human skin contains a population of resident T cells, which have mainly a skin homing effector memory phenotype (Clark et al., 2006; Seneschal et al., 2012). A proportion of skin resident T cells have a regulatory phenotype, thus promoting tolerance to self-antigens and normal flora (Seneschal et al., 2012). Cutaneous T cells are ideally placed to respond to local antigen presentation by DCs.

In human skin, DCs are found in the dermis (dermal DCs) and in the epidermis, where they are known as Langerhans’ cells (LCs). They may be distinguished according to expression of various membrane markers (Chu et al., 2012; Eisenwort et al., 2011; Merad et al., 2008; Segura et al., 2012; Zaba et al., 2009) (Table 1.1).
Table 1.1 Phenotype of dendritic cells in human skin.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Membrane marker expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal LC</td>
<td>Langerin⁺, CD1a⁺, CD11c⁺, HLA-DR⁺, EpCAM⁺, TROP2⁺</td>
</tr>
<tr>
<td>Dermal DC</td>
<td>CD1c⁺, CD1a⁺, CD11c⁺, HLA-DR⁺, CD14⁻</td>
</tr>
<tr>
<td></td>
<td>CD1c⁺, CD14⁺, CD141⁺, HLA-DR⁺, CD1a⁻</td>
</tr>
<tr>
<td>Plasmacytoid DC</td>
<td>CD123⁺, BDCA-2⁺, HLA-DR⁺, CD11c⁻</td>
</tr>
</tbody>
</table>

Abbreviations: CD, cluster of differentiation; EpCAM, epithelial-cellular adhesion molecule; HLA-DR, human leukocyte antigen-DR.

1.4 Immunosenescence and the SENIEUR Protocol

Changes in the immune system associated with advancing age are often referred to as immunosenescence. One of the inherent difficulties in studying immunosenescence in humans is the influence of confounding factors such as malnutrition, disease, lifestyle factors, and medications. The SENIEUR protocol was introduced in an attempt to standardise the study of the effects of ageing per se (Ligthart et al., 1984). The exclusion criteria are based upon clinical findings, laboratory data, and medication prescribed for a defined disease or known to influence the immune system. When applied to residents in a care home only 10% fulfilled these stringent criteria (Ligthart et al., 1984). In a more recent study of T cell phenotype in aged individuals (mean age 90 years), only 9.4% of the study population fulfilled the SENIEUR criteria, although a significant number were living independently (Nilsson et al., 2003).

There has been considerable debate regarding the merits of the SENIEUR protocol in ageing research. Several authors have criticised the tool for excluding the study of the frail elderly who are most at risk of illness, and have proposed alternative solutions such as using exclusion criteria tailored to each study (Castle et al., 2001; Ershler, 2001; Miller, 2001): this latter approach has been adopted for this research project (exclusion criteria are described in chapter 2).
1.4.1 Immunosenescence

Ageing affects both the innate and the adaptive immune systems resulting in an increased susceptibility to infectious disease and a reduced response to vaccination. Changes in the innate immune system include: an increase in the ratio of myeloid to lymphoid progenitors; impaired neutrophil chemotaxis and phagocytosis; impaired natural killer cell cytotoxicity; and a reduction in monocyte pro-inflammatory cytokine production following toll-like receptor (TLR) induction (Shaw et al., 2010). Macrophages from aged mice also produce reduced amounts of pro-inflammatory cytokines upon stimulation, and have a reduced expression of a number of TLRs, which could potentially impair their functional ability (Boehmer et al., 2004; Renshaw et al., 2002).

In both man and mouse, age-related thymic atrophy leads to a reduced ability to produce naïve T cells, and a corresponding shift in the balance of T cells from a naïve to a memory phenotype (Singh and Singh, 1979; Xu et al., 1993). The associated reduction in the T cell receptor repertoire may at least in part account for the reduced response to vaccination and infectious challenges seen in elderly individuals (Naylor et al., 2005; Yager et al., 2008). Furthermore, the inversion of the ratio of CD4+ to CD8+ T cells that occurs with age has been linked to adverse health outcomes including increased mortality and increased risk of hospital acquired infection (Plonquet et al., 2011; Strindhall et al., 2007).

Previous studies have also shown a reduction in the number and percentage of circulating B cells in aged humans (Frasca and Blomberg, 2009; Paganelli et al., 1992). In addition, the B cell/plasma cell repertoire is reduced with age, which coupled with clonal expansions of more than one cell, has been associated with frailty (Dunn-Walters and Ademokun, 2010). Specific antibody titre following influenza vaccine also declines with age (Murasko et al., 2002).

1.4.2 Cell Senescence

Normal cells undergo a finite number of divisions after which they become senescent. This process is thought to serve as a defence mechanism against the development of malignancy, but may also be involved in the ageing process (Collado et al., 2007). Telomeres, consisting of numerous G rich repeats at the end of chromosomes, prevent
chromosomes from degrading or fusing with neighbouring chromosomes. During normal deoxyribonucleic acid (DNA) replication, telomeres become progressively shorter until eventually the cell enters replicative senescence. The enzyme telomerase maintains telomeres, and is of particular importance in cells that undergo frequent cell division (e.g. epidermal stem cells). Dyskeratosis congenita, which is associated with a mutation affecting telomerase, is associated with abnormal nails, and skin pigmentation and premature ageing (Buckingham and Klingelhutz, 2011). Other premature ageing syndromes, which often have prominent cutaneous features, are also associated with premature cell senescence (Capell et al., 2009). Furthermore, skin samples from aged individuals demonstrate increased keratinocyte staining for β-galactosidase (a marker of cell senescence) compared with younger individuals (Dimri et al., 1995).

1.4.3 Ageing of Dendritic Cells

One area that has been relatively overlooked in the context of immunity and ageing is the role of the DC. Dendritic cells can be broadly divided into two subtypes. The first consists of the myeloid and plasmacytoid DCs found in circulating blood, and the second consists of DCs found in tissues and organs (Agrawal et al., 2007b). Plasmacytoid dendritic cells (pDC) arise from lymphoid and common DC progenitors (Reizis et al., 2011), and they secrete relatively large quantities of interferon (IFN) -α in response to certain infectious challenges. There is controversy in the literature as to whether the number of pDCs changes with age with some authors reporting a reduction in aged humans (Jing et al., 2009; Panda et al., 2010; Shodell and Siegal, 2002), and others reporting no change (Agrawal et al., 2007a; Della Bella et al., 2007). Possible explanations for the discrepancy in the results of the different studies include variations in the size of the study populations, and differences in experimental protocols (such as number of cells acquired for flow cytometric analysis). Perhaps it is of greater relevance that there is evidence to suggest an age related functional impairment of pDCs in both man and mouse, with a reduction in IFN-α secretion in response to certain viruses (Jing et al., 2009; Stout-Delgado et al., 2008). The number of circulating myeloid DCs is reportedly reduced in elderly individuals (Della Bella et al., 2007; Jing et al., 2009), and myeloid and pDCs from individuals
over 65 years of age have been shown to produce lower levels of pro-inflammatory cytokines following treatment with TLR ligands (Panda et al., 2010).

Due to the difficulty in studying the function of human DCs in vivo, an in vitro model using monocyte derived DCs (MoDCs) has been developed. Monocytes isolated from peripheral blood are cultured with interleukin (IL)-4 and granulocyte macrophage colony stimulating factor (GM-CSF) to produce DCs (Sallusto and Lanzavecchia, 1994). Generation of MoDCs and the expression of characteristic DC markers (including markers of activation) does not appear reduced in the elderly, however, there are contrasting reports regarding their functional ability (Jing et al., 2009; Steger et al., 1996). Some studies have found no differences in antigen presentation, response to influenza vaccine, and ability to stimulate senescent T cells in MoDCs from young compared with old individuals (Lung et al., 2000; Saurwein-Teissl et al., 1998; Steger et al., 1996). In contrast, Agrawal et al found that MoDCs from aged individuals had reduced phagocytic ability and an impaired migratory response to cc chemokine ligand (CCL)19, the ligand for cc chemokine receptor (CCR)7, which acts as a homing signal directing DCs to lymphoid tissue (Agrawal et al., 2007a).

Results from murine studies suggest age associated deficits in cultured DCs. Paula et al reported that the DC yield from bone marrow precursor cells cultured with GM-CSF and IL-4 was significantly reduced in aged mice as was the maturation response to lipopolysaccharide (LPS) stimulation (Paula et al., 2009). Other studies have revealed further functional impairment including reduced ability to stimulate ovalbumin specific CD4+ and CD8+ T cell responses; reduced migration in vivo; defective anti-tumour activity and reduced expression of DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin) (Grolleau-Julius et al., 2006; Grolleau-Julius et al., 2008). These findings are consistent with those of Sharma et al, who also found that DCs from aged mice had reduced anti-tumour activity (Sharma et al., 2006).

Although the majority of studies on the effect of ageing on DCs have been conducted in vitro, two recent in vivo studies in mice have demonstrated an age related impairment in DC function resulting in a reduced immune response. Zhao et al found that, whilst respiratory DCs from aged mice were able to take up antigen and stimulate naive T cells, their migration was impaired compared to younger mice (Zhao et al., 2011). As a result, following infection with a respiratory virus, aged mice had a reduced virus specific CD8+ T cell response. The decrease in migration was at least in part due to increased levels of prostaglandin D2 (PGD2), such that treatment
with a PGD2 antagonist restored migration. One action of the PGD2 antagonist was to increase the number of CCR7 expressing respiratory DCs in the aged mice, thus providing a mechanism for the restoration of migration. In the second study, Pereira et al showed that in vivo transfer of T cells from young or aged mice to young mice resulted in T cell expansion and differentiation, whereas when T cells from young or aged mice were transferred to aged mice the response was reduced (Pereira et al., 2011). Dendritic cells from aged mice failed to upregulate CD40 expression, and production of IFN-γ, IL-6, and tumour necrosis factor (TNF)-α in response to stimulation. The authors concluded that age associated changes in T cell function were at least partly related to reduced DC function.

In humans, one area where there is convincing evidence for an in vivo age related change in the DC population is the skin, specifically the LC (Bhushan et al., 2002; Cumberbatch et al., 2002; Gilchrest et al., 1982).

### 1.4.4 Immunosenescence and the Skin Immune System

Ageing has demonstrable effects on the skin immune system. In addition to reduced barrier function, which may itself lead to increased susceptibility to infection, Agius et al found a reduced local T cell response to cutaneous antigens in aged individuals (Agius et al., 2009). In conjunction with this, they detected a reduced synthesis of TNF-α by macrophages in the skin of aged individuals, which corresponded with a reduction in expression of markers required for T cell recruitment.

As for the immune system in general, evidence for the effects of ageing on the cutaneous DC population is scarce, despite the important role that these cells play in forming a bridge between innate and adaptive immunity. Previous work has established an age-related reduction in LC numbers in both man and mouse (Bhushan et al., 2002; Choi and Sauder, 1987; Cumberbatch et al., 2002; Gilchrest et al., 1982). In addition, there is a significantly greater reduction in LC number in chronically sun-exposed sites when compared with sun-protected sites (Thiers et al., 1984). Interestingly, there is also evidence to suggest that LC numbers are reduced in cutaneous malignancies, including squamous cell and basal cell carcinoma – both of which are more common with increasing age (Gibson et al., 1998; Schreiner et al., 1995; Takahara et al., 2009). The basis for the observed reduction in LC numbers
with age has not yet been elucidated. The text that follows will centre on the current understanding of LC biology with a focus on age-associated alterations.

1.5 The Epidermal Langerhans’ Cell

The German pathologist Paul Langerhans, 1847-1888, first described the epidermal cell that now bears his name whilst studying the nerve supply of the skin. Whilst still a medical student, he used the gold chloride staining method to identify non-pigmentary epidermal cells with prominent dendrites (Langerhans, 1868; Sakula, 1988). Soon after, whilst studying the rabbit pancreas, he identified distinct clumps of cells which are now known as the “islets of Langerhans” (Ebling, 1980).

Langerhans’ cells resident in the epidermis act as the outermost sentinels of the adaptive immune system sampling the external environment for danger, and are adept at processing antigen. Following an external challenge, a proportion of LCs are mobilised and then migrate to local skin-draining lymph nodes acquiring at the same time immunostimulatory properties that facilitate antigen presentation to T cells (Schuler and Steinman, 1985) (Figure 1.1).
Figure 1.1 Langerhans’ cell (LC) migration. Following antigenic stimulation, the LC migrates from the epidermis to the lymph node via dermal lymphatics. At the lymph node the LC presents the antigen to T lymphocytes. (Antigen, red; LC, light grey; T lymphocyte, dark grey).

Langerhans’ cells within the epidermis have a characteristic phenotype. They contain Birbeck granules which are “tennis racket” shaped intracytoplasmic organelles (Birbeck et al., 1961). They also express langerin, a member of the c-type lectin family which is thought to be involved in Birbeck granule formation (Tripp et al., 2004; Valladeau et al., 2000). Langerin can be detected on the LC surface but is also internalised via endocytosis (Mc Dermott et al., 2002). The function of the Birbeck granule has not been fully elucidated, but it is thought that in conjunction with langerin, it is involved in antigen processing (Valladeau et al., 2000). Consistent with this de Witte et al found that LCs exposed to human immunodeficiency virus (HIV) bound HIV virions through langerin, which was then internalised to Birbeck granules. The LCs inhibited T cell infection with HIV, however, if langerin was blocked with an inhibitor, HIV transmission was enhanced (de Witte et al., 2007). Langerhans’
cells also express DEC-205 another c-type lectin receptor which is important for the uptake of extracellular proteins (Ebner et al., 2004).

In addition, LCs express a variety of other cell markers and receptors including CD1a, CD1lc, CCL3, E-cadherin, major histocompatibility complex (MHC) class I and II, receptors for immunoglobulin (Ig)E and IgG, IL-1 receptor I (IL-1RI), IL-1 receptor II (IL-1RII), IL-6 receptor, TNF receptor (TNF-R) 2, and IFN-γ receptor (Astier et al., 1994; Bieber et al., 1992; De Panfilis et al., 1989; Larregina et al., 1996; Tang et al., 1993; Valladeau et al., 2000). In humans CD1a is often used as a marker to identify LCs, and is thought to be important in binding lipid antigens. De Jong et al reported that CD1a/lipid complexes on LCs are recognised by a subset of CD1a autoreactive CD4+ T cells which home to the skin and produce IL-22 (de Jong et al., 2010). Most recently Eisenwort et al showed that human LCs express TROP2, an EpCam like molecule that is not expressed on dermal DCs (Eisenwort et al., 2011).

1.5.1 The Role of Langerhans’ Cells and Dermal Dendritic Cells

Defining the specific role of LCs in the cutaneous immune response has been difficult, particularly due to problems in differentiating the effects of LCs from dermal DCs. Over the past few years a growing body of research has been published on this subject.

1.5.1.1 The Dermal Dendritic Cell

Resident populations of dermal DCs were described by two groups in 1993 (Lenz et al., 1993; Nestle et al., 1993). Lenz et al isolated dermal DCs from dermal explants that expressed CD1a and HLA-DR. The dermal DCs did not contain Birbeck granules and did not express langerin. Nestle et al identified distinct populations of dermal DCs that were capable of stimulating allogeneic T cell proliferation. The cells expressed either CD1a or CD14. They also found a population of CD1a/CD14− cells that expressed factor XIIIa, however, it is now thought that these cells represent macrophages (Zaba et al., 2007; Zaba et al., 2009). Subsequent studies have revealed that dermal DCs can play an important part in the immune response to certain pathogens (Allan et al., 2003; Itano et al., 2003), although their function in relation to LCs remains incompletely understood. The development of experimental mouse
models in which LCs may be ablated by exposure to diphtheria toxin (DT) led to some interesting findings. Temporary depletion of LCs was found by one group to have no significant effect on the development of contact sensitisation to dinitrofluorobenzene (Kissenpfennig et al., 2005), whereas a different group found the response to trinitrochlorobenzene and oxazolone was reduced (Bennett et al., 2007; Bennett et al., 2005). The first group hypothesized that dermal DCs, which arrived at local lymph nodes prior to LCs, were important in initiating the immune response. It was perhaps unexpected that Kaplan et al., using a DT model in which mice have a congenital absence of LCs; found contact sensitization to be increased (Kaplan et al., 2005). The authors suggested that LCs may have a regulatory role, although others have argued that mice lacking LCs from birth may have alterations in the broader immune system, for example in the T cell repertoire (Bennett et al., 2007).

The discovery of a murine langerin+ dermal DC capable of migrating to local lymph nodes and stimulating T cell proliferation at least in part explained the observed variation in results (Bursch et al., 2007; Poulin et al., 2007). These cells express CD103, but not Ep-CAM. Like LCs, the langerin+ dermal DCs are ablated following exposure to DT, but appear to recover more quickly (Wang et al., 2008), and in contrast to LCs, they are derived from circulating precursors in the steady state (Ginhoux et al., 2007).

The presence and phenotype of a dermal langerin+ DC population in humans has not yet been established, in fact two studies have reported that there is no human equivalent to the murine dermal langerin+ DC (Eisenwort et al., 2011; Klechevsky et al., 2008).

1.5.1.2 Functional Studies of Langerhans’ Cells and Dermal Dendritic Cells

There is evidence to suggest that in humans LCs are more efficient than dermal DCs at priming naive T cells (Furio et al., 2010; Klechevsky et al., 2008; van der Aar et al., 2011). Klechevsky et al compared LCs and dermal DCs isolated from human skin explants and blood derived LCs, and found that CD8+ T cells primed by LCs had superior cytotoxic function than those primed by dermal DCs. The CD14+ dermal DCs were important in the B cell response (Klechevsky et al., 2008). Van der Aar et al reported that human LCs primed with influenza virus or polyriboinosinic polyribocytidylic acid were superior in terms of their ability to stimulate naive CD8+
T cells compared to dermal DCs. The LC stimulated CD8\(^+\) T cells also produced more granzyme B which is important for cytotoxicity (van der Aar et al., 2011). Interestingly, Banchereau et al showed that LCs were more potent inducers of CD8\(^+\) T cell proliferation due to production of IL-15, which was not produced by CD14\(^+\) dermal DCs (Banchereau et al., 2012). Dermal DCs expressed IL-10, which had an inhibitory effect upon T cell proliferation. Epstein-Barr virus pulsed LCs were also better than dermal DCs at stimulating antigen specific memory CD8\(^+\) T cell proliferation and IFN-\(\lambda\) production (Polak et al., 2012). The effect in this study was dependent upon CD70 expression on LCs. In contrast to these findings, another study demonstrated that although human LCs could capture measles virus via langerin, and present antigen to CD4\(^+\) T cells, they could not cross present inactivated virus particles or apoptotic infected cells to CD8\(^+\) T cells (van der Vlist et al., 2011). The use of different viral antigens in these studies may at least partly explain the different findings.

Skin DCs also have a role in maintaining self tolerance in steady state conditions. Human LCs cultured in vitro with skin resident memory T cells induced the proliferation of CD4\(^+\) T cells, most of which were T regulatory cells (Tregs). The Tregs could inhibit autologous T effector memory cell proliferation. However, in the presence of Candida albicans, proliferation of antigen specific T effector memory cells, which produced IFN-\(\lambda\) and IL-17, occurred (Seneschal et al., 2012). Of note, within the skin, Tregs were located near to or in contact with LCs. A population of human dermal DCs that express CD14 and CD141 and produce IL-10 have also been demonstrated to induce Tregs (Chu et al., 2012).

There have been a number of studies comparing dermal DCs and LCs in mice, and there is an ongoing debate as to whether murine LCs can cross present antigen to CD8\(^+\) T cells. Bedoui et al reported that, although all skin DCs could present viral and self antigen to CD4\(^+\) T cells, only CD103\(^+\) dermal DCs could cross present to CD8\(^+\) T cells (Bedoui et al., 2009). Other studies have used the DT-langerin mouse model to show that non-langerin positive dermal DCs and not LCs are important in cross presentation and activation of CD8\(^+\) T cells (Furmanov et al., 2010; Stoecklinger et al., 2011). Since after ablation langerin positive dermal DCs repopulate the skin some time before LCs, it is possible to differentiate between the effects of the 2 cell types. Interestingly, Bennett et al found that LCs were required for the activation of CD8\(^+\) T cells in a mouse model of graft versus host disease. Although in the absence of LCs
primed CD8+ T cells accessed the skin, they did not cause disease (Bennett et al., 2011). Using gene gun immunisation in mice at different time points post ablation of langerin+ cells, a different study reported that the two langerin+ cell types have distinct immune functions (Nagao et al., 2009). The LC was important in the regulation of the humoral response via IgG1 production, whereas the dermal langerin+ DC was important for acute IgG2a/c and IgG2b production.

A number of mouse studies have also recently been published which attempt to define the roles of the different skin DC subsets in CHS. Selective ablation of langerin+ dermal DCs or LCs did not affect the CHS response in one study; however, ablation of both subtypes did significantly reduce the response (Honda et al., 2010). In accordance with these findings, Edelson et al found that in mice without langerin+ dermal DCs (but normal LCs) the CHS response was maintained (Edelson et al., 2010). Two studies have shown a reduction in the CHS response in the absence of LCs (using different methods of LC ablation), although in one of these studies the CHS response was maintained if a higher dose of hapten was applied (possibly because at the higher dose hapten reaches the dermal DCs) (Noordegraaf et al., 2010; Zahner et al., 2011). In contrast, one study using mice generated to allow selective ablation of LCs prior to sensitisation, demonstrated an increase in the CHS response (Bobr et al., 2010). The conflicting results suggest that variations in methodology, such as the strains of mice and the dose of hapten used, can have a significant impact on the outcome.

In summary, there appears to be significant differences between the cutaneous DC populations in man and mouse, both in terms of the phenotype and functional ability of the DCs. Findings from murine studies are not necessarily translatable to humans, particularly in the absence of a langerin+ dermal DC population in humans. It is likely, however, that in both species there is a degree of functional redundancy within the dendritic cell population such that the dermal DCs can perform some of the functions of the LCs and vice versa.

1.5.2 Origin of the Langerhans’ Cell

Langerhans’ cell development is dependent upon the presence of transforming growth factor-beta (TGF-β) 1, such that TGF-β1 null mice have no LCs (Borkowski et al., 1996). Using mice with a LC specific knockout of the gene for TGF-β1 or its
signalling receptor (TGF-β receptor II), Kaplan et al have shown specifically that LC-derived TGF-β1 is required for the development of LCs, and that TGF-β acts on the LC directly rather than via an intermediate cell type (Kaplan et al., 2007). In addition, Kel et al have demonstrated that as well as being required for LC development, TGF-β is also required to retain immature LCs in the epidermis of mice (Kel et al., 2010). Using a mouse model where the TGF-β receptor was selectively knocked out on CD11c+ cells, they found that although LC were present at birth (albeit in reduced numbers) within a few days they upregulated markers of maturation and migration, and began to disappear from the epidermis.

A study of human embryonic and foetal skin has shown that epidermal CD45+CD1c+ DCs are present in embryonic skin (Schuster et al., 2009). The active form of TGF-β1 was detectable in the epidermis by 9 weeks’ gestation, preceding the expression of langerin on the CD45+CD1c+ cells which occurred after approximately 11 weeks’ gestation. CD1a expression occurred at an estimated gestational age of 13 weeks. The CD45+CD1c+ cells could function as antigen presenting cells in that they were able to phagocytose bacterial antigen, upregulate markers of maturation, and stimulate proliferation of allogeneic T cells.

Until recently, the immediate LC precursor in vivo was unknown. Initially, it was thought that LCs were derived from a single precursor, but it is now known that maintenance of the LC population depends on the local environment (Merad et al., 2002). During inflammation and/or injury, LCs appear to be replaced by blood-derived precursors, whereas in the steady state they are replaced by local precursors (Merad et al., 2002).

Although the source of the local precursor has not been fully elucidated, LCs may be capable of undergoing mitosis in the epidermis. This could represent one mechanism through which steady state numbers are maintained (Czernielewski et al., 1985). Consistent with this finding, a recent report of a hand allograft recipient demonstrated that the LCs in the allograft remained of donor origin even after 10 years. In one biopsy, a LC was observed in mitosis. Interestingly there were no episodes of rejection, whereas in a previous similar case following an episode of rejection, recipient LCs were identified in the allograft (Kanitakis et al., 2011). In a different study, when human skin was grafted onto mice lacking B, T and natural killer cells, although LCs initially disappeared, they reappeared several weeks later. Dual staining with langerin and Ki67 (a marker associated with cellular proliferation) revealed an
increase in Ki67⁺ LCs in grafted skin suggesting that the LCs were self-renewing. In contrast, dermal dendritic cells were present throughout (Hemmerling et al., 2011).

### 1.5.3 In vitro Methods of Langerhans’ Cell Culture

*In vitro*, in cytokine-supplemented media, LC-like cells can be cultured from CD34⁺ haematopoietic stem cells obtained from human cord blood, or from GM-CSF treated adults (Caux et al., 1992; Gatti et al., 2000). As mentioned earlier, TGF-β1 is essential for LC development (Borkowski et al., 1996), and in the presence of this cytokine, GM-CSF and TNF-α, CD14⁺CD1a⁻ cells develop into LC-like cells that express CD1a, E-cadherin and MHC class II (HLA-DR), whilst more mature stem cell derived CD1a⁺ DCs develop into LC-like cells with or without the addition of TGF-β1 (Jaksits et al., 1999). Larregina et al were able to generate LC-like cells from a dermal population of CD14⁺ cells using a human skin explant model. They collected CD14⁺ cells that migrated from dermal explants and cultured them in the presence of TGFβ-1 (Larregina et al., 2001). The resultant cells expressed CD1a, E-cadherin and langerin. Human CD14⁺ monocytes and CD1a⁺CD11c⁺ DCs from peripheral blood can also give rise to LC-like cells in the presence of TGFβ-1, IL-4 and GM-CSF; the cells express CD1a, E-cadherin and langerin (Geissmann et al., 1998; Ito et al., 1999).

### 1.5.4 In vivo Study of Langerhans’ Cell Ontogony

In 2006, Ginhoux et al proposed that during inflammation LCs are repopulated from circulating monocytes. In a series of experiments using a colony stimulating factor 1 receptor (CSF-1R) null mouse model, and latex bead labelling of circulating monocytes, the authors found that CSF-1R is required for LC development, and that in mice Gr1⁺ monocytes travel to inflamed skin and differentiate into LCs (Ginhoux et al., 2006).

Based on these observations, it could be hypothesized that the reduction in LC numbers seen in aged individuals is due to a reduction in the frequency of precursor cells. This could be a reduction in circulating or local precursors, or a combination of the two. Della Bella et al found an increased number of circulating monocytes in elderly humans. Although monocytes from aged individuals seem able to differentiate into DCs in cytokine supplemented media (Della Bella et al., 2007), their ability to
differentiate into LC-like cells has not been studied. An age-related reduction in haematopoietic growth factors, in particular GM-CSF, may be of relevance (Buchanan et al., 1996).

The skin resident LC precursor has not yet been identified; although LCs undergo mitosis it may be that this is not sufficient to maintain LC numbers in the steady state. There have been no studies to specifically address whether LCs of aged individuals are capable of undergoing mitosis.

1.5.5 Langerhans’ Cell Migration

Langerhans’ cells migrate to skin draining lymph nodes in steady state conditions. These “steady state” LCs are semi-mature in that they do not up-regulate all markers of maturation required for an efficient T cell effector response, and are thought to be important for maintenance of self-tolerance (Hemmi et al., 2001; Kissenpfennig et al., 2005; Ohl et al., 2004). In contrast, following a stimulus such as exposure to a contact allergen, UV radiation, or a mechanical trauma, a larger proportion of LCs is mobilised (Gilchrest et al., 1982; Macatonia et al., 1987; Ruutu et al., 2011). The initiation and regulation of LC mobilisation and migration are orchestrated by cytokines and chemokines. Langerhans’ cells express a range of cytokine receptors, integrins and adhesion molecules. They are also able to produce an array of cytokines and induce cytokine production by other cells.

1.5.5.1 The Role of Cytokines in Langerhans’ Cell Migration: Tumour Necrosis Factor-α

Previous work has revealed that both IL-1β and TNF-α provide mandatory signals for LC migration in an interdependent manner. Tumour necrosis factor-α, a pro-inflammatory cytokine, is produced and released in the epidermis by activated keratinocytes, for example after exposure to a contact sensitiser (Kock et al., 1990; Wang et al., 1997). Systemic (intravenous or intraperitoneal) and intradermal injection of TNF-α triggers LC migration in mice (Cumberbatch et al., 1994; Cumberbatch and Kimber, 1992; Roake et al., 1995). Intradermal TNF-α injection also provokes LC migration in humans as measured by a reduction of CD1a+ cells in
epidermal sheets compared with control sites receiving an injection of saline alone (Cumberbatch et al., 1999b). Interestingly, when mice were injected with human TNF-α these effects were not observed, and this was found to be due to the species specificity of the TNF-R2 (Cumberbatch et al., 1994).

Two forms of TNF-R have been identified (TNF-R1 and 2); TNF-R1 was initially believed to be responsible for mediating most of the biological activity of TNF-α. However, when Kondo et al studied the effect of a contact allergen (dinitrofluorobenzene) on TNF-R1 deficient mice, they found that the response to the contact allergen was increased (Kondo et al., 1995b). Using a TNF-R2 knockout mouse, Wang et al confirmed that the absence of TNF-R2 rather than TNF-R1 led to a reduction in hapten bearing cells in local lymph nodes (Wang et al., 1997). Subsequently, Larregina et al using flow cytometry demonstrated that human LCs isolated from epidermal cell suspensions expressed TNF-R2 but not TNF-R1 (Larregina et al., 1996). More recently, Berthier-Vergnes et al reported that exposure of freshly isolated human LCs to TNF-α led to enhanced expression of co-stimulatory molecules including CD83, CD86 and CD40 and induced LC secretion of IL-12 and cx chemokine ligand (CXCL)10. These effects were also mediated through TNF-R2 (Berthier-Vergnes et al., 2005).

1.5.5.2 Interleukin-1β

Interleukin-1β is an important pro-inflammatory cytokine, and the most studied member of the IL-1 family. The inactive pro-form of IL-1β is usually converted into the active molecule via the action of caspase-1, although caspase-1 independent IL-1β production can also occur (Dinarello, 2011a). In humans, systemic administration of IL-1β causes systemic features including fever, anorexia, myalgia, and arthralgia. Interestingly, the cryopyrinopathy associated periodic syndromes, which ultimately lead to increased secretion of IL-1β, are characterised by similar symptoms and often have cutaneous manifestations (Dinarello, 2011a). In contrast, mice deficient in IL-1β have impaired CHS reactions, and impaired acute phase responses to non-microbial inflammatory stimuli (Fantuzzi and Dinarello, 1996; Shornick et al., 1996). It is noteworthy that addition of high doses of antigen seems to bypass the requirement for IL-1β to induce CHS in the knockout mouse (Shornick et al., 1996). One possible explanation is that at higher doses of antigen, dermal DCs are able to initiate the
immune response, which would therefore imply that migration of dermal DCs is not dependent upon IL-1β.

In 1997, Cumberbatch et al showed that systemic injection (intraperitoneal) of neutralising anti-TNF-α or anti-IL-1β antibodies into mice followed by application of a contact sensitiser led to a reduction in the number of LCs migrating from the skin to local lymph nodes compared with controls. Furthermore, anti-TNF-α antibodies abrogated the migration of LC in response to intradermal IL-1β and vice versa. One interesting finding was that in the presence of the anti-TNF-α antibodies and intradermal IL-1β, the LCs visualised in epidermal sheets had an activated appearance with prominent dendrites, yet after intradermal TNF-α and systemic administration of neutralising anti-IL-1β, the LCs appeared rounded and detached from adjacent keratinocytes. The authors hypothesized that this observation was due to TNF-α induced changes in adhesion molecule expression, in particular E-cadherin (Cumberbatch et al., 1997b). Using a combination of mouse and human skin explants, Stoitzner et al corroborated the findings of Cumberbatch et al (Stoitzner et al., 1999).

Langerhans’ cells appear to be the principle source of IL-1β in the mouse epidermis (Heufler et al., 1992). In a study by Enk et al, addition of IL-1β to keratinocyte cultures caused upregulation of TNF, IL10 and IL1A, but not IL1B messenger ribonucleic acid (mRNA) (Enk et al., 1993a). When IL-1β was added to LC containing epidermal cell suspensions, IL1B mRNA was upregulated, therefore the authors of the study concluded that IL-1β also has an autocrine function. More recently, using a transgenic mouse expressing red fluorescent protein under the control of the IL-1β promoter, Matsushima et al also found LCs were a source of IL-1β (Matsushima et al., 2010).

Cumberbatch et al further investigated the kinetics of the cytokine response to intradermal injection of IL-1β and TNF-α in the mouse. A significant reduction in MHC class II+ cells in epidermal sheets was observed within 30 minutes of TNF-α injection compared with 2 hours with IL-1β. These differences were also reflected in the numbers of DCs in the draining lymph nodes, which were increased more rapidly after TNF-α treatment (Cumberbatch et al., 1997a). Based on these observations, the authors proposed the following paradigm for LC migration: following stimulation, LCs produce IL-1β that acts in an autocrine manner to provide one signal for migration and also triggers production of TNF-α by adjacent keratinocytes. The TNF-α then acts upon LCs in a paracrine fashion to provide a second stimulus for migration.
(Cumberbatch et al., 1997a) (Figure 1.2). The same group have conducted in vivo studies in man showing that intradermal IL-1β can induce LC migration and also increase levels of TNF-α (as measured in suction blister fluid) (Cumberbatch et al., 2003). Taken together these data suggest that regulation of LC mobilisation in man and mouse is virtually identical.

**Figure 1.2** The role of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in Langerhans’ cell (LC) migration. (Keratinocyte, K, tan; LC, grey; antigen, red.)

The production of IL-1β in human epidermis is more complex. Although LCs appear to be a source of IL-1β (Morhenn et al., 1992; Rambukkana et al., 1996), keratinocytes have also been shown to contain IL1B mRNA (Kupper et al., 1986). Analysis of lysates from keratinocyte cultures found that the IL-1β was in the inactive form (proIL1-β) (Mizutani et al., 1991). In contrast, Nylander-Lundqvist et al found biologically active IL-1β in extracts from plantar stratum corneum, which presumably contains only keratinocytes. (Nylander-Lundqvist et al., 1996). Subsequent studies by Zepter et al suggest that unstimulated keratinocytes produce little active IL-1β; however, on exposure to a stimulus (a contact sensitizer) some active cytokine is formed by keratinocyte-derived caspase-1. In their study, supernatants from the keratinocyte culture also contained active IL-1β, suggesting IL-1β can be secreted from keratinocytes (Zepter et al., 1997). Consistent with these findings, a more recent
study reported that UVB irradiation of cultured human keratinocytes promoted release of active IL-1β, and that the active form of IL-1β was produced via the action of keratinocyte derived caspase-1 (Feldmeyer et al., 2007). One possible explanation for these findings is that keratinocyte injury leads to release of IL-1β, which can be processed to the active form to trigger an immune response.

1.5.5.3 Caspase-1

As discussed, IL-1β activity in the skin is at least partly regulated by the action of caspase-1. Active caspase-1 is formed in innate immune complexes known as inflammasomes (Feldmeyer et al., 2010). Langerhans’ cells in mice express mRNA for caspase-1 (CASP1), and following stimulation, both CASP1 and IL1B mRNA are upregulated (Ariizumi et al., 1995). Moreover, caspase-1 knockout mice have a markedly reduced response to contact allergens as measured by enumeration of LCs in epidermal sheets (Antonopoulos et al., 2001). Mice deficient in components of the inflammasome also have impaired CHS responses (Watanabe et al., 2007). In humans, keratinocytes express caspase-1 (Feldmeyer et al., 2007; Johansen et al., 2007) and LCs have been shown to express NACHT leucine-rich-repeat protein 1 (NALP-1), a component of the inflammasome which activates caspase-1 (Kummer et al., 2007).

1.5.5.4 Interleukin-1 Receptors and Interleukin-1 Receptor Antagonist

There are 9 distinct genes encoding the IL-1 receptor family members. All IL-1 receptors share the toll-IL-1 receptor domain with the TLRs, which are an important component of the innate immune system. Interleukin- receptor I and IL-1RII serve as receptors for IL-1β (Dinarello, 2009; Dinarello, 2011b). Interleukin-1RI is a functioning (signal transducing) receptor that in mouse epidermis is found mainly on LCs, although keratinocytes have been shown to possess intracellular IL-1RI (through which IL-1β is able to stimulate the production by keratinocytes of TNF-α) (Cumberbatch et al., 1998; Cumberbatch et al., 1999a; Kampgen et al., 1994). Human keratinocytes and LCs have been shown to express IL-1RI (Eller et al., 1995; Larregina et al., 1996).
The second receptor, IL-1RII, is thought to act as a decoy receptor (Colotta et al., 1993) in so far as it is not able to deliver a signal across the plasma membrane. Several studies have confirmed the presence of IL1R2 mRNA in cultured human keratinocytes derived from neonatal foreskin (Eller et al., 1995; Groves et al., 1995; Groves et al., 1994). The IL-1RII is expressed on the surface of human keratinocytes and is also released in a soluble form, but does not appear to have a functional role in terms of signal transduction (Groves et al., 1995). A study of transgenic mice, which over express IL-1RII in keratinocytes, has shown that increased expression of IL-1RII is associated with a reduced local inflammatory response to topical application of phorbol 13-myristate 12-acetate (PMA) suggesting that the receptor serves to regulate IL-1 mediated inflammation (Rauschmayr et al., 1997). There is also evidence to suggest that IL-1RII is expressed on murine and human LCs (Cumberbatch et al., 1998; Larregina et al., 1996).

Research using mice exposed by intradermal injection to neutralising anti IL-1RI, or anti IL-1RII antibodies followed by a topical contact sensitiser or TNF-α, found that only blockade of IL-1RI led to a reduction in DCs in draining lymph nodes (Cumberbatch et al., 1999a). In addition, knockout mice lacking IL-1R1 were found to exhibit a reduced inflammatory response to topical application of PMA (Palmer et al., 2007). In humans, local treatment with recombinant IL-1R1 after exposure to an allergen reduced the allergic response at the treatment site in terms of induration and itch (Mullarkey et al., 1994), again confirming that IL-1R1 is the functional receptor.

Another key regulator of IL-1β activity is the IL-1 receptor antagonist (IL-1Ra), which binds and acts mainly through the IL-1RI (Hannum et al., 1990; Palmer et al., 2007), although there is some evidence that it can bind to IL-RII on certain cell types (Dripps et al., 1991b). While IL-1Ra is able to bind to the IL-1RI, it does not appear to induce signal transduction (Dripps et al., 1991a). Mice lacking IL-1Ra have an abnormal phenotype with sparse hair, scaling and inflammatory skin lesions. In contrast, mice which produce increased levels of IL-1Ra have a reduced cutaneous response to PMA (Palmer et al., 2007).

With regard to human skin, Bigler et al have shown that human keratinocytes cultured from neonatal foreskin can produce IL-1Ra (Bigler et al., 1992). Additionally, when IL-1Ra is administered intradermally to mice it can inhibit both the sensitisation and elicitation phases of CHS (Kondo et al., 1995a). Kutsch et al also showed that neonatal keratinocytes can produce IL-1Ra mRNA and protein: interestingly,
keratinocytes cultured with TNF-α produced higher levels of IL-1Ra (Kutsch et al., 1993).

1.5.5.5 Interleukin 10

The anti-inflammatory cytokine IL-10 is involved in the regulation of LC mobilisation (Wang et al., 1999). The principal source of IL-10 in the epidermis is the keratinocyte (Enk and Katz, 1992), although LCs can also produce IL-10 (Igyarto et al., 2009; Yoshiki et al., 2010). Human blood derived CD1a⁺ DCs cultured with alloreactive T cells in the presence of IL-10 induced less T cell proliferation than did controls. Comparison of supernatants from the DC/T cell cultures revealed reduced levels of TNF-α and IFN-γ (Caux et al., 1994). Similar studies with human epidermal derived LCs also demonstrated an inhibition of T cell proliferation when IL-10 was added to the culture media. The same group showed that addition of IL-1β reduced the suppressive action of IL-10, although TNF-α did not (Peguet-Navarro et al., 1994). When Enk et al further studied the role of IL-10 in mouse derived epidermal cell suspensions they found that the T helper response was abrogated (Enk et al., 1993b). Subsequent in vivo mouse work by the same group found that the ear swelling response to a contact sensitizer was reduced by prior intradermal injection of IL-10, and that this effect was maintained upon rechallenge two weeks later. Analysis of epidermal cell suspensions derived after IL-10 injection found reduced levels of IL1B, TNFA and IL1A mRNA; the reduction in IL1B, being most marked (Enk et al., 1994). The authors hypothesized that IL-10 leads to inhibition of IL-1β release from LCs and that LCs under the influence of IL-10 may be tolerance inducing. Consistent with these findings, Igyarto et al recently reported that LCs can suppress the CHS response in mice via IL-10 dependent inhibition of hapten specific T cells (Igyarto et al., 2009). Ultraviolet B radiation has also been shown to stimulate IL-10 production by murine LCs, which is involved in the induction of T regulatory cells (Yoshiki et al., 2010). In a human skin explant model, de Gruijl et al found that émigrés collected from explants treated with IL-10 had a more immature phenotype with a reduced T cell stimulatory ability (de Gruijl et al., 2006).

There is also some evidence that IL-10 may alter the expression of CD44 (a cell surface protein involved in inter-cellular communication) and intercellular adhesion
molecule (ICAM)-1, also known as CD54, on the surface of LCs (Chatelain et al., 1998; Osada et al., 1995). CD54 is found in low levels on unstimulated CD1a+ cells, however, it is upregulated on exposure to IFN-γ and a combination of TNF-α and IL-1α. Addition of IL-10 to culture media can reduce the expression of CD54 on CD1a+ cells from epidermal cell suspensions, and can also prevent the IFN-γ/TNF-α and IL-1α induced upregulation (Chatelain et al., 1998).

Wang et al have used an IL-10 knockout mouse model to show the regulatory effects of IL-10. Although the knockout mouse had similar numbers of LCs compared to wild type, the levels of TNF and IL1B mRNA in epidermal cell suspensions were increased, and exposure to a contact sensitiser led to an increased ear swelling response and an increased number of hapten bearing cells in skin draining lymph nodes. Interestingly, injection of neutralising TNF-α antibodies or IL-1Ra reduced the CHS response (Wang et al., 1999). Taken together these findings suggest that a balance between the pro-inflammatory cytokines (IL-1β and TNF-α) and the anti-inflammatory cytokine IL-10 is involved in the regulation of LC mobilisation.

1.5.5.6 The Role of Adhesion Molecules and Matrix Metalloproteinases in Langerhans’ Cell Migration

Adhesion molecules are required to both retain LCs within the epidermis and to facilitate their interaction with the ECM and basement membrane. E-cadherin mediates the adhesion of LCs to surrounding keratinocytes (Tang et al., 1993). In studies in mice, Cumberbatch et al demonstrated that E-cadherin is expressed by both LCs and keratinocytes, but not by DCs isolated from draining lymph nodes (Cumberbatch et al., 1996). Langerhans’ cells express α6 and β1 integrins, which facilitate adhesion to components of the ECM, in particular laminin. Studies on LCs isolated from human and murine epidermis have shown that anti-α6 integrin antibodies reduce binding to laminin, and prevent migration to draining lymph nodes (Le Varlet et al., 1992; Price et al., 1997) suggesting that this integrin is required for LC migration. More recently, Maddaluno et al have shown that LCs express the adhesion molecule L1, and that L1 is important for the interaction between the LC and the lymphatic endothelium (Maddaluno et al., 2009).
Production of MMPs, in particular MMP-9, is thought to allow LCs to traverse the dermo-epidermal junction (Noirey et al., 2002; Ratzinger et al., 2002). Inhibition of MMPs restricts LC migration from murine and human epidermal explants; furthermore LC emigration is reduced in explant cultures from MMP-9 gene deficient mice (Ratzinger et al., 2002). Interestingly, a recent report suggests that in both man and mouse LCs can migrate through pre-existing pores in the basement membrane. Exposure of human skin to a contact allergen, irritant, or UV radiation led to an increase in pore diameter with cells presumed to be migrating LCs visualised passing through (Oakford et al., 2011). The mechanism mediating the change in the pore size has not been elucidated.

Tight junctions help maintain the barrier function of the epidermis and prevent loss of water. Claudin-1, an important component of tight junctions, is expressed on keratinocytes and LCs. Although claudin-1 is not thought to play a role in LC migration, LC dendrites which express claudin-1 are able to penetrate tight junctions and uptake antigen via endocytosis (Kubo et al., 2009; Zimmerli et al., 2008).

1.5.5.7 The Role of Chemokines in Langerhans’ Cell Migration

Chemokines are known to be important effector molecules in LC trafficking. Immature DCs express several chemokine receptors including CCR2 and CCR6, which appear to be required for recruitment of bone marrow derived LC precursors to the skin. Experiments using lethally irradiated mice reconstituted with CCR2/-/- bone marrow have shown that following an inflammatory stimulus mice lacking CCR2 have significantly reduced LC numbers compared with wild-type controls. The LC numbers returned slowly to control levels over a period of weeks suggesting this is not the only mechanism by which LCs are recruited into the epidermis (Merad et al., 2002). A similar study by the same group found that irradiated mice reconstituted with CCR6/-/- bone marrow had reduced LCs after 6 weeks compared with mice reconstituted with normal bone marrow (Merad et al., 2004).

Upon stimulation, DCs down-regulate expression of the chemokine receptors CCR1 and CCR5. In addition, stimulated DCs do not respond to the ligands for CCR1 and CCR5 (CCL4 and CCL5) (Sozzani et al., 1998). This may be important in allowing activated LCs to leave the epidermis. In combination with this, activated LCs upregulate expression of CCR7, and culture of mouse ear skin with CCL21 (one of
the ligands for CCR7) leads to an increased number of MHC class II+ émigrés in culture media (Saeki et al., 1999). Stimulated DCs also migrate in response to CCL19 (another ligand for CCR7) (Sozzani et al., 1998), although there is evidence to suggest that CCL19 is dispensable for skin DC migration (Britschgi et al., 2010).

The latest research also supports the role of CCR7 and the ligand CCL21 in DC migration. Lymphatic endothelial cells have been shown to contain CCL21, and CCR7 has been shown to be required for DCs to dock and transmigrate into lymphatics (Tal et al., 2011). CCL21 secretion from dermal lymphatic endothelial cells can be induced by TNF-α (Johnson and Jackson, 2010) as can CCR7 expression on monocyte derived LCs (Geissmann et al., 2002). In addition CCR7 appears to be important for LC migration both in the steady state and during inflammation (Ohl et al., 2004).

Ouwehand et al have proposed a two-step model for LCs migrating from the epidermis to the lymph node following exposure to a contact allergen. In the first step, they demonstrated that cx chemokine receptor (CXCR) 4 and its ligand CXCL12 are needed for LCs to migrate from the epidermis to the dermis. Using human skin explants they found CXCL12 localised within the dermis. Neutralising antibodies for CXCL12 blocked LC migration, and LC-like cells obtained from a human cell line (MUTZ-3) upregulated CXCR4 within 16 hours of exposure to nickel sulphate and also migrated towards fibroblasts in culture. In the second step, LCs increased production of CCR7 and travelled to dermal lymphatics and then localised within skin draining lymph nodes (Figure 1.3). (Ouwehand et al., 2008). Subsequently, the same group have shown that LC migration following exposure to a topical irritant is dependent upon CCL2 and CCL5 secreted by dermal fibroblasts rather than CXCL12 and CCL19/CCL21 (Ouwehand et al., 2011). Of note, TNF-α induced increased fibroblast secretion of CCL2 and CCL5, and neutralisation of TNF-α blocked irritant induced LC migration. Interestingly, the migratory LCs remained immature with no upregulation of CD83 suggesting that they may play a tolerogenic role upon irritant exposure. This may also explain the apparent discrepancy between the findings of this study and those of Sozzani et al who reported a lack of response to CCL5, since in the latter study the DCs were stimulated to mature (Sozzani et al., 1998).
Figure 1.3 Summary of the key cytokines and chemokines involved in Langerhans’ cell migration upon exposure to a contact allergen. The pro-inflammatory cytokines, IL-1β and TNF-α, and the anti-inflammatory cytokine, IL-10, regulate LC mobilisation and migration. Upon stimulation, CCR1 and CCR5 are down-regulated, whilst CCR7 and CXCR4 are up-regulated to facilitate LC movement from the epidermis to the draining lymph node. (LC, green; T lymphocyte, yellow; chemokine ligands in brackets).

1.6 The Influence of Ageing on Langerhans’ Cell Migration

As mentioned earlier, research has established that there is a reduction in LC number in aged skin, furthermore LCs in younger individuals appear to have longer more branched dendrites (Gilchrest et al., 1982; Thiers et al., 1984). However, there have been very few studies to address how ageing affects LC mobilisation and migration. Initial work by Sprecher et al found that DCs within epidermal cell suspensions (presumed to be LCs) from aged mice induced less T cell proliferation when cultured with allogeneic T cells. In addition, when the aged mice were immunised with OVA and then epidermal cell suspensions prepared and cultured with T cells in the presence of ovalbumin there was again a reduced T cell proliferation suggesting impaired antigen presentation (Sprecher et al., 1990). It may be that the reduced density of LCs in the aged mice accounted for the changes in the functional response rather than a
defect on a per cell basis, however, Cumberbatch *et al* were able to demonstrate reduced LC migration in aged mice exposed to a contact allergen compared with their younger counterparts, as measured by reduction in LCs in epidermal sheets and accumulation of DCs in draining lymph nodes. Aged mice were able to mount a similar elicitation response to the allergen upon rechallenge. This led the authors to hypothesize that the reduced delivery of antigen to the lymph node in the aged mice is still sufficient to provoke sensitisation, at least in conditions where the amount of antigen available is not limiting (Cumberbatch *et al.*, 2002). To investigate further the possible mechanism(s) for the observed reduction in LC migration in aged skin they performed intradermal injections of TNF-α and IL-1β and measured LC numbers in epidermal sheets. Although LC migration in response to TNF-α was significantly reduced in aged mice, the response to IL-1β was unchanged compared with younger mice. Subsequent studies by the same group confirmed this is the case in humans also (Bhushan *et al.*, 2002; Bhushan *et al.*, 2004). They offered several possible explanations for this apparent defect in IL-1β in aged skin including: reduced transcription or translation of IL-1β; a reduced ability to convert proIL-1β into the bioactive form; and alteration in the balance of the IL-1 receptors and IL-1Ra. Previous studies have identified an age related reduction in IL-1α in the epidermis of aged mice (Sauder *et al.*, 1989; Ye *et al.*, 2002), however in these studies IL-1β levels were not measured. Notably in the study by Ye *et al*, IL-1Ra mRNA and protein levels were reduced in the skin of aged mice, whilst the level of IL-1RII was increased suggesting that there could be an alteration in IL-1β signalling, although in the absence of a measurement of IL-1β level this cannot be confirmed (Ye *et al.*, 2002). Furthermore, these findings have not been confirmed in human skin.

Whilst IL-1β seems to be a key factor in the changes that occur in LC migration with age, Corsini *et al* have identified a reduction in TNF-α production following LPS stimulation of epidermis from aged rats (Corsini *et al.*, 2008), and Agius *et al* reported a reduction in TNF-α synthesis by macrophages in aged human skin (Agius *et al.*, 2009).

In addition, it could be hypothesized that increased levels of the inhibitory cytokine, IL-10, contribute to impaired LC migration in aged individuals. While there have been no studies to address this question, one investigation has shown that peripheral blood mononuclear cells (PBMCs) from older individuals stimulated with staphylococcus enterotoxin B have a reduced proliferative response and produce more
IL-10 (Castle et al., 1999). In a different study, unstimulated cultured PBMCs from older individuals produced equivalent levels of IL-10 compared with their young counterparts (Llorente et al., 1997). Studies of LPS stimulated macrophages from aged mice revealed a reduction in the expression of IL-1β and TNF-α genes and an increased expression of the IL-10 gene (Chelvarajan et al., 2006). Furthermore, flow cytometric analyses of monocytes from aged men and women demonstrated a reduced proportion of cells co-expressing IL-1β and TNF-α in aged women (compared with younger women), but an increased proportion in aged men (compared with younger men) (Pietschmann et al., 2003). Previously, the production of IL-1 by cultured monocytes has been found to be unchanged or reduced in aged individuals (McLachlan et al., 1995; Rudd and Banerjee, 1989). Whether these findings are translatable to the skin DC population remains to be seen.

1.7 The Study of Dendritic Cells Using Skin Explant Models

The successful culture of mammalian skin ex vivo has been well documented. Studies of human skin explants (incorporating both epidermis and dermis) conducted over 50 years ago demonstrated proliferation of epidermal cells and maintenance of skin architecture over a 7-10 day culture period (Elmets et al., 1982; Flaxman and Harper, 1975; Reaven and Cox, 1965; Reinertson, 1961; Sarkany et al., 1965). More recently, skin explants have been used to study cutaneous DCs, and as discussed earlier, have proved useful in defining the role of LCs in the cutaneous immune response. The following text provides some background on the use of the skin explant model to study DCs.

In 1990, Larsen et al reported on LC migration from murine explants prepared from dorsal ear skin. Within 4 hours of culture, there was a demonstrable increase in LC size and Ia expression. Langerhans’ cells appeared to leave the epidermis, which corresponded with an increase in Ia⁺ cells in the dermis (LCs were also visualised in dermal lymphatics). Cytospin of the culture fluid revealed a significant proportion of cells expressing Ia. Notably, LCs from cultured skin had an increased ability to stimulate lymphocytes compared to those from freshly obtained skin. Taken together, these data suggest that the trauma of the explant preparation results in spontaneous LC maturation and migration (Larsen et al., 1990). In accordance with these findings, Weinlich et al reported migration of dendritic cells from murine dorsal ear explants.
Langerhans’ cell migration occurred spontaneously in culture, and could be increased by prior in vivo exposure to a topical contact sensitiser. Electron microscopy confirmed the presence of migrating DCs in the dermis (some of which were Birbeck granule containing LCs) and in dermal lymphatics. A range of markers were used to identify the LCs, including adenosine diphosphatase, MHC class II, and anti DEC-205 to show that the decrease in LC number was not due to a change in expression of phenotypic markers, (Weinlich et al., 1998). Langerhans’ cells have also been shown to migrate from explants composed of epidermis alone. Using electron microscopy, LCs were visualised detaching from keratinocytes and penetrating the basement membrane. In explants with epidermis and dermis, LCs were seen entering the lymphatics through gaps in the endothelial lining. Interestingly, migration was seen to occur within 5 hours of culture (Stoitzner et al., 2002).

Studies in humans have corroborated that skin explant culture is associated with spontaneous LC migration (Elmets et al., 1982; Jacobs et al., 2001; Lukas et al., 1996; Rambukkana et al., 1995). Lukas et al cultured split thickness skin grafts in transwell inner chambers for 2 days. Comparison of epidermal sheets taken before and after culture, revealed a significant drop in LC number (ranging from 46-66% reduction). Again, a range of markers were used to identify LCs (HLA-DR, Lag, CD1a) to confirm that a true reduction in LC number rather than a change in phenotype had occurred (Lukas et al., 1996). Phenotypic analyses of DCs that have emigrated from skin explants have demonstrated that the cells have a mature phenotype consistent with changes that occur in vivo. Expression of markers of maturation including CD83, CD86, CCR7 and CD54 have been reported (de Gruijl et al., 2006; Ebner et al., 2004; Jacobs et al., 2006; Rambukkana et al., 1996).

More recently, skin explant studies have been used to demonstrate the functional ability of LCs. In one study, the authors were able to show that LCs from murine and human explants were able to capture, and in the case of the murine explants, present antigen (Flacher et al., 2010). An intradermal injection of ovalbumin coupled to anti-DEC205 was given 4 hours prior to epidermal explant preparation and culture for 3 days. After this time the migratory LCs from the media were cultured with ovalbumin specific allogeneic T cells with a resultant T cell proliferation. Culture of human whole skin explants in medium containing either anti-Langerin or anti-DEC205 antibody led to labelling of LCs with each antibody. Therefore, LCs are able to internalise relatively large molecules injected into the dermis or introduced to culture
medium. In a second study, using intradermal injection of ovalbumin into whole human skin explants followed by a 2 day culture and analysis of émigrés using flow cytometry, or intradermal injection of fluorescently labelled ovalbumin, LCs were shown to be more effective at ovalbumin uptake than dermal DCs (Bond et al., 2009). Henri et al found that murine skin explant derived LCs could present antigen to CD4+ and CD8+ T cells, and using a conditional LC knockout model, they showed that dermal DCs were equally effective at antigen presentation (Henri et al., 2007).

There has also been some interest in the use of human skin explants to study contact sensitisers. Topical exposure of skin explants to toxic concentrations of an irritant or non toxic concentration of a contact sensitiser followed by culture provoked an increase in CD1a+ LCs in the culture media compared to control. However, an increase in the percentage of mature LCs was seen in response to irritation, compared to sub-toxic concentrations of allergen (Jacobs et al., 2006). In the aforementioned study the decrease in CD1a+ cells in frozen tissue sections correlated well with the number of CD1a+ émigrés. These findings again suggest that LCs do not down-regulate CD1a upon migration, or at least that CD1a expression is maintained at a detectable level. Previously, Prignano et al found that CD1a expression was reduced on LCs that had migrated from epidermal explants compared to LCs within the epidermis, however the number of CD1a+ cells was not affected (Prignano et al., 2001).

### 1.8 Retinoic Acid

Vitamin A (retinol) is essential for good health and immunity: deficiency is associated with increased risk of death and impaired resistance to infection (Black et al., 2008; Mayo-Wilson et al., 2011). It is metabolised via the action of retinal dehydrogenases to produce retinoic acid (RA). The effects of RA are mediated through binding to the retinoid nuclear hormone receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR). Several different cell types can synthesize RA, including skin DCs (langerin negative), splenic DCs, gut epithelia, and gut stromal cells (Guilliams et al., 2010; Hall et al., 2011).
1.8.1 Retinoic Acid and the Immune System

Retinoic acid has both pro- and anti-inflammatory actions. It is important in the generation of inducible Treg cells; the differentiation and survival of Th17 cells; the regulation of effector T cells; and has been shown to play a role in the development of CD4+ T cell mediated immunity (Hall et al., 2011; Pino-Lagos et al., 2011). In murine studies, subcutaneous immunisation with ovalbumin in the presence of RA led to an up-regulation of chemokines and integrins on T cells in skin draining lymph nodes that was consistent with a gut homing rather than a skin homing phenotype. Furthermore, there was an increased antigen specific IgA response in the RA supplemented group, suggesting that RA may have a role in augmenting intestinal protection following immunisation (Hammerschmidt et al., 2011).

Geissmann et al studied the effects of retinoic acid in vitro on human monocyte derived LC-like cells. Interestingly, the cultured LC-like cells expressed mRNA for all RARs and RXRs. Addition of retinoids to cultures of immature LC-like cells led to apoptosis (which was mediated through RAR). However, stimulation of the LC-like cells by the addition of TNF-α, or IL-1β inhibited the apoptotic effects of retinoids, led to enhanced expression of CD86 and MHC class II, and enhanced antigen presentation to T cells (mediated through RXR and RAR/RXR heterodimers) (Geissmann et al., 2003). These findings add further weight to the suggestion that RA could have role in immunisation strategies.

1.8.2 Retinoic Acid Receptors in Skin

Retinoic acid has a range of effects on the skin and is used both systemically and topically in the treatment of a variety of skin conditions including psoriasis, acne and photoageing. Prior to the identification of the RXR, RAR-γ was found to be the most abundant RAR in adult human skin (Elder et al., 1991). Subsequently, RAR-α, β and γ have been identified in normal human epidermis: in particular LCs were shown to express RXR-α (Reichrath et al., 1997; Reichrath et al., 1995). Intrinsic skin ageing is associated with an increase in keratinocyte RAR-α expression. Interestingly, in vitro transfection of COS-1 cells with the RAR-α gene caused an increase in MMP-1 expression, which along with RAR-α expression, could be reduced by the addition of RA. One possible explanation for these observations is that skin ageing is associated
with a relative reduction in retinoid availability, and receptor expression is increased to compensate (Watson et al., 2004).

1.8.3 The Effects of All-trans Retinoic Acid on Human Skin

The effects of topical all-trans RA (the predominant form of RA) upon the skin depend upon several factors including the concentration of RA used, age of the subject, use of occlusion, and whether treatment occurs in vivo or in vitro. Epidermal thickening is one of the most prominent effects observed in vivo (Bhawan et al., 1991; Fisher et al., 1991). Varani et al have studied the effect of all-trans RA on full thickness human skin explants from adult and neonatal skin. Explants from adults underwent necrosis by 8 days in culture. Addition of calcium or all-trans RA to the culture medium led to some preservation of the epidermal and dermal structures with the addition of both having the greatest effect, including an increase in epidermal thickness (Varani et al., 1993). In contrast, RA did not have any additional effect on explants from neonatal foreskin compared to calcium alone and did not lead to expansion of the epidermis. Cultured keratinocytes and fibroblasts derived from neonatal and adult skin also responded differently to RA. Keratinocyte proliferation occurred in neonatal keratinocytes with or without RA, and in adult derived keratinocytes only with RA. The same was true in adult derived fibroblasts, however, addition of RA to neonatal fibroblast cultures reduced cell growth (Varani et al., 1994). In vivo, the features of photoprotected adult human skin treated with 0.1% all trans RA under occlusion for 4 days have been shown to include a visible erythema, increased epidermal thickness, spongiosis, a sparse perivascular lymphocytic infiltrate in the dermis, and increased TGF-β1 expression. These changes were not specific to RA and were similar to that observed with the irritant sodium lauryl sulphate (SLS). However, RA treatment caused an increase in mucin deposition, whereas SLS did not, suggesting that RA effects are not entirely due to irritancy (Fisher et al., 1992).

1.8.4 The Effects of Retinoic Acid on Langerhans’ Cells

There are conflicting reports in the literature on the effect of retinoids on LC frequency. A number of studies have found that treatment with topical or systemic
retinoid does not affect LC frequency in man or mouse (Koulu and Jansen, 1982; Meunier et al., 1994; Williams and Hill, 1991). Meunier et al used topical 0.1% all trans RA over a 4 day protocol on adult human skin. The RA was not fully occluded, which reduced the irritant effect. Biopsies taken after 4 days of RA treatment revealed an increased expression of CD11c and HLA-DR on LCs, although LC numbers were unchanged. Langerhans’ cells from RA treated skin caused an increase in alloreactive T cell proliferation compared to control. These findings are similar to those reported by Geissmann et al (see earlier) and suggest that RA may induce LC maturation (Geissmann et al., 2003; Meunier et al., 1994).

In contrast, Fisher et al reported a slight reduction in LCs in RA treated skin, although this did not reach significance (Fisher et al., 1991). In this study the RA was applied for 4 days and kept under occlusion throughout, leading to a notable retinoid induced irritant effect which could have caused LC migration, thus explaining the reduction in LCs. Barnadas et al also reported a decrease in LCs in photodamaged skin treated with topical RA for 6 months. The decrease was only evident when LCs were counted as units per epidermal surface (not when counted as unit per epidermal length), suggesting the apparent decrease may actually be due to the retinoid induced epidermal hyperplasia rather than a decrease in number of LCs per se (Barnadas et al., 1995).

In a similar study of photodamaged skin treated with topical RA for 6 months (albeit at a lower concentration), there was a significant increase in the number of CD1a+ cells in the epidermis and dermis (Murphy et al., 1998). These findings are consistent with the effects of systemic and topical retinoid therapy on LC frequency in renal transplant recipients (Carneiro et al., 2005; Rook et al., 1995).

As reported by Meunier et al in 1994, in addition to mediating effects on LC number, RA can also affect LC function (Meunier et al., 1994). Murine studies have shown that topical and systemic retinoids can prevent ultraviolet B induced LC depletion, and when added to epidermal cell cultures can enhance LC ability to stimulate allogeneic T lymphocytes (Dunlop et al., 1994; Ho et al., 1991).

1.8.5 The Effect of Retinoic Acid on Cutaneous Interleukin-1

Several studies have reported the effects of RA on IL-1α and IL-1β production in the skin. In two studies (one in man, one in mouse), addition of RA to keratinocyte
cultures caused an increase in levels of IL-1, although there was no differentiation between IL-1α and IL-1β (Blanton et al., 1989; Tokura et al., 1992). Elder et al also found that RA, even at very low concentration, induced IL1B mRNA production by cultured keratinocytes as early as four hours post treatment (Elder et al., 1991), and human gingival tissue explants treated with retinol over a 72 hour culture period released increased levels of interleukin-1 (again there was no differentiation between IL-1α and IL-1β) compared to controls (Walsh et al., 1985).

In vivo, topical treatment of photoprotected skin with 0.1% RA under occlusion for four days led to a slight decrease in IL-1β mRNA and protein, although levels of IL-1α were unchanged (Fisher et al., 1991). This is in contrast to the results observed by Gruaz et al who reported no change in levels of IL-1β following daily application of RA for 4 days; however occlusion of either the vehicle or RA was associated with an increase in IL-1β (Gruaz et al., 1990).

It is evident that different methods of application and duration of RA treatment can mediate different effects upon LCs and the skin in general which should be considered when using RA as a therapeutic agent.

### 1.9 Conclusions

Over recent years, the understanding of LC biology has increased considerably. Key features of LC mobilisation and migration have been identified, and additionally dermal DCs have become an area of research interest. The importance of these cells in the immune response is clear, yet they remain relatively neglected in the study of immunogerontology. Further work is required to establish how the ageing process affects skin DC function, and to allow development of targeted therapies.

### 1.10 Aims and Hypothesis of the Research Project

The aims of this project were to investigate the mechanisms by which LC number and function are perturbed in aged skin. The reduced number of epidermal LCs in the elderly may be due to a reduction in circulating CD14+ monocyte precursor cells, or a relative inability of the precursors to differentiate into LCs. Furthermore, it is possible that the reduced number of epidermal LCs is paralleled by a reduced number of dermal DCs, which if so could indicate a common precursor defect.
Langerhans’ cell migration in the aged can be at least partially restored by exogenous IL-1β; therefore one particular aim was to investigate the effect of age on epidermal IL-1β availability.

The hypotheses of the research project were that the reduced number of epidermal LCs observed in the elderly is at least in part due to a relative inability of circulating precursors to differentiate into functional LCs, and that LC migration is impaired due to a reduced availability of IL-1β.

1.11 Experimental Strategy

All experiments were performed in healthy aged (70 years and over) and healthy young volunteers (30 years and under), the latter acting as controls. Skin biopsies were taken from sun protected buttock skin. To determine whether there is a change in the number or function of circulating precursor cells monocytes were isolated by magnetic activated cell sorting (MACS) of PBMCs separated from venous blood samples. The monocytes were then cultured in media containing GM-CSF, IL-4 and TGF-β. The resultant monocyte derived LCs (MoLCs) and their response to stimulation with LPS (positive control), TNF-α, and IL-1β was characterised by flow cytometry. The relative expression of the LC marker CD1a and markers of LC activation including CD86, CD54, and HLA-DR were measured. Production of cytokines and chemokines of interest (including IL-1β, TNF-α, IL-1Ra, IL-10, IL-6, CCL-3 and CCL-4) were evaluated using cytokine bead array. A transwell migration assay was also used to study the migratory ability of MoLCs cultured from monocytes of young and aged volunteers.

Epidermal cell suspensions prepared from the skin of young and aged individuals were dual-labeled with langerin and markers of LC activation including HLA-DR and CD86. Flow cytometry was used to quantify the percentage of cells within the epidermis that are langerin+ and the relative expression of the markers activation. Characterisation of dermal DCs was attempted initially using flow cytometric analysis of dermal cell suspensions prepared from 6mm punch biopsies. The cells were labeled with DC markers including CD1a, CD11c, and HLA-DR. Skin biopsies were then analysed using immunohistochemistry to identify both the LC and dermal DC populations.
The levels of IL-1β and caspase-1 mRNA and protein were measured in epidermal samples using polymerase chain reaction (PCR) and western blot respectively. Interleukin-1RI and IL-1RII expression were studied using flow cytometry of epidermal cell suspensions, whilst levels of IL-1Ra were determined using enzyme linked immunosorbent assay (ELISA) of explant supernatants. Epidermal explants were used to study LC migration ex vivo both in terms of spontaneous migration and the response to addition of the cytokines IL-1β and TNF-α to the culture media. The effect of topical all trans RA under occlusion for 72 hours on LC migration was also investigated using the epidermal explant model.
2. Materials and Methods

2.1 Ethical Approval

Studies were conducted with approval from local Research Ethics Committees (REC, reference numbers 08/H1014/92, 09/H1012/5, 09/H1012/21, and 11/NW/0186) and the University of Manchester Ethics Committee. All participants were required to give written, informed consent.

2.2 Subjects

Healthy volunteers were used for all experiments and were recruited from an existing database at the Dermatology Centre, Salford Royal Hospital, or via advertisements at local Universities, libraries, health centres, and hospitals. The following inclusion and exclusion criteria applied:

Inclusion criteria
- 30 years of age or under (for young subjects)
- 70 years of age or over (for aged subjects)
- Written informed consent

Exclusion criteria
- Active skin disease
- Active febrile illness
- Use of an experimental drug or device in preceding 30 days prior to study inclusion
- History of keloid scarring
- Pregnant or lactating females
- Use of significant medication (for example, immunosuppressants) which may affect study results

The number of biopsies taken for each experiment and the volunteer characteristics are described in Table 2.1.
Table 2.1 Summary table of volunteer demographics and numbers of samples used per experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chapter</th>
<th>Number of biopsies</th>
<th>Number of subjects</th>
<th>Sex of subjects</th>
<th>Age range of subjects (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Young</td>
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<tr>
<td>Phenotype of MoLCs</td>
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<td>12</td>
<td>2 female 4 male</td>
<td>2 female 4 male</td>
</tr>
<tr>
<td>Transwell migration assay</td>
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<td>0</td>
<td>10</td>
<td>3 female 2 male</td>
<td>3 female 2 male</td>
</tr>
<tr>
<td>Epidermal cell suspensions</td>
<td>4</td>
<td>2</td>
<td>30</td>
<td>8 female 9 male</td>
<td>6 female 7 male</td>
</tr>
<tr>
<td>Dermal cell suspensions</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>4 female 3 male</td>
<td>2 female 1 male</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>3 female 2 male</td>
<td>3 female 2 male</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
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<td>2</td>
<td>18</td>
<td>4 female 5 male</td>
<td>3 female 6 male</td>
</tr>
<tr>
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<td>2</td>
<td>6</td>
<td>3 female 1 female 2 male</td>
<td>1 female 2 male</td>
</tr>
<tr>
<td>Interleukin-1β ELISA</td>
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<td>2</td>
<td>24</td>
<td>7 female 5 male</td>
<td>5 female 7 male</td>
</tr>
<tr>
<td>Epidermal explants</td>
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<td>2 or 4</td>
<td>30</td>
<td>12 female 4 male</td>
<td>10 female 4 male</td>
</tr>
<tr>
<td>Retinoic acid patch test</td>
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<td>4</td>
<td>11</td>
<td>3 female 1 male</td>
<td>5 female 2 male</td>
</tr>
</tbody>
</table>

Abbreviations: MoLCs, monocyte derived Langerhans’ cells; ELISA, enzyme linked immunosorbent assay.

2.3 Culture of Immature Dendritic Cells from Peripheral Blood Monocytes

Preparation of Peripheral Blood Mononuclear Cells

One hundred and twenty ml of venous blood were taken into 10ml plastic lithium heparin vacutainers (3S healthcare, Enfield, UK). Blood was then diluted 1:1 with Dulbecco’s phosphate buffered saline (PBS, Invitrogen, Paisley, UK) pre-warmed to 37°C in fresh tubes. The diluted blood was layered onto Ficoll-Paque (GE Healthcare Ltd, Buckinghamshire, UK) again pre-warmed to 37°C and centrifuged for 40 minutes (min) at 400 g (Sorvall® legend RT), at room temperature (RT). The resultant “white layer” of lymphocytes at the interface was pipetted into 50ml tubes and made up to 45ml with 0.5M ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Dorset, UK)
suspended in PBS. The cells were centrifuged at 283 g, for 5 min, at RT, and the wash process repeated a further three times. After the final wash the cells were resuspended in 10ml of 0.5M EDTA in PBS and a viable cell count performed using a haemocytometer. Trypan-blue (0.4% solution, Sigma-Aldrich) was used to exclude non-viable cells. Following this, the cells were washed and resuspended in 30μl of MACS buffer (comprising 0.2% heat inactivated foetal calf serum (FCS, Invitrogen), and 2mM EDTA in PBS) per 10⁷ cells. Five μl of the suspension was removed and diluted with 745μl of fluorescence-activated cell sorting (FACS) buffer (comprising 5% FCS in PBS- PBS from Fisher Scientific, Loughborough, UK) and kept on ice for later flow cytometric analyses.

Isolation of Monocytes

The cells were incubated with 10μl of Fc receptor (FcR) blocking reagent and 10μl biotin-antibody cocktail (both from human monocyte isolation kit, Miltenyi Biotec, Surrey, UK) per 10⁷ cells at 4°C for 10 min. Next, a further 30μl of MACS buffer and 20μl of anti-biotin microbeads per 10⁷ cells were added and the mixture incubated for a further 15 min at 4°C. The cells were washed by adding 2ml MACS buffer and centrifuging at 300 g for 10 min at RT. The cells were then resuspended in 500μl MACS buffer and were applied to the MACS column (LS MACS cell separation column, Miltenyi Biotec) inside the magnetic field. The column was rinsed three times with 3ml of MACS buffer and the flow through collected in a 10ml round bottomed tube. The cells were centrifuged at 241 g for 5 min at RT and resuspended in 5ml of MACS buffer. A cell count was performed as described previously and 250μl of the suspension removed, made up to 750μl with FACS buffer, and put on ice for later flow cytometric analysis. The remaining suspension was centrifuged at 241 g for 5 min at RT.

Culture of Monocytes

The cells were resuspended at a density of 10⁶ per ml of pre-warmed culture medium (RPMI 1640, containing 25mM HEPES, 100μg/ml streptomycin, 100units/ml ampicillin, and 29.2mg/ml glutamine, all Gibco, Invitrogen) containing 10% filtered heat inactivated FCS. The following cytokines were added to the culture medium: recombinant human IL-4 at 10ng/ml (R&D Systems, Abingdon, UK endotoxin level <1.0 endotoxin unit (EU) per 1μg), recombinant TGF-β at 10ng/ml (R&D Systems,
endotoxin level <1.0 EU per 1μg), and GM-CSF at 250ng/ml (R&D Systems, endotoxin level <1.0 EU per 1μg). The cells were cultured at 1ml per well in a 24 well flat bottomed plate at 37°C in a humidified incubator with 5% CO₂.

2.4 Staining and Flow Cytometric Analysis of Peripheral Blood Monocytes

One hundred μl of cells taken pre- and post- MACS isolation were added to 96 well plates and made up to 200μl with FACS buffer. The plates were centrifuged at 241 g for 5 min at 4°C. Cells were then incubated with 100μl of the following antibodies with the appropriate isotype control used at an equivalent concentration: CD3 (2μg/ml, eBioscience, Hatfield, UK, clone OKT3) isotype control mouse IgG2a (Dako, Stockport, UK) CD14 (10μg/ml, eBioscience, clone 61D3), isotype control mouse IgG1 (BD Bioscience, Oxford, UK) and CD19 (10μg/ml, Chemicon International, Billerica, MA, USA, clone HD37) isotype control mouse IgG1 (BD Bioscience) for 30 min on ice. Two wells were incubated with FACS buffer alone to act as a control for non-specific binding of the secondary antibody. The cells were washed with 100μl FACS buffer and centrifuged at 241 g for 5 min at 4°C, followed by two further washes with 200μl of FACS buffer. The cells were incubated with 100μl of fluorescein isothiocyanate (FITC) labelled polyclonal goat anti-mouse Ig (20μg/ml, Dako) for 30 min on ice in the dark, and then washed three times as before. Following the final wash, cells were resuspended in 100μl of sodium azide buffer (containing 0.5% of 10% sodium azide and 1% FCS in PBS). Cells were analysed by flow cytometry using a FACSCalibur and CellQuest Pro software (Becton-Dickenson, NJ, USA). Twenty thousand cells were acquired per sample and non-viable cells excluded using 1mg/ml propidium iodide (PI, Sigma-Aldrich).

2.5 Feeding and Stimulation of Cultured Human Monocyte Derived Langerhans’ Cells

Cells were fed on day 3 of culture. Five hundred μl of media was removed carefully from each well and placed in a 15ml falcon tube, which was then centrifuged at 168 g for 5 min at RT. The supernatant was removed carefully and the pellet resuspended in 500μl per well of pre-warmed culture media containing 10% FCS as described above. Interleukin-4, GM-CSF, and TGF-β were added to the media as before, and 500μl
was then added to each well. The cells were cultured for a further 2 days at 37°C in a humidified incubator with 5% CO₂.

After this time, the cells were removed by gentle scraping and mixing with a pipette. The contents of all the wells were combined into a 50ml falcon tube and centrifuged at 168 g for 5 min at RT. The cells were resuspended in 3ml of fresh pre-warmed culture media containing 10% FCS, and a viable cell count performed using a haemocytometer and trypan-blue to exclude non-viable cells. The cells were resuspended in culture media at $1 \times 10^6$ cells/ml and IL-4 and GM-CSF added at 10ng/ml and 250ng/ml respectively. One ml of the suspension was added to each well in a new 24 well flat bottomed culture plate. Cells were stimulated with 100ng/ml, 10ng/ml, or 1ng/ml of recombinant human TNF-α (R&D Systems, endotoxin level <1.0 EU per 1μg) or recombinant human IL-1β (R&D Systems, endotoxin level <1.0 EU per 1μg). Lipopolysaccharide from Escherichia coli serotype 0 (Sigma-Aldrich) was used as a positive control at 1μg/ml, and medium alone as a negative control. The cells were then cultured for a further 24 hours prior to staining and analysis.

### 2.6 Analysis of Human Monocyte Derived Langerhans’ Cells using Flow Cytometry

Cells were harvested as described above, placed in 1.5ml Eppendorf tubes, and centrifuged using a Microfuge®22R (Beckman Coulter, High Wickham, UK) at 176 g for 5 min at RT. Supernatants were removed, aliquoted, and kept at -70°C for later analysis using cytokine bead array. The cells treated with LPS, media, and 10ng/ml or 100ng/ml of TNF-α or IL-1β were resuspended in 900μl of FACS buffer and aliquoted 100μl per well into a 96 well plate. A further 100μl of FACS buffer was added to each well and the plate centrifuged at 241 g for 5 min at 4°C. Cells were then incubated with 100μl of the following antibodies with the appropriate isotype control used at an equivalent concentration: HLA-DR (2μg/ml Dako, clone DK22, isotype control mouse IgG2a), CD1a (2μg/ml Dako, clone NA 1/34, isotype control mouse IgG2a), CD54 (10μg/ml, BD Bioscience, clone HA58, isotype control mouse IgG1), CD86 (10μg/ml, BD Bioscience, clone 2331(FUN-1), isotype control mouse IgG1), and CD14 (10μg/ml), for 30 min on ice. Four wells were incubated with FACS buffer alone to act as a control for non-specific binding of the secondary antibody. After 30 min the cells were washed with 100μl FACS buffer and centrifuged at 241 g for 5 min
at 4°C, followed by 2 further washes with 200μl of FACS buffer. The cells were then incubated with 100μl of FITC labelled polyclonal goat anti-mouse Ig (20μg/ml) for 30 min on ice in the dark, after which they were washed three times. Following the final wash, cells were resuspended in 100μl of sodium azide buffer, and analysed via flow cytometry as before.

2.7 Analysis of Culture Supernatants Using Cytokine Bead Array and ELISA

The level of IL-1β, TNF-α, IL-1Ra, IL-10, CCL3 and CCL4 secreted from MoLCs was measured in the culture supernatants using the Bio-Plex Pro™ Human Cytokine Standard according to the manufacturer’s instructions (Bio-Rad Laboratories), and data acquired using the Luminex system (Luminex 100; MiraiBio Hitachi Genetic Systems). The level of IL-6 in the culture supernatants was measured using a sandwich ELISA kit according to the manufacturer’s instructions (R&D systems). In brief, 96 well plates were coated with 100μl of IL-6 antibody (2μg/ml) overnight and then washed three times with wash buffer containing 0.05% Tween 20 (Sigma-Aldrich) in PBS. The plates were then incubated with 300μl of block containing 1% bovine serum albumin (BSA, Sigma Aldrich)/PBS for 1 hour and washed. Next the plates were incubated for 2 hours with recombinant IL-6 in 2-fold serial dilutions with 1% BSA/PBS as the standard and the samples. The wash steps were repeated and the plates incubated with the detection antibody (50ng/ml of biotinylated goat anti-human IL-6) for 2 hours followed again by three washes. Streptavidin- horseradish peroxidise (HRP) diluted in 1% BSA/PBS (to recommended dilution of 1 in 200) was added to the plates for 20 min in the dark, after which the plates were washed and incubated with the substrate solution (containing hydrogen peroxide and tetramethylbenzidine) for a further 20 min. The reaction was stopped using 2 N sulphuric acid and the optical density of each well determined using a microplate reader (Titertek Multiskan, Pforzheim, Germany) set at 450nm. Data was analysed using Genesis software (Thermo Scientific, Wilmington, DE, USA).

2.8 Transwell Migration Assay Using Monocyte Derived Langerhans’ Cells

Monocyte derived LCs that had been stimulated with LPS, IL-1β, TNF-α, or control medium were removed carefully from 24-well plates as described above. The cells
were placed in 1.5ml Eppendorf tubes, and centrifuged using a Microfuge®22R at 176 g for 5 min at RT. The supernatant was removed and the cells resuspended in 500μl of pre-warmed culture medium containing 10% FCS and a cell count performed using an electronic cell counter (CASY Model Cell Counter, Roche, Burgess Hill, UK). The cells were again centrifuged and resuspended at a concentration of 2 x 10^6 cells per ml of medium. A total of 595.2μl of 1% FCS/RPMI was added to the central wells of a 24-well plate followed by 4.8 μl 0.5% BSA/PBS (control wells), or 4.8μl of 25μg/ml recombinant human CCL19 (final concentration 200ng/ml, R&D Systems, endotoxin level <1.0 EU per 1μg). Costar Transwell inserts with a 5μm pore size and 6.5mm membrane diameter (Fisher Scientific) were then placed above the medium and 2x 10^5 cells in 100 μl of medium added. The cells were then incubated at 37°C in a humidified incubator with 5% CO_2 for 3 hours. The medium from the bottom of each well and the underside of the Transwell insert was carefully removed into 1.5ml Eppendorf tubes and cell counts performed using the electronic cell counter.

2.9 Preparation and Analysis of Epidermal Cell Suspensions

Two 6mm diameter punch biopsies (Stiefel, Healthcare Equipment and Supplies Co, Egham, Surrey, UK) were taken from sun protected buttock skin and placed in PBS. Excess fat was trimmed from the biopsies, which were then washed in PBS before being incubated in 0.5% trypsin- 0.2% EDTA (Gibco, Invitrogen) for 1 hour at 37°C. Following this, the biopsies were washed in a 5ml sterilin tube containing RPMI culture medium and 20% FCS. The biopsies were then transferred to a clean petri dish and the epidermis scraped off using a scalpel (Swann-Morton blade No.20, Fisher Scientific). The fragments of epidermis were transferred onto 200 mesh stainless steel gauze and cut into smaller pieces using the scalpel. A syringe plunger was used to push cells through the gauze to create a single cell suspension by mechanical disaggregation and the resulting cell suspension was placed in a 15ml falcon tube and topped up to 10ml with medium. The cells were centrifuged at 168 g at 4°C for 5 min and resuspended in 0.8ml of medium. Two hundred μl of the suspension was pipetted into each of four wells of a 96 well plate; the plate was subsequently centrifuged at 241 g for 5 min at 4°C. One hundred μl of 10% FCS in PBS was used to quench the enzyme for 10 min on ice. The cells were then washed with 100μl of FACS buffer and centrifuged as above.
After washing, the cells were incubated with either anti-HLA-DR (at 2μg/ml) or anti-CD86 (at 10μg/ml) and the appropriate isotype control at an equivalent concentration for 1 hour on ice. The cells were washed three times with FACS buffer as previously described and then incubated with phycoerythrin (PE) labelled polyclonal goat anti-mouse Ig (4μg/ml, Immunokontakt, Frankfurt, Germany) for 30 min on ice in the dark. Next, cells were washed and fixed in 100μl 2% paraformaldehyde (PFA, Sigma-Aldrich) and incubated at RT for 10min in the dark. Again, cells were washed with FACS buffer before a 10 min incubation with 0.1% saponin (Sigma-Aldrich) at RT for 10 min in the dark. The cells were washed and then blocked for 10 min with purified polyclonal rat IgG (AbD Serotec, Kidlington, UK) used at 20μg/ml in FACS buffer. After a further wash step the cells were incubated with Alexa Fluor 488 conjugated anti-langerin antibody (10μg/ml, clone 929F3.01, Cambridge Bioscience, Cambridge, UK, isotype control rat IgG2a) or the appropriate isotype control (both made up in 0.1% saponin) for 45 min at RT in the dark. The cells were washed three times, resuspended in 100μl of sodium azide buffer, and analysed via flow cytometry using a FACSCalibur and CellQuest Pro software. One hundred thousand cells were acquired.

2.10 Preparation and Analysis of Dermal Cell Suspensions

After removal of the epidermis from the 6mm punch biopsies (described above) the dermis was cut into small pieces and placed in a 5ml sterilin containing 1mg/ml of type IV collagenase (Sigma-Aldrich) and incubated at RT for 2 hours. Next, the dermis was washed in RPMI media containing 10% FCS and then transferred onto steel gauze in a petri dish and cut into smaller pieces. As with the epidermis, a syringe plunger was used to push cells through the gauze and the resulting cell suspension was placed in a 15ml falcon tube and topped up to 10ml with media. The cells were centrifuged at 168 g at 4°C for 5 min and resuspended in 0.4ml of 10% FCS RPMI media. Two hundred μl were pipetted into each of two wells of a 96 well plate and the plate centrifuged at 241 g for 5 min at 4°C. Ten percent FCS in PBS was used to quench as described previously and the cells were then incubated with either allophycocyanin (APC) conjugated mouse anti-human CD11c (10μg/ml, ebioscience, clone 3.9, isotype control mouse IgG1), HLA-DR (at 2μg/ml), or the appropriate isotype controls for 1 hour. After washing, cells incubated with anti-HLA-DR were
stained with PE conjugated polyclonal goat anti-mouse Ig for 30 min in the dark. Normal mouse serum diluted 1:10 with FACS buffer was then used to block. Finally, cells were washed and incubated with FITC conjugated mouse anti-human CD1a (20μg/ml, BD bioscience, clone HI149, isotype control IgG1) or the appropriate isotype control for 45 min. After washing, cells were resuspended in sodium azide buffer as before. Up to 1x10^5 cells were acquired on the flow cytometer: non-viable cells were excluded using 7- aminoactinomycin D (AAD, BD Bioscience).

2.11 Identification of Skin Dendritic Cells Using Immunofluorescence

Whole 6mm skin biopsies were snap frozen in Optimal Cutting Temperature embedding compound (Fisher Scientific) and stored at -70 °C until required. Five μm vertical cryosections were prepared using a Cryostat (Bright Instruments) and placed on gelatine coated slides and again stored at -70 °C. Prior to staining slides were removed from the freezer and allowed to air dry. A wax pen (Vector laboratories, Peterborough, UK) was used to draw around each section and the slides were then placed in 4% PFA for fixation for 10 min. The slides were washed in tris buffered saline (TBS, Fisher Scientific) for 30 min after which they were placed in 0.5% Triton X-100 (Sigma-Aldrich) for solubilisation for 10 min. A further 5 min wash was then performed using TBS. After washing, 1% BSA/TBS was applied to each section to block for 30 min. The block was removed by gently tapping the sides of the slides and mouse anti-human langerin diluted in 1% BSA/TBS (4μg/ml, clone DCGM4, Beckman Coulter) applied to the slides for 1 hour at RT. The slides were then washed in TBS for 5 min, after which Alexa Fluor 647 rabbit anti-mouse IgG (10μg/ml in TBS, Invitrogen) was applied. The slides were washed for 5 min as above, then mouse anti-human CD1a diluted in 1% BSA/TBS (10μg/ml, clone NA1/34-HLK, Santa Cruz, Heidelberg, Germany) was applied for 1 hour at RT. Following a further wash, Alexa Fluor 488 rabbit anti-mouse IgG (10μg/ml in TBS, Invitrogen) was applied for 30 min at RT. The sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) diluted 1 in 1000 with TBS (Invitrogen). The slides were washed in TBS for 5 min and then mounted using VECTASHIELD® HardSet™ mounting medium (Vector laboratories). Negative controls without the primary antibodies were performed in parallel. The sections were viewed on a Keyence fluorescence microscope at x200 magnification (Coventry, UK).
2.12 Polymerase Chain Reaction

*Messenger Ribonucleic Acid Extraction*
Two 6mm punch biopsies were taken from each volunteer and snap frozen in liquid nitrogen before storage at -70°C until required. The epidermis was removed from each biopsy using a scalpel, and cut into small pieces before being placed in 1ml of Trizol® (Invitrogen). The samples were then homogenised on ice for 1 min. Two hundred µl of PCR grade chloroform (Sigma-Aldrich) was added and the tube shaken for 15 seconds (sec), followed by a 2 min incubation at RT. The samples were centrifuged at 12000 g for 15 min at 4°C and 400µl of the colourless aqueous phase removed. An equal volume of 70% ethanol was added to each sample and mRNA extracted using the Purelink RNA extraction kit (Invitrogen) according to the manufacturer’s instructions. Contaminating DNA was removed using DNA-free™ DNase treatment and removal reagents (Ambion®, Invitrogen) again according to the manufacturer’s instructions. Messenger RNA from the young and old age individuals were pooled into two groups. The concentration and purity of mRNA in each individual and each pooled sample was then measured using a NanoDrop spectrophotometer which calculated the ratio of absorbance at 260 and 280nm (Thermo Scientific). The integrity of the RNA was confirmed using gel electrophoresis of RNA on a 1% agarose gel containing ethidium bromide (Sigma-Aldrich).

Reverse transcription was performed using 1µg of mRNA. A high capacity RNA-to-cDNA kit (Invitrogen) was used according to the manufacturer’s instructions. Negative controls (no RNA or no enzyme) were also included. The reaction volume on the thermal cycler (Gene Amp PCR System, Applied Biosystems) was set to 20µl and samples were run at 37°C for 1 hour followed by 95°C for 5 min. The samples were then held at 4°C and stored at -20°C prior to use.

*Real-time Quantitative Reverse Transcription Polymerase Chain Reaction*
Interleukin-1β and *CASPI* mRNA expression was analysed using quantitative PCR. Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and keratin 14 (*KRT-14*) were used as housekeeping genes. Taqman Gene Expression Assays containing the appropriate probes and primers were used (Table 2.2, Applied Biosystems). Five µl of cDNA
were added to 10μl of Taqman Universal PCR Mastermix (Applied Biosystems) and 5μl of each primer diluted to 1:4 in RNase free H$_2$O. Each sample was run in triplicate in a 96 well PCR plate, and negative controls both without sample and without reagents were run in parallel. The PCR reaction was carried out according to the manufacturer’s instructions using a StepOne Real-Time PCR System (Applied Biosystems), and data analysed using the Sequence Detection System Software (Applied Biosystems).

The cycle threshold (Ct) was calculated for the genes of interest and the housekeeping genes. Relative expression of $IL1B$ and $CASP1$ was evaluated using the delta delta Ct method. Firstly the difference between the gene of interest and the housekeeping gene was calculated for each age group (delta Ct value), followed by the difference between the delta values (delta-delta Ct value). The fold expression was then calculated using the formula $2^{\text{delta delta Ct}}$ with the level of expression in the old group expressed relative to the young group.

**Table 2.2** Taqman gene expression assays used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>$IL1B$</td>
<td>Hs01555410_m1</td>
</tr>
<tr>
<td>$CASP1$</td>
<td>Hs00354836_m1</td>
</tr>
<tr>
<td>$HPRT$</td>
<td>Hs99999909_m1</td>
</tr>
<tr>
<td>$GAPDH$</td>
<td>Hs99999905_m1</td>
</tr>
<tr>
<td>$KRT14$</td>
<td>Hs00265033_m1</td>
</tr>
</tbody>
</table>

**2.13 Measurement of Epidermal Interleukin-1β and Caspase-1 Protein**

Two 6mm punch biopsies were taken from each volunteer and snap frozen in liquid nitrogen then stored at -70°C until required. The epidermis was removed from each biopsy using a scalpel, and cut into small pieces and placed in 1ml of lysis buffer (Invitrogen) with 10μl of protease inhibitor cocktail I (Merck Millipore, Darmstadt, Germany) in a 5ml sterilin. The epidermis was then homogenised on ice for 1 min. The samples were transferred to 1.5ml eppendorf tubes and centrifuged at 12000 g for 10 min at 4°C. The supernatant was removed and the process repeated. Samples were concentrated to 200μl using Amicon Ultra-0.5 centrifugal filter devices (Millipore)
and the protein concentration determined using a DC protein assay according to the manufacturer’s instructions (Bio-Rad).

**Western Blot- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis**

Initially samples were diluted 1:1 with laemmli buffer (BioRad). However, the protein concentration in the epidermal lysates was relatively low, and therefore to minimise dilution a four times concentrated laemmli sample buffer was prepared (Table 2.3), and the samples diluted 4:1.

**Table 2.3** Reagents used to prepare a four times concentrated laemmli buffer.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base (Biorad)</td>
<td>0.2M</td>
<td>5ml</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS, Sigma-Aldrich)</td>
<td>8%</td>
<td>5ml</td>
</tr>
<tr>
<td>Glycerol (Fisher Scientific)</td>
<td>-</td>
<td>10ml</td>
</tr>
<tr>
<td>Bromophenol blue (Biorad)</td>
<td>0.4%</td>
<td>1ml</td>
</tr>
<tr>
<td>Deionised water</td>
<td>-</td>
<td>4ml</td>
</tr>
</tbody>
</table>

The samples were then mixed with laemmli buffer containing 5% mercaptoethanol (Invitrogen) and heated at 80°C for 10 min. A 10% resolving gel (Table 2.4) was prepared in a 20ml universal tube and poured between 2 glass plates (comprising a 0.75mm spacer plate and short plate) which had been clamped together in a casting frame and held within a casting stand (BioRad). After the resolving gel had set (approximately 45 min) the stacking gel (Table 2.4) was poured into the gel cassette to the top of the short plate, and a 10-well comb carefully placed between the spacers at the top of the spacer plate. The gel was allowed to set for approximately 45 minutes and the comb carefully removed.
Table 2.4 Reagents used to prepare resolving and stacking gels for western blot.

<table>
<thead>
<tr>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O 2.5ml</td>
<td>H₂O 1.625ml</td>
</tr>
<tr>
<td>Lower gel buffer (1.5M Tris HCl pH8.8, 0.4% SDS in 500ml H₂O) 1.5ml</td>
<td>Upper gel buffer (0.5M Tris HCl pH6.8, 0.4% SDS in 500ml H₂O) 625μl</td>
</tr>
<tr>
<td>Acrylamide solution (Sigma-Aldrich) 2ml</td>
<td>Acrylamide solution 250μl</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED, Sigma-Aldrich) 9μl</td>
<td>TEMED 3.5μl</td>
</tr>
<tr>
<td>10% ammonium persulphate (Sigma-Aldrich) 21.6μl</td>
<td>10% ammonium persulphate 7.5μl</td>
</tr>
</tbody>
</table>

The gel cassette was placed into the electrode assembly and then into the electrophoresis tank (Mini PROTEAN 3, BioRad). A tris glycine running buffer (BioRad) was used. A pre-stained protein colour marker (Invitrogen) was loaded into the first well. Samples containing 10μg of protein were loaded into the remaining wells using a pipette and gel tips (Starlab, Milton Keynes, UK). The power was set to 120 volts for the first 5min followed by 80 volts for approximately 1 hour.

**Protein Transfer**

The gel was carefully removed from the gel cassette using a gel releaser (BioRad) in a tray containing transfer buffer (10x stock solution containing 0.25M tris pH8.3 and 1.92M glycine, diluted with deionised H₂O, followed by addition of 200ml of methanol to each 800ml of diluted buffer). A nitrocellulose membrane (Sigma-Aldrich), two pieces of filter paper and two fibre pads were pre-soaked in transfer buffer. One fibre pad was placed on the black side of the gel cassette, followed by a piece of filter paper and then the gel. Care was taken to ensure the gel was flat against the filter paper. The nitrocellulose was then placed carefully over the gel followed by filter paper and a fibre pad. After the filter paper had been applied on top of the membrane, a roller was used to ensure all air bubbles had been removed. The gel
cassette was placed in the tank with the black side of the cassette facing the black electrode. The transfer buffer and an ice block (for cooling) were added to the tank and the power was set to 100 volts for 1 hour.

Chemiluminescent Detection

Following the transfer the nitrocellulose membrane was placed in block for 1 hour on a Belly Dancer™ shaker (Table 2.5). The membrane was then washed for 3 min in TBS containing 0.1% Tween (TBST). The wash step was repeated a further four times and the membrane was then incubated overnight at 4°C in the primary antibody conjugated to HRP as detailed Table 2.5). The membrane was then washed in TBST (15x 1 min washes) before being incubated in the secondary antibody for 1 hour at RT on the shaker (Table 2.5). The wash steps were repeated and the membrane was incubated in chemiluminescent substrate for 1 min (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). The membrane was then wrapped in cling film and exposed to film in the dark (Amersham Hyperfilm ECL, Scientific Laboratory Supplies, Yorks, UK) for 5 sec, 30 sec, 1 min and 5 min as needed, after which the films were developed.

Recombinant human IL-1β (R&D Systems) and human caspase-1 (Millipore) were used as positive controls, and β-actin was used as a control for equal protein loading. Initial experiments performed using the polyclonal caspase-1 and β-actin antibodies resulted in the appearance of multiple bands on the blots, which persisted despite changing concentrations of the primary and secondary antibodies. The problem was solved by using monoclonal primary antibodies which resulted in single bands corresponding to the size of the proteins in question.
Table 2.5 Summary of blocking agents and antibodies used in western blot.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Block</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-1β</td>
<td>5% skimmed milk (Sigma-Aldrich) in TBST</td>
<td>Polyclonal goat anti-human (R&amp;D Systems) 0.1μg/ml in 0.1% BSA in TBST</td>
<td>Polyclonal donkey anti-sheep/goat:HRP (AbD Serotec) 0.1μg/ml in 5% skimmed milk in TBST</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>5% skimmed milk in TBST</td>
<td>Polyclonal goat anti-human (Abcam) 0.5μg/ml in 0.1% BSA in TBST</td>
<td>Polyclonal donkey anti-sheep/goat:HRP 0.1μg/ml in 5% skimmed milk in TBST</td>
</tr>
<tr>
<td></td>
<td>5% BSA in TBST</td>
<td>Rabbit anti-human (clone D7F10, Cell Signaling Technology Inc, Massachusetts, USA) in 5% BSA in TBST</td>
<td>Polyclonal goat anti-rabbit:HRP (Abcam) 0.4μg/ml in 5% skimmed milk in TBST</td>
</tr>
<tr>
<td>β-actin</td>
<td>5% BSA in TBST</td>
<td>Polyclonal rabbit anti-human (Abcam) 0.2μg/ml in 0.1% BSA in TBST</td>
<td>Polyclonal goat anti-rabbit:HRP, 0.4μg/ml in 5% skimmed milk in TBST</td>
</tr>
<tr>
<td></td>
<td>5% BSA in TBST</td>
<td>Mouse anti-human (mAbcam 8226, Abcam) 0.1μg/ml in 5% BSA in TBST</td>
<td>Polyclonal sheep anti-mouse:HRP (AbD Serotec) 0.1μg/ml in 5% skimmed milk in TBST</td>
</tr>
</tbody>
</table>

Measurement of Epidermal Interleukin-1β Using ELISA

The levels of IL-1β protein in epidermal homogenates were also measured using a sandwich ELISA kit as described earlier (R&D systems). The epidermis from two 6mm punch biopsies was prepared as above, and the protein concentration determined using a DC protein assay according to the manufacturer’s instructions (Bio-Rad). The level of IL-1β in the epidermal homogenates as measured by ELISA was then corrected for protein content.
2.14 Analysis of Interleukin-1 Receptor I and II Expression on Epidermal Cells Using Flow Cytometry

Epidermal cell suspensions were prepared as detailed earlier. Human IgG (10μg/ml, Sigma-Aldrich) was used to block for 15 min at RT, after which a PE labelled polyclonal goat anti-human IL-1RI antibody, or monoclonal mouse anti-human IL-1RII antibody (both 25μg/ml) or the appropriate isotype controls were applied (all R&D Systems) for 30 min at RT. The cells were then fixed and permeabilised as described, and stained with the Alexa Fluor 488 conjugated anti-langerin antibody. Intracellular expression of IL-1RII was also measured. The cells were fixed and permeabilised followed by a blocking step with human IgG. The IL-1RII antibody was then applied and the cells washed and stained with Alexa Fluor 488 conjugated anti-langerin antibody as before. After washing, cells were resuspended in sodium azide buffer and up to 1x10^5 cells were acquired on the flow cytometer.

2.15 Epidermal Explant Preparation and Langerhans’ Cell Enumeration

Whole 6mm punch biopsies were washed in sterile PBS then incubated in 0.02M EDTA dissolved in sterile PBS at 37°C for 2 hours. The biopsies were then washed in sterile PBS and the epidermis carefully removed using tweezers. The epidermis was then washed in pre-warmed RPMI culture media containing 10% FCS and double strength antibiotic (200μg/ml streptomycin, 200units/ml ampicillin), 29.2mg/ml glutamine (all from Gibco, Invitrogen), and 2.5μg/ml amphotericin B (Sigma-Aldrich). The wash was then repeated twice with media containing normal strength antibiotic and glutamine (without amphotericin). One epidermal sheet was fixed in acetone at -20°C for 20 min (designated time zero, T0) after which the sheet was placed in PBS and stored at 4°C for a maximum of 72 hours. Five hundred μl of media containing 10% FCS was pipetted into a 24-well plate and the remaining epidermal sheet(s) carefully floated on top of the media (dermal side down) to maintain an air interface. The sheets were then incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours (designated time 24, T24). The sheets were then fixed in acetone as described.

To identify the LCs, the sheets were stained with mouse anti-human CD1a (clone NA1/34-HLK, Abcam) or rat anti-human langerin (clone 929F3.01, Cambridge...
Bioscience) both at 10μg/ml in 0.1% BSA/PBS for 1 hour at RT. Initial experiments were also performed with the appropriate isotype controls. The sheets were then washed in PBS for 10 min and incubated for 45 min in the dark at RT with either FITC labelled polyclonal goat anti-mouse IgG (20μg/ml, Dako), or Alexa Fluor 488 goat anti-rat IgG (10μg/ml, Invitrogen), depending on the primary antibody used. The sheets were washed as before and carefully mounted using VECTASHIELD® mounting medium (Vector laboratories) on Twin Frost microscope slides (Fisher Scientific). Coverslips were placed over the sheets and pressure applied to remove any air bubbles. The coverslips were then sealed with nail varnish. Langerhans’ cells were enumerated using a fluorescence microscope (BX50, Olympus) with a calibrated grid at 40x magnification (0.32 × 0.213 mm at 40x magnification). Sheets were counted in a blinded fashion such that the counter was unaware of whether the sheet corresponded to T0 or T24. Fifty consecutive fields were counted in each sheet avoiding the edges, and the mean number of LCs per mm² was then calculated. By comparing LC counts at T0 and T24, it was possible to calculate the proportion of LCs that had migrated from the explants following the culture period. Photographic images were taken using a colour charge-coupled device camera (RS CoolSNAP; Photometrics, Tucson, AZ, USA) and viewed using the MetaMorph Imaging System (version 4.01; Princeton Instruments, Buckinghamshire, UK). In some experiments, epidermal explants were cultured for either 2, 6 or 24 hours to determine the kinetics of LC migration.

The Effect of Interleukin-1β and Tumour Necrosis Factor-α on Cultured Epidermal Explants

Some epidermal explants were cultured in culture media containing either 2000U of human recombinant IL-1β or 5000U TNF-α (both Insight Biotechnology, Middlesex, UK) for 2 or 24 hours.

Characterisation of Emigrated Langerhans’ Cells

Following the 24 hour culture, the media was carefully removed from each well and placed in a 1.5ml eppendorf, which was then centrifuged at 176 g for 5 min at RT. The supernatant was removed and stored at -70°C. The remaining cells were then fixed and permeabilised as detailed earlier and washed with FACS buffer. Cells were then stained with Alexa Fluor 488 conjugated anti-langerin antibody (10μg/ml) for 45
min in the dark at RT. Cells were counterstained with DAPI diluted 1 in 1000, pipetted onto a microscope slide, and mounted using VECTASHIELD® mounting medium. The cells were visualised using a fluorescence microscope as before. In a further attempt to characterise the émigrés, cells obtained following the culture period were incubated in 100μl of FACS buffer for 10 min in a 96 well round bottomed plate, after which 100μl of mouse anti-human FITC-labelled CD1a antibody at a final concentration of 5μg/ml (eBioscience) was added to the cells for 45 min at 4°C in the dark. The cells were then centrifuged at 241 g for 5 min at 4°C and resuspended in 100μl of sodium azide buffer. The samples were analysed via flow cytometry as described earlier.

2.16 Retinoic Acid Patch Test

Two Finn chambers (Bio-Diagnostics Ltd, Worcestershire, UK) containing 50μl of 0.025% tretinoin cream (Retin A, Janssen-Cilag, Buckinghamshire, UK), and two containing 50μl of aqueous cream BP (Co-pharma Ltd, Watford, UK) were applied to sun protected buttock skin and secured with waterproof dressings. The aqueous cream served as a control. After 96 hours the patches were removed and 6mm punch biopsies were taken from the four patch sites. Epidermal sheets were prepared as described earlier and one sheet from each treatment (tretinoin or aqueous cream) cultured for 24 hours. The remaining sheet was fixed immediately in acetone to provide a baseline LC count (T0). Langerhans’ cell counts were performed as before and the percentage LC migration was calculated.

2.17 Measurement of Interleukin-1β and Interleukin-1 Receptor Antagonist Secretion from Epidermal Explants

The culture supernatants from epidermal explants were analysed using IL-1β and IL-1Ra sandwich ELISA kits as described earlier (both R&D Systems).

2.18 Statistical Analysis

Data analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The unpaired Student’s t-test was used for
comparisons between two groups (e.g. LC counts in epidermal sheets from young versus old volunteers). For comparison of multiple groups within the young or the old subject group (e.g. the effect of IL-1β and TNF-α versus media alone on LC migration from epidermal explants in the old group), a one-way analysis of variance (ANOVA) with Tukeys post-hoc test was used. For comparison of multiple groups within both the young and old groups (e.g. the effect of LPS, TNF-α and IL-1β on CD1a expression on MoLCs from both young and old volunteers), a two-way ANOVA with Bonferroni post-hoc test was used (*p<0.05, **p<0.01, ***p<0.001).
3. The Phenotype and Function of Monocyte Derived Langerhans’ Cells from Young and Aged Volunteers

Previous work has shown that during inflammation, LCs are replaced by circulating monocyte precursors (Ginhoux et al., 2006) Furthermore, LC-like cells can be cultured from CD14⁺ monocytes in the presence of TGF-β, IL-4 and GM-CSF (Geissmann et al., 1998). One possible hypothesis to explain the observed reduction in LC number in aged individuals is that there is a failure of monocytes to differentiate into LCs. In addition, MoLCs from aged individuals may be functionally impaired, thus explaining the impairment observed in epidermal LCs in vivo (Bhushan et al., 2002). A series of experiments was performed to investigate these hypotheses.

3.1 Culture of Immature Dendritic Cells from Peripheral Blood Monocytes

The ability of peripheral blood monocytes to differentiate into LCs was studied in six young (age range 20-30 years, mean 25.5) and six old (age range 70-78 years, mean 74.2) healthy volunteers. The groups were sex matched with 2 females and 4 males per group.

CD14⁺ monocytes were separated from PBMCs by negative selection using MACS. The cells were analysed by flow cytometry pre- and post-MACS separation to determine the percentage of CD14⁺ cells (Figures 3.1 A, B and 3.2). The mean percentage of CD14⁺ cells pre-separation was 24.0 ± 1.6% in the young group and 22.0 ± 1.0% in the old group compared to 91.4 ± 1.3% and 90.5 ± 1.8% respectively post-separation. There were no significant differences between the two age groups regarding the percentage CD14⁺ cells present at baseline or post-MACS.
Figure 3.1 Flow cytometric analysis of CD14 positivity on cells pre- (A) and post- (B) magnetic activated cell sorting (MACS). Green lines; CD14, pink lines; isotype control, data from a single experiment representative of 12 experiments.

Figure 3.2 Mean percentage CD14$^+$ cells pre- and post- magnetic activated cell sorting (MACS), n=6 both age groups, data shown as mean ± SEM.

Following MACS purification a cell count was performed to determine the monocyte yield (Figure 3.3). The mean yield of monocytes per ml of venous blood was slightly lower in the old age group, however, this was not statistically significant.
Figure 3.3 Monocyte yield following magnetic activated cell sorting (MACS) per ml of venous blood (VB) taken, n=6 both age groups, bar to demonstrate mean.

The monocytes were cultured in cytokine-supplemented media containing GM-CSF, IL-4 and TGF-β. Previous work has demonstrated that stimulation of moLCs with IL-1β, TNF-α, and LPS can cause upregulation of markers of activation (Geissmann et al., 1999). Therefore, to determine whether MoLCs differ functionally between young and old donors, on day 5 of culture cells were stimulated with 10, or 100ng/ml of IL-1β and TNF-α, or with 1μg/ml LPS.

To establish if there was any deficit in the ability of blood derived monocytes from aged individuals to differentiate into LCs a cell count was performed prior to stimulation (Figure 3.4). The mean number of cells in the young group was slightly higher; however the difference was not statistically significant.
**Figure 3.4** Yield of monocyte derived Langerhans’ cells (MoLCs) on day 5 of culture, n=6 both age groups, bar to demonstrate mean.

### 3.2 Analysis of Monocyte Derived Langerhans’ Cells Using Flow Cytometry

On day 6 of culture, the MoLCs were analysed using flow cytometry to compare baseline phenotype and response to stimulation with LPS, IL-1β, or TNF-α between the young and old groups. The cell populations from the two age groups were similar with regard to size, granularity and viability (Figure 3.5).
Figure 3.5 On day 6 of culture monocyte derived Langerhans’ cells were analysed using flow cytometry. The populations from young and old donors were compared with regard to size (forward scatter, FSC-H) and granularity (side scatter, SSC-H), column A. Non-viable cells were gated out using propidium iodide (PI) staining, column B. Data shown from media control treated cells stained for CD1a from one representative young and old individual.

The response to LPS and to 100ng/ml and 10ng/ml of TNF-α, or IL-1β was determined by examining the relative expression of markers of LC maturation/activation, recorded as mean fluorescence intensity (MFI). The percentage of cells positive for each marker was also recorded. The markers examined included CD1a (as a marker of LCs), CD14, MHC class II (HLA-DR), the costimulatory molecule CD86, and the adhesion molecule CD54. The percentage of non-viable cells was analysed using PI and ranged from 3.2-16.5% (mean young group, 9.2 ± 2.0%; mean old group, 6.4 ± 0.7%).
3.2.1 CD1a Expression on Monocyte Derived Langerhans’ Cells

To assess whether cultured cells had differentiated appropriately towards a LC phenotype, CD1a expression was measured. Consistent with previous reports (Geissmann et al., 1998) a high proportion of cultured cells in both age groups expressed CD1a (Figure 3.6). Although there was a trend towards an increased proportion of CD1a+ cells in the old group this was not statistically significant. Stimulation did not significantly alter the percentage of CD1a+ cells.

**Figure 3.6** The percentage of monocyte derived Langerhans’ cells expressing CD1a following stimulation with 1μg/ml of LPS, or 100ng/ml of IL-1β or TNF-α (media alone used as negative control), n=6 both age groups, data shown as mean ± SEM.

There was a non-significant trend towards upregulation of expression of CD1a on stimulation with LPS, TNF-α, and IL-1β as measured by changes in MFI in both age groups. There was notable inter-individual variation in level of CD1a expression and response to stimulation (Figures 3.7 and 3.8). No significant differences were observed between the two age groups either at baseline, or in response to stimulation.
Figure 3.7 Representative histograms of a young and an old individual demonstrating expression of CD1a by monocyte derived Langerhans’ cells by medium controls (green line), and following stimulation with IL-1β (blue line) and TNF-α (orange line). Isotype control shown in pink.
Figure 3.8 Expression of CD1a by monocyte derived Langerhans’ cells from young and old volunteers as measured by mean fluorescence intensity (MFI) of cells in media alone (negative control) and following stimulation with 1μg/ml LPS, 100ng/ml TNF-α, and 100ng/ml IL-1β, n=6 both age groups.

3.2.2 CD14 Expression on Monocyte Derived Langerhans’ Cells

The proportion of cells expressing the monocyte marker CD14 was measured in order to characterise further the level of differentiation of the cultured cells. For the purposes of these experiments MFI levels of less than 10 were considered to be
negative (Figure 3.9). None of the MoLCs cultured from young individuals expressed CD14 at baseline or upon stimulation. Of the MoLCs cultured from monocytes of old individuals, there was a detectable level of CD14 following stimulation with LPS in two individual experiments only. In the first case the MFI of CD14 following stimulation with LPS was 16 (96.2% cells CD14$^+$), and in the second case the MFI was 22 (2.3% cells CD14$^+$). These levels of CD14 expression were lower than that observed after initial monocyte isolation (MFI 30 and 66 respectively). Notably in both cases the CD1a expression upon stimulation with LPS was considerably higher than that for CD14 (MFI 358, 96.3% cells CD1a$^+$; MFI 189, 95.0% cells CD1a$^+$).

**Figure 3.9** Representative histogram from a young volunteer demonstrating lack of CD14 expression by monocyte derived Langerhans’ cells (media control). CD14, mean fluorescence intensity (MFI) 4.2, green line; isotype control, MFI 3.7, pink line.

### 3.2.3 HLA-DR Expression on Monocyte Derived Langerhans’ Cells

Langerhans’ cells and both unstimulated (immature) and stimulated MoLCs express MHC class II (Geissmann et al., 1998). To study whether MoLCs derived from old individuals display the expected phenotype and response to stimulation, the cells were stained for HLA-DR. The percentage of HLA-DR$^+$ cells was high in stimulated and unstimulated cells in both age groups (Figure 3.10). Interestingly, stimulation with TNF-$\alpha$, and IL-1$\beta$ reduced the proportion of HLA-DR$^+$ cells in the old age group,
although this did not reach statistical significance when compared with the media control. This trend was not reflected in the MFI levels, which increased in both age groups following stimulation (Figure 3.11), however, only the increase in MFI following stimulation with LPS and IL-1β in the young and old group was statistically significant (p<0.05). There was no significant difference in the baseline levels of HLA-DR between age groups. When comparing fold change in MFI between stimulants, IL-1β had a more marked effect than TNF-α on HLA-DR expression in the old group (p<0.05) but not in the young group.

**Figure 3.10** The percentage of monocyte derived Langerhans’ cells expressing HLA-DR following stimulation with 1μg/ml of LPS, or 100ng/ml of IL-1β or TNF-α (media alone used as negative control), n=6 both age groups, data shown as mean ± SEM.
Figure 3.11 Expression of HLA-DR in monocyte derived Langerhans’ cells from young and old volunteers as measured by mean fluorescence intensity (MFI) of cells in media alone (negative control) and following stimulation with 1μg/ml LPS, 100ng/ml TNF-α, and 100ng/ml IL-1β, n=6 both age groups. Significant increase in MFI following stimulation: * p<0.05, ** p<0.01, ***, p<0.001.

### 3.2.4 CD86 Expression on Monocyte Derived Langerhans’ Cells

To study whether MoLCs from aged individuals responded appropriately to stimulation, the levels of CD86 and CD54 expression were measured. As expected, only a relatively small proportion of unstimulated MoLCs expressed at low levels the
co-stimulatory molecule CD86 in both age groups (Figure 3.12). The percentage of CD86+ cells was highly variable between individuals (mean 22.3 ± 12.3% in young group media control, compared with 15.3 ± 15.3% in old group). Although there was a trend towards an increased proportion of CD86+ cells following stimulation, in particular with LPS and IL-1β, this was not statistically significant.

![Figure 3.12](image)

**Figure 3.12** The percentage of monocyte derived Langerhans’ cells expressing CD86 following stimulation with 1μg/ml of LPS, or 100ng/ml of IL-1β or TNF-α (media used as negative control), n=6 both age groups, data shown as mean ± SEM.

Consistent with previous reports, there was a trend towards upregulation of CD86 expression following stimulation (Geissmann et al., 1999), although the increases in MFI following stimulation with LPS and IL-1β in the old group were the only statistically significant changes (p<0.01, Figure 3.13). Again, IL-1β was a more potent stimulator of CD86 expression than TNF-α in the old age group (p<0.05).
Figure 3.13 Expression of CD86 in monocyte derived Langerhans’ cells from young and old volunteers as measured by mean fluorescence intensity (MFI) of cells in media alone (negative control) and following stimulation with 1μg/ml LPS, 100ng/ml TNF-α, and 100ng/ml IL-1β, n=6 both age groups. Significant increase in MFI following stimulation: ** p<0.01.
3.2.5 CD54 Expression on Monocyte Derived Langerhans’ Cells

The majority of unstimulated and stimulated MoLCs expressed the adhesion molecule CD54 (Figures 3.14 and 3.15). There was a significant up-regulation in the relative level of CD54 expression following stimulation with LPS, TNF-α, and IL-1β that was unaffected by age (p<0.01). Lipopolysaccharide induced a greater increase in CD54 expression than did TNF-α in the old group (p<0.05), otherwise there were no significant differences between the effects of the different stimulants used.

![Graph showing CD54 expression following stimulation with various agents](image)

**Figure 3.14** The percentage of monocyte derived Langerhans’ cells expressing CD54 following stimulation with 1μg/ml of LPS, or 100ng/ml of TNF-α or IL-1β (media used as negative control), n=6 both age groups, data shown as mean ± SEM.
Figure 3.15 Expression of CD54 in monocyte derived Langerhans’ cells from young and old volunteers as measured by mean fluorescence intensity (MFI) of cells in media alone (negative control) and following stimulation with 1μg/ml LPS, 100ng/ml TNF-α, and 100ng/ml IL-1β, n=6 both age groups. Significant increase in MFI following stimulation: ** p<0.01, *** p<0.001).
3.2.6 Response of Monocyte Derived Langerhans’ Cells to Stimulation with Lower Doses of Interleukin-1β and Tumour necrosis factor-α

To investigate the possibility that MoLCs derived from old individuals are less responsive to lower doses of IL-1β and TNF-α than those derived from young individuals, MoLCs stimulated with 10ng/ml of IL-1β and TNF-α were studied using flow cytometry (n=2 both age groups). The response to stimulation (as measured by MFI of cells stained for CD1a, HLA-DR, CD54 and CD86) with the lower doses of IL-1β and TNF-α was not significantly different between the age groups (data not shown).

3.3 Measurement of Cytokine and Chemokine Secretion from Monocyte Derived Langerhans’ Cells

There is some evidence to suggest that secretion of pro-inflammatory cytokines from DCs is altered in aged humans and mice (Agrawal et al., 2007a; Pereira et al., 2011), however, there have been no studies to address whether the same applies to human MoLCs. The levels of cytokines and chemokines involved in LC migration, both with and without stimulation (with IL-1β and TNF-α), were therefore measured in the culture supernatants using cytokine bead array (n=6 both age groups, Figure 3.16). In addition, levels of IL-6 secretion were measured by ELISA (n=3 both age groups, Figure 3.16).
Figure 3.16 Cytokine secretion from unstimulated (media) and stimulated (100ng/ml IL-1β or 100ng/ml TNF-α) monocyte derived Langerhans’ cells from young and old volunteers measured by cytokine bead array (n=6 both age groups) or enzyme linked immunosorbent assay (IL-6, n=3 both age groups). Data shown as mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001.
There was minimal detectable IL-1β secretion from MoLCs derived from both young and aged volunteers, and values obtained were below the accurate limit of detection of the cytokine bead array. Unstimulated MoLCs from both age groups secreted only low levels of all other cytokines and chemokines measured other than IL-1Ra. Following stimulation with IL-1β, there was a trend towards an increased secretion of all cytokines and chemokines measured by MoLCs from both the young and old; however, this only reached statistical significance in the following groups: TNF-α secretion (young and old), IL-10 secretion (young), CCL3 secretion (young), and CCL4 secretion (young and old). Stimulation with TNF-α did not have any significant effect on cytokine or chemokine secretion, furthermore there were no significant differences between the young and old groups in terms of levels of cytokines or chemokines secreted by unstimulated and stimulated MoLCs.

3.4 Migratory Response of Monocyte Derived Langerhans’ Cells to a Chemokine Ligand

To explore the possibility that MoLCs derived from aged individuals maintain a normal phenotype, but are functionally impaired, a transwell migration assay was performed. Previous studies have established that CCR7 is important for LC homing to skin draining lymph nodes (Ohl et al., 2004; Ouwehand et al., 2008), and that cultured MoLCs express CCR7 (Geissmann et al., 2002), therefore the migratory response of unstimulated and stimulated MoLCs to CCL19 (a ligand for CCR7) was studied. The percentage of MoLCs that migrated was calculated (Figure 3.17). A proportion of both unstimulated and stimulated MoLCs from both age groups migrated in the absence of the chemokine ligand, CCL19. In the young group, the mean “spontaneous” migration (i.e. to control media) of unstimulated MoLCs was 14.8 ± 1.2% compared with 11.5 ± 2.1% in the old group (both n=5). In the old age group, only cells stimulated with IL-1β showed a significantly increased migratory response to CCL19 compared with control media (mean 24.3 ± 2.2% migration to CCL19, compared to 8.8 ± 2.2% control media, p<0.001). In the young age group, stimulation with IL-1β and TNF-α significantly increased migration to CCL19, although IL-1β had a more marked effect than TNF-α (IL-1β mean 29.6 ± 3.2% migration to CCL19, compared with 11.7 ± 3.0% control media; TNF-α mean 23.6 ± 1.4% to CCL19, compared with 17.1 ± 1.3% control media). Stimulation with LPS
did not increase migration to CCL19 compared with control media, although there was a trend towards increased migration in both age groups. There were no significant differences between the age groups in terms of the percentage migration of unstimulated or stimulated MoLCs.

![Graphs showing percentage migration of unstimulated and stimulated MoLCs from young and old volunteers in response to CCL19 or control media.](image)

**Figure 3.17** Percentage migration of unstimulated (media, n=5 both age groups) and stimulated (1μg/ml LPS or 100ng/ml TNF-α, n=3 both age groups, or 100ng/ml IL-1β n=5 both age groups) monocyte derived Langerhans cells from young and old volunteers in response to the chemokine ligand CCL19, or control media. Data shown as mean ± SEM, * p<0.05, *** p<0.001.
3.5 Discussion

The present investigations were designed to investigate whether, in aged individuals, there was a reduction in circulating monocyte precursors, or a failure of monocytes to differentiate into functional DCs. There was no demonstrable difference in the number of circulating monocyte precursors, or in their capacity to differentiate into MoLCs and respond to stimulation in terms of phenotypic maturation. Furthermore, cytokine secretion was unimpaired in MoLCs derived from aged volunteers. The migratory response following stimulation with IL-1β was equivalent in both age groups, however stimulation with TNF-α only enhanced migration in the young group.

3.5.1 Volunteer Selection

All old individuals recruited for this research were living independently and able to travel to and from the hospital. The stringent SENIEUR criteria were not applied, as previously discussed. Some old individuals were taking prescribed medication including lipid-lowering and antihypertensive drugs. During analysis of the results, no apparent differences emerged between old individuals who were taking medication, and those who were not (separate statistical analyses were not performed due to the small numbers). It should be noted, however, that there is evidence that certain medications can affect DC function. For example, in one study using monocyte-derived DCs a variety of anti-inflammatory drugs, including diclofenac, dexamethasone and vitamin D₃, reduced DC maturation in response to nickel sulphate (Toebak et al., 2008).

Sex hormones can also influence the cutaneous immune response (Kanda and Watanabe, 2005), and therefore the age groups were sex-matched. There were no obvious differences in the results from male and female subjects in either age group.

3.5.2 Monocyte Yield

There was no significant difference between age groups in the mean yield of monocytes following isolation by MACS. This is in contrast to a report by Della Bella et al who found an increase in the number of peripheral blood monocytes in old
individuals compared with their younger counterparts (Della Bella et al., 2007). In this latter study, the monocyte counts were higher per ml of blood in both the young and the aged compared to the current study. There are several possible explanations for these discrepancies. Firstly, in the latter study the volunteers ranged in age from 20-92 years and the definition for old was greater than 60 years (compared with the current study of two distinct groups of 30 years and under, and 70 years and over). Secondly, in the present study the monocytes were isolated from PBMCs using MACS, whereas Della Bella et al used flow cytometry to identify and enumerate monocytes. Finally, the sample size in the current study was smaller, and it is possible that a larger sample size would yield different results.

3.5.3 Yield and Phenotype of Monocyte Derived Langerhans’ Cells

Although cultured MoLCs share many characteristics of their epidermal counterparts, and are a useful \textit{in vitro} research tool, they are not identical. For example, a recent study demonstrated that MoLCs do not express EpCam whilst LCs isolated from the skin do (Eisenwort et al., 2011). It is also important to consider that \textit{in vitro} culture may not be representative of the conditions \textit{in vivo}. For example, the availability of growth factors, including GM-CSF, may be reduced particularly in aged individuals (Buchanan et al., 1996), and there may also be skin-specific micro environmental effects.

In the present study, the ability of peripheral blood monocytes from old individuals to differentiate into MoLCs in culture was not impaired in that the yield of cells was equivalent between the age groups. There have been no previous reports on the culture yield of MoLCs in the aged, however, several studies have been published on the effect of age on circulating myeloid DCs and cultured MoDCs; the latter are considered to resemble circulating myeloid DCs. Della Bella et al reported an age-related reduction in circulating myeloid DC number (Della Bella et al., 2007) whilst Jing et al found a reduction in the frequency of circulating myeloid DCs in frail but not healthy individuals aged 60 and over. The frail elderly had at least one disabling disease and had assistance with activities of daily living. Similar to the current study, the healthy elderly were living independently, and some were taking medications, for example antihypertensive agents (Jing et al., 2009). Interestingly, they found no difference in the yield of cultured MoDCs between the healthy young and aged. Other
groups have also reported that there is no age-related reduction in the ability of monocytes to differentiate into DCs in culture (Agrawal et al., 2007a; Lung et al., 2000).

Given that monocytes from aged individuals could differentiate into LCs in culture, it was important to determine whether the cells had the appropriate phenotype and response to stimulation. Consistent with previous studies, a high proportion of the MoLCs expressed CD1a and HLA-DR (Geissmann et al., 1998; Jaksits et al., 1999). As expected, CD14 was down-regulated following culture. In two cases (both from old volunteers), low-level expression of CD14 was noted on MoLCs following stimulation with LPS, but not in the corresponding media control-treated cells. However, these cells also expressed CD1a demonstrating that they had differentiated accordingly. During the culture process, populations of CD14+/CD1a+ cells may be present but are usually not detectable by day 6 (Geissmann et al., 1998). One possible reason for this observation is that LPS, which is known to increase CD14 expression (Jiang et al., 2000), upregulated low levels of CD14 that were present on the unstimulated cells.

3.5.4 Effect of Stimulation upon Monocyte Derived Langerhans’ Cells

As expected, stimulation with LPS and IL-1β caused a significant increase in HLA-DR expression in both age groups (Geissmann et al., 1999). In the earlier study by Geissmann et al., IL-1β appeared to be a more potent stimulator of HLA-DR expression than did TNF-α, which corroborates the current work. There were no differences between age groups in terms of the percentage of HLA-DR+ cells or level of expression in unstimulated or stimulated MoLCs. These findings are similar to those of previous studies of MoDCs in young and aged individuals which detected no age-related differences in HLA-DR expression on unstimulated cultured DCs (Agrawal et al., 2007a; Jing et al., 2009; Lung et al., 2000). It is important to note, however, that these DCs are not necessarily equivalent to MoLCs in terms of receptor expression and cytokine secretion (Geissmann et al., 1999; Rajkovic et al., 2011; Takeuchi et al., 2003).

Only a small percentage of unstimulated MoLCs expressed low levels of CD86. Although there was a trend towards higher levels of CD86 expression in the young age group at baseline, this was not significant. In the study by Geissmann et al,
unstimulated (immature) MoLCs did not express CD86; however, stimulation with TNF-α, IL-1β and LPS led to upregulation of CD86 expression (Geissmann et al., 1999). The proportion of CD86+ cells reported following stimulation with LPS was slightly higher than in the current study (approximately 60% positive compared with 44% in the young group and 48% in the old group). This may be because in this study cells were stimulated for a shorter time period before analysis (24 hours compared with 40 hours in Geissmann’s study). Additionally, in the current study, although stimulation generally increased relative expression of CD86, this was only statistically significant in the old age group following stimulation with LPS and IL-1β. This may be due to the small sample size and degree of inter-individual variation, particularly in the media (negative control) samples from the young individuals. Studies of MoDCs in aged compared with young individuals also failed to show any differences in expression of CD86. Stimulation with LPS or whole inactivated influenza virus caused equivalent upregulation of CD86 expression on DCs from both young and old age groups (Agrawal et al., 2007a; Lung et al., 2000). In contrast, one investigation of peripheral blood DCs from young and old individuals demonstrated a higher proportion of CD86+ cells in old individuals (>60 years), although stimulation with LPS induced an equivalent increase in MFI levels and proportion of CD86+ cells in both age groups (Della Bella et al., 2007). The authors hypothesised that the DCs may be in a more activated state because of the proinflammatory conditions that are associated with ageing. The results of this study may not be comparable to the current investigations as the DCs were isolated directly from peripheral blood.

In both age groups, the vast majority of MoLCs were CD54+, which is similar to earlier findings by Strobl et al who cultured LCs from CD34+ progenitors (Strobl et al., 1996). Rajkovic et al also found that a high proportion of MoLCs expressed CD54, however there was little change in CD54 expression post-stimulation with a cocktail of proinflammatory cytokines (Rajkovic et al., 2011). In contrast, Saurwein-Teissl et al cultured DCs from PBMCs from young and aged individuals, and demonstrated that stimulation with influenza vaccine lead to a comparable upregulation of CD54 (and HLA-DR) in both groups (Saurwein-Teissl et al., 1998). Steger et al also studied DCs cultured from PBMCs in young and elderly individuals. The yield of DCs was higher in the old group, yet as reported in the present study, there were no differences in the phenotype of the cultured DCs which expressed HLA-DR and CD54 (Steger et al., 1996). The authors hypothesised that a reduction in
emigration of DCs from the circulation to peripheral tissues (such as the skin) due to changes in the ECM or endothelium may account for the increased yield of DCs in the old group. Of note, the elderly volunteers (>65 years) fulfilled the SENIEUR criteria, and therefore may not be comparable to those studied in the current research. The results of the present investigations correspond to those of Agarwal et al and Lung et al who cultured DCs from monocytes of young and aged individuals (Agawal et al., 2007a; Lung et al., 2000). Neither of these studies employed the SENIEUR criteria in volunteer selection, and there were no differences in the yield or phenotype of MoDCs (regarding expression of HLA-DR, CD86 and CD54) between age groups. Agarwal et al stimulated DCs with LPS and again found no significant difference in CD86 and HLA-DR expression between the age groups, whereas Lung et al used whole inactivated influenza virus, and found an equivalent increase in CD54 and CD86 expression in both young and old groups.

3.5.5 Cytokine Secretion by Monocyte Derived Langerhans’ Cells

Cultured MoLCs and DCs secrete a variety of cytokines and chemokines which are important in regulating the inflammatory response. Monocyte derived LCs have been shown to secrete TNF-α, IL-6, IL-10, IL-12, IL-23 and IL-27 (Geissmann et al., 1999; Rajkovic et al., 2011). In the current study the levels of selected cytokines and chemokines known to be important in LC migration and mobilisation and the inflammatory response were measured. Previous investigations have shown that immature MoLCs secrete little or no IL-10, whereas stimulation of MoLCs with peptidoglycan or a cocktail of proinflammatory mediators, but not LPS, can increase IL-10 secretion (Rajkovic et al., 2011; Takeuchi et al., 2003). One possible hypothesis to explain impaired LC migration in aged individuals is an excess production of the inhibitory cytokine IL-10; however, there were no differences in IL-10 secretion between the age groups. As in earlier studies unstimulated MoLCs from young and aged individuals secreted little detectable IL-10, although IL-1β caused a significant increase in IL-10 secretion from MoLCs from young individuals (there was a non-significant trend towards increased IL-10 secretion in the aged group) suggesting that these cells can respond appropriately to stimulation. In contrast to the findings of Rajkovic et al, the unstimulated MoLCs from both age groups secreted minimal IL-6 and TNF-α. The supernatants in the current study were
from media from MoLCs that had been cultured for 24 hours, whereas in the study by Rajkovic et al the MoLCs were cultured for 48 hours and could thus have secreted more cytokine in that time (Rajkovic et al., 2011). Stimulation with IL-1β caused an increase in TNF-α secretion in both age groups, which is in keeping with changes that occur during epidermal LC mobilisation. Interleukin-1β and IL-1Ra secretion were not measured in earlier studies of MoLCs, however, similar to the results presented, studies have shown that MoDCs secrete minimal IL-1β, and activated LCs which migrate from epidermal explants secrete little or no detectable IL-1β (Jing et al., 2009; Klechevsky et al., 2008). Furthermore, suction blister fluids from normal skin contain very low levels of IL-1β but high levels of IL-1Ra (Janssens et al., 2009). In the current investigations, the level of IL-1Ra secretion from MoLCs from both young and old individuals was significantly higher than that of IL-1β. This was not unexpected, since previous studies have shown that in order to inhibit the effect of IL-1β, a significant excess of IL-1Ra is required (Banda et al., 2005; Bigler et al., 1992).

The levels of CCL3 and CCL4 (which have a role in recruitment of T cells, monocytes and neutrophils (Kunstfeld et al., 1998; Lee et al., 2000; Taub et al., 1993)) secreted by MoLCs increased significantly upon stimulation with IL-1β, but not TNF-α, in the young age group, although in the old age group only the increase in CCL4 secretion following stimulation with IL-1β was significant. This may be due to the small numbers included in the study and inter-individual variation rather than a true failure to upregulate CCL3 secretion, as on an individual basis there was an increase in CCL3 secretion on stimulation with IL-1β in all experiments in the old group. It may seem counterintuitive that whilst the receptors for CCL3 and CCL4 (CCR1 and 5) are down-regulated on DCs upon stimulation (Sallusto et al., 1998; Sozzani et al., 1998), the secretion of the ligands increases. However, stimulated DCs have been shown to be unresponsive to CCL3 (Sozzani et al., 1998), and therefore in this circumstance the chemokine ligands will be involved in chemotaxis of other leucocytes.

In this study there were no differences in cytokine secretion between the young and old groups, and MoLCs from aged individuals could produce both proinflammatory cytokines and cytokines specifically involved in LC mobilisation. In agreement with these findings, studies in which MoDCs from aged individuals were stimulated with whole inactivated influenza vaccine or poly I:C found no differences in cytokine
secretion between young and old age groups (Jing et al., 2009; Lung et al., 2000; Saurwein-Teissl et al., 1998). Several studies have, however, demonstrated that MoDCs from aged individuals secrete increased levels of TNF-α, IL-6 and IFN-α in response to stimulation with LPS and apoptotic cells (Agrawal et al., 2007a; Agrawal et al., 2009; Ciaramella et al., 2011). This suggests that the choice of stimulant to mature the DCs may influence cytokine secretion in the different age groups, and it is therefore possible that if different stimulants had been used then the current study would have yielded different results. Although as discussed earlier, MoDCs are not necessarily equivalent to MoLCs in terms of cytokine secretion.

3.5.6 Migratory Response of Human Monocyte Derived Langerhans’ Cells to a Chemokine Ligand

In this investigation, MoLCs derived from both young and old individuals stimulated with IL-1β migrated towards the chemokine ligand CCL19. Activated LCs and MoLCs express CCR7 and migrate towards the corresponding ligands CCL19 and CCL21 (Geissmann et al., 2002; Saeki et al., 1999; Sozzani et al., 1998). The migratory response following stimulation with IL-1β in the aged was not unexpected as previous studies have shown that intradermal injection of IL-1β promotes LC migration from the skin of both young and aged humans and mice (Bhushan et al., 2004; Cumberbatch et al., 2002). Geissmann et al have shown that MoLCs stimulated with TNF-α for 48 hours (compared to 24 hours in this study) migrate in response to CCL19 and CCL21 (Geissmann et al., 2002). Interestingly, in the current investigations, stimulation with TNF-α only promoted migration to CCL19 in the young group. This is again consistent with observations in aged compared with young skin (Bhushan et al., 2002). Possible explanations for this observation could include that MoLCs derived from aged individuals fail to produce adequate IL-1β to induce migration, or that there is a change in the balance of IL-1β and related cytokines including IL-1Ra and IL-10. The level of IL-1β and IL-10 secretion from the MoLCs was uniformly low making it difficult to confirm these hypotheses.

There are no studies comparing the migratory response of MoLCs derived from young and old donors, however, one study has shown that migration of stimulated MoDCs from old donors to CCL19 is impaired (Agrawal et al., 2007a). In the aforementioned study, MoDCs were stimulated with LPS and the number of cells that migrated
towards CCL19 in a transwell system was measured. In contrast, in the current study there were no age related differences in the migratory response to CCL19 between the age groups and stimulation with LPS did not promote migration in either age group. These differences may be due to inherent differences in the cell types, or it may be that in the current study a larger sample size would have yielded different results.

3.5.7 Comparison of the Effects of Interleukin-1β and Tumour Necrosis Factor-α

Interestingly, in this study the IL-1β used was a more potent stimulator of MoLCs than the TNF-α (used at the same concentration) with regard to upregulation of expression of certain markers of maturation, secretion of IL-10, CCL3 and CCL4, and migration towards a chemokine ligand. This may be due to different biological activity of the cytokines, and also corroborates the findings of Geissmann et al who showed that stimulation with IL-1β led to a higher proportion of HLA-DR+ and CD86+ MoLCs compared to TNF-α (Geissmann et al., 1999).

3.5.8 Conclusions

Peripheral blood monocytes from aged individuals were not impaired in terms of their ability to develop into LCs in culture. With age, the MoLCs also maintained a normal phenotype in terms of expression of markers of maturation and secretion of cytokines. However, the migratory response to a chemokine ligand was reduced in the old age group. Taken together, these data suggest that in the aged there is no marked defect in circulating precursors, or in their ability to differentiate into LCs given the appropriate cytokine environment. Thus it is possible that the defect observed in vivo with respect to LC migration is not at the level of the LC, but that changes in the local environment (such as availability of cytokines required for migration) are more important.

3.5.9 Limitations of the Study

The main limitation of the study was the small sample size. In certain experiments, for example measurement of the level of expression of markers of LC maturation by flow cytometry, there was a significant degree of inter-individual variation which meant
that a larger sample size would have been required to detect a difference. However, similar studies in humans have also used relatively small sample sizes and it could be argued that biologically significant differences would be apparent even with small numbers.

Another limitation of the study is that only selected markers of MoLC phenotype and function were measured in response to selected stimulants. It is possible that an age related change would have become apparent if other markers had been measured or different stimulants used. For example, one study of MoDCs found a difference in IFN-α secretion between cells derived from young and aged individuals (Agrawal et al., 2009), which was not measured in the current study. In each experiment only a limited number of end points could be studied therefore an attempt was made to select those most relevant to LC function, and also those most likely to yield reliable results.
4. The Phenotype of Langerhans’ Cells and Dermal Dendritic Cells and the Influence of Ageing on Epidermal Interleukin-1β

To determine whether LCs from elderly individuals have an altered phenotype (consistent with reduced migratory potential), LCs from epidermal cell suspensions were analysed using flow cytometry to study expression of markers of activation. Experiments were also performed to compare the number of dermal DCs in young and aged individuals, since there is limited evidence available on the changes that occur in the dermal DC population with age. (Quatresooz and Pierard, 2009). It is possible that the reduced number of LCs in the aged corresponds with a reduction in dermal DCs, as it has been shown that dermal DCs can give rise to LC-like cells in vitro (Larregina et al., 2001).

Earlier reports suggest that impaired Langerhans’ cell migration in aged individuals may be restored by exogenous IL-1β (Bhushan et al., 2004). There are several possible explanations for this observation including, reduced transcription or translation of IL-1β in the epidermis of aged individuals; a reduction in the levels of the enzyme caspase-1, which processes proIL-1β into the bioactive form; and a change in the balance of the IL-1 receptors and IL-1Ra. A series of experiments were performed to address these possibilities.

4.1 The Phenotype of Langerhans’ Cells

Epidermal cell suspensions were prepared from 6mm punch biopsies taken from 11 young (age range 19-29 years, mean 22.6 years) and 9 old volunteers (age range 73-91 years, mean 78.4 years). There were 5 females in both groups. Epidermis was separated from dermis using 0.5% trypsin-EDTA and mashed through steel gauze to create a single cell suspension. Cells were dual-stained for langerin and MHC class II (HLA-DR), or langerin and CD86.

The flow cytometer was set to acquire 1.5 x10⁵ cells, however, no samples contained sufficient cells to reach the target cell count. The mean cell yield as measured by minimum number of cells acquired on the flow cytometer was 9.29 ± 0.61 x10⁴ in the young group and 9.20 ± 1.10 x10⁴ in the old group (Figure 4.1).


**Figure 4.1** Yield of cells (x10^4) from epidermal cell suspensions prepared from young (n=11) and old (n=9) volunteers and measured via flow cytometry, bar to demonstrate mean.

4.1.1 Langerin Expression on Epidermal Cells

Previous studies (using counts from epidermal sheets stained for CD1a) have demonstrated a reduction in LC numbers in elderly human skin (Bhushan et al., 2002; Bhushan et al., 2004). In the current study, the number of cells analysed via flow cytometry varied between volunteers, therefore rather than calculating the absolute number of langerin^+^ cells, the percentage of langerin^+^ cells was recorded. There was no significant difference between the percentage of langerin^+^ cells in the young and old groups, although there was a trend towards a reduction in the young group (Figures 4.2 and 4.3).
Figure 4.2 Representative density plots of epidermal cell suspensions from a young and old individual stained for langerin and analysed using flow cytometry. Population of langerin⁺ cells visible in lower right quadrant.

Figure 4.3 Epidermal cell suspensions from young (n=11) and old (n=9) volunteers were stained for langerin and the percentage of langerin-expressing cells was determined using flow cytometry, bar to demonstrate mean.
4.1.2 Langerin and HLA-DR Expression on Epidermal Cells

To determine whether epidermal LCs from young and old individuals expressed equivalent levels of HLA-DR, cells were dual-stained for langerin and HLA-DR (n=5 young group, n=4 old group, Figure 4.4). The percentages of langerin and HLA-DR$^+$ cells were recorded, and the relative level of HLA-DR expression was also measured (Figure 4.5). In both age groups the majority of LCs expressed HLA-DR (Figure 4.6); however, within the total cell population the percentage of HLA-DR$^+$ cells was higher than the percentage of langerin$^+$ cells in both age groups suggesting that other epidermal cells, including keratinocytes and T cells, also expressed HLA-DR (Figure 4.7), which is in keeping with the data shown in Figure 4.4.

![Figure 4.4](image)

**Figure 4.4** Representative density plots of epidermal cell suspensions from a young, and an old individual, stained for langerin and HLA-DR and analysed using flow cytometry. Populations of langerin and HLA-DR$^+$ cells visible in upper right quadrants in plots on the right, evidence of lower levels of HLA-DR expression by some langerin$^+$ cells is also evident.
**Figure 4.5** Representative histogram of an epidermal cell suspension from a young volunteer stained for langerin and HLA-DR and analysed using flow cytometry. HLA-DR, green; isotype control, pink.

**Figure 4.6** Epidermal cell suspensions from young (n=5) and old (n=4) individuals were dual-stained for HLA-DR and langerin and the percentage of langerin expressing cells that also expressed HLA-DR was calculated. Data shown as mean ± SEM.
**Figure 4.7** The percentage of langerin$^+$ cells within epidermal cell suspensions from young (n=5) and old volunteers (n=4) was compared with the percentage of HLA-DR$^+$ cells using flow cytometry. Data shown as mean ± SEM.

The level of expression of HLA-DR on LCs, as measured by MFI, was not significantly different between the age groups, however, there was a significant degree of inter-individual variation in the old group (Figure 4.8).

**Figure 4.8** Mean fluorescence intensity (MFI) of HLA-DR on Langerhans’ cells in epidermal cell suspensions derived from young (n=4) and old (n=3) volunteers analysed using flow cytometry. Data shown as mean ± SEM.
4.1.3 Langerin and CD86 Expression on Epidermal Cells

Similar to the MoLCs, in both age groups only a small proportion of LCs identified by langerin staining also expressed CD86 (Figure 4.9). In addition, the level of CD86 expression was relatively low in both age groups (Figure 4.10).

Figure 4.9 Epidermal cell suspensions from young (n=6) and old (n=5) volunteers were dual-stained with langerin and CD86. The percentage of langerin\(^+\) cells that also expressed CD86 was measured using flow cytometry, bar to demonstrate mean.
Figure 4.10 Representative density plots of epidermal cell suspensions from a young and old individual stained for langerin and CD86 and analysed using flow cytometry. Populations of langerin$^+$ cells with low level CD86 expression visible in upper right gates of plots in the right column.

4.2 Analysis of Dermal Cell Suspensions Using Flow Cytometry

A further objective was to study whether dermal DC numbers and phenotype are altered in aged individuals. Following removal of the epidermis from 6mm punch biopsies (2 biopsies per volunteer), the dermis was digested using type IV collagenase and a cell suspension prepared. Previous studies have shown that a significant proportion of resident dermal DCs express CD1a (Angel et al., 2006; Klechevsky et al., 2008; Zaba et al., 2009). Due to the limited amount of tissue available only two markers could be used per experiment, therefore cells were dual stained with CD1a and HLA-DR, or with CD1a and CD11c, and analysed using flow cytometry. In an attempt to increase the cell yield, type I collagenase was used as an alternative digestion method in one experiment; however, this resulted in a reduced cell yield (1.3 x10$^4$ cells), thus type IV collagenase was used for the remaining experiments.
The flow cytometer was set to acquire 1 x10^5 cells, however the mean yield of cells was 5.8 ± 1.0 x10^4 in the young group (n=7) compared with 3.9 ± 0.7 x10^4 in the old group (n=3, Figure 4.11).

![Graph showing cell yield from dermal cell suspensions]

**Figure 4.11** Yield of cells (x10^4) from dermal cell suspensions prepared from young (n=7) and old (n=3) volunteers and measured via flow cytometry, bar to demonstrate mean.

It was not possible to compare the numbers of cells expressing each marker as the number of cells acquired on the flow cytometer varied between each individual. The mean percentage of HLA-DR^+ cells in dermal cell suspensions from the young group (n=5) was 3.6 ± 1.1%, compared with 1.8 ± 0.3% in the old group (n=2). A small population of cells co-expressing CD1a and HLA-DR was detectable in both age groups: mean 0.2 ± 0.1%, young group and 0.1 ± 0.1%, old group (Figures 4.12 and 4.13). There was no detectable CD11c on cells from two young and one old volunteer, which is consistent with reports that CD11c is sensitive to trypsinisation (Stutte et al., 2008). Due to the low cell yields and small proportion of cells expressing the markers of interest within the dermal cell suspensions these experiments were not pursued further.
Figure 4.12 Representative density plots of dermal cell suspensions from a young and old individual stained for HLA-DR and CD1a and analysed using flow cytometry. Small populations of HLA-DR and CD1a+ cells visible in upper right gated areas of plots in the right column, HLA-DR+/CD1a- cells in upper left gated area.

![Density plots](image)

**Figure 4.13** Dermal cell suspensions from young (n=4) and old (n=2) individuals were dual-stained for HLA-DR and CD1a and the percentage of positive cells analysed using flow cytometry. Data shown as mean ± SEM.
4.3 Identification of Dermal Dendritic Cells Using Immunofluorescence

Vertical cryosections from 6mm punch biopsies were stained with CD1a and langerin in a further attempt to identify dermal DCs within the skin of young and old volunteers (n=5 both groups, age range young group 20-25 years, mean 22.2 years; age range old group 70-75 years, mean 72.8 years). The number of cells within the dermis expressing either CD1a and langerin or CD1a alone was recorded in 3 high power fields (hpf) for each volunteer (Figure 4.14). Again there were few dermal DCs within each section (particularly relative to the numbers of LCs evident within the epidermis), although there was a clear distinction between CD1a+ dermal DCs and cells co-expressing CD1a and langerin (the latter presumed to be migrating LCs). In both age groups the level of CD1a expression on dermal DCs appeared to be lower than that on LCs (Figures 4.15 and 4.16). There was no difference in the number of CD1a+ cells within the dermis between the age groups. There was a reduction in the number of cells co-expressing CD1a and langerin in the dermis in the old group (mean 1.50 ± 0.36 cells young group compared with mean 0.36 ± 0.17 cells old group, p<0.01). In one of the sections prepared from a biopsy of an old volunteer there was a number of CD1a+/langerin+ cells which appeared to be clustered around a skin appendage. No other sections from either young or old individuals contained an equivalent appendage; therefore this section was excluded from the analysis (Figure 4.17). The number of epidermal LCs also appeared reduced in the old individuals although this was not formally quantified.
Figure 4.14 The number of cells within vertical cryosections of skin biopsies from young and old volunteers, n=5 both groups, co-expressing langerin and CD1a, or expressing CD1a alone was determined using immunofluorescence. Bar to demonstrate mean, ** p<0.01.
Figure 4.15 Photomicrographs of vertical cryosections of a 6mm punch biopsy from a representative young volunteer stained with langerin (red), CD1a (green), and 4’,6-diamidino-2-phenylindole (blue). The bottom right image shows the overlay of the 3 markers where cells co-expressing langerin and CD1a appear yellow. Numerous Langerhans’ cells are evident within the epidermis. Langerhans’ cell within the dermis shown with thick arrows, CD1a⁺ dermal DC shown with thin arrows.
Figure 4.16 Photomicrographs of vertical cryosections of a 6mm punch biopsy from a representative old volunteer stained with langerin (red), CD1a (green), and 4’,6-diamidino-2-phenylindole (blue). The bottom right image shows the overlay of the 3 markers, where cells co-expressing langerin and CD1a appear yellow. Langerhans’ cell within the dermis shown with thick arrows.
Figure 4.17 Photomicrograph of a vertical cryosection of a 6mm punch biopsy from an old volunteer stained with langerin (red), CD1a (green), and 4',6-diamidino-2-phenylindole (blue). The image shows the overlay of the 3 markers, where cells co-expressing langerin and CD1a appear yellow. Cluster of Langerhans’ cells associated with a skin appendage is shown with a thick arrow.

4.4 The Influence of Ageing on Epidermal Interleukin-1β

A series of experiments was performed to investigate the effect of age on epidermal IL-1β. The mean age of the young volunteers was 23.4 years, range 18-30 years, compared with a mean age of 74.4 years for the old volunteers, range 70-91 years.

4.4.1 Measurement of Epidermal Levels of Interleukin-1β and Caspase-1

The levels of *IL1B* and *CASP1* mRNA were measured in the epidermis of young and old volunteers using quantitative real-time PCR. Following RNA extraction, the samples from each age group were pooled (n=9 both groups). Three housekeeping genes were used as endogenous controls: *GAPDH, HPRT*, and *KRT14*. The fold change in *IL1B* and *CASP1* mRNA was calculated using the mean of the values obtained with each housekeeping gene. The expression of *IL1B* and *CASP1* mRNA was similar in the age groups (expression of *IL1B* and *CASP1* mRNA in the old group was 1.12 and 1.08 respectively when calculated relative to expression in the young
There was little difference between the housekeeping genes although KRT14 appeared the most consistent between the age groups.

![Graph showing the expression of IL1B and CASP1 mRNA in epidermal samples (RNA pooled from 9 individuals in each age group) normalised to the housekeeping genes, GAPDH, HPRT, and KRT14, was measured. Level of expression in old group shown relative to expression in young group.](image)

**Figure 4.18** The expression of *IL1B* and *CASP1* mRNA in epidermal samples (RNA pooled from 9 individuals in each age group) normalised to the housekeeping genes, GAPDH, HPRT, and KRT14, was measured. Level of expression in old group shown relative to expression in young group.

Initially, attempts were made to measure the levels of IL-1β protein in the epidermis of young and old individuals using western blot so as to allow differentiation between the levels of the inactive precursor and the active form of the cytokine. However, it was not possible to detect IL-1β in the epidermal homogenates from either age group. The positive control for the antibody, recombinant human IL-1β, was detected consistently and by using reducing concentrations of the IL-1β, the limit of detection using western blot was found to be 32 pg/ml. Further experiments were performed using ELISA to measure IL-1β levels in epidermal homogenates (n=12 both groups). Although IL-1β was detectable, the levels were uniformly low (below the limit of detection of the western blot) and there was no significant difference between the age groups (Figure 4.19). The level of IL-1β was corrected for total protein content within the epidermal homogenates which again was not significantly different between the young and the old groups (Figure 4.20).
Figure 4.19 The levels of IL-1β protein in epidermal homogenates prepared from biopsies taken from young and old volunteers (n=12 both groups) were measured using enzyme linked immunosorbent assay. The values shown are corrected for total protein content, bar to demonstrate mean.

Figure 4.20 The levels of protein in epidermal homogenates (n=12 both groups) as measured using a Lowry assay.

The level of caspase-1 protein within epidermal homogenates was measured using western blot (n=3 both age groups) with β-actin as a loading control. Initial attempts to measure caspase-1 using a polyclonal anti-caspase-1 antibody resulted in the appearance of multiple bands (data not shown). A monoclonal antibody was used for future experiments, which resulted in a single band at 45kDa corresponding to the
unprocessed form of caspase-1 (Figure 4.21). There were no obvious differences between the age groups. Recombinant caspase-1 was used as a positive control for the antibody.

![Caspase-1 and β-actin blots](image)

**Figure 4.21** The levels of caspase-1 protein in epidermal homogenates prepared from biopsies taken from young and old volunteers (n=3 both groups) were measured using western blot, β-actin was used as a loading control.

### 4.4.2 Measurement of Interleukin-1β and Interleukin-1 Receptor Antagonist Secretion from Epidermal Explants

To determine whether IL-1β and IL-1Ra secretion are altered in the epidermis of old individuals, the levels of IL-1β and IL-1Ra were measured in supernatants of epidermal explants cultured for 24 hours. There was no detectable IL-1β, however, there was a significant reduction in the level of IL-1Ra in explant supernatants from old volunteers as measured by ELISA (mean old group 4611 ± 749 pg/ml, n=9, compared with mean young group 8149 ± 1131 pg/ml, n=7, p<0.05, Figure 4.22).
**Figure 4.22** The levels of IL-1Ra protein in supernatants of cultured epidermal explants prepared from young (n=7) and old (n=9) volunteers were measured using enzyme linked immunosorbent assay. Data shown as mean ± SEM, * p<0.05.

### 4.4.3 Analysis of Interleukin-1 Receptor I and II Expression on Epidermal Cells

To determine whether LCs from within the epidermis of young and old individuals expressed equivalent levels of IL-1RI, cells were dual-stained for langerin and IL-1RI (n=3 both groups). In both age groups a similar proportion of the LCs expressed low levels of IL-1RI (Figures 4.23 and 4.24). There was also no difference in the level of expression of IL-1RI on LCs between the age groups in terms of MFI (Figure 4.25). A small proportion of the non-langerin expressing cells (presumed to be keratinocytes) in both age groups also expressed low levels of IL-1RI (Figures 4.24 and 4.26). There was a non-significant increase in the percentage of langerin(IL-1RI⁺ cells in the old group.
Figure 4.23 Epidermal cell suspensions from young and old volunteers (n=3 both groups) were dual-stained with langerin and IL-1RI and the percentage of langerin\(^+\) cells that also expressed IL-1RI was calculated. Data shown as mean ± SEM.

Figure 4.24 Representative density plots of epidermal cell suspensions from a young and old individual stained for langerin and IL-1RI and analysed using flow cytometry. Population of langerin\(^+\) cells expressing IL-1RI in upper right gates of plots in the right column, and population of langerin\(^-\) cells expressing IL-1RI visible in left gate of plots in the right column.
**Figure 4.25** Mean fluorescence intensity (MFI) of IL-1RI on Langerhans’ cells in epidermal cell suspensions derived from young and old volunteers, n=3 both groups, analysed using flow cytometry. Data shown as mean ± SEM.

![Graph showing MFI of IL-1RI](image)

**Figure 4.26** Epidermal cell suspensions from young and old volunteers were dual-stained with langerin and IL-1RI, n=3 both groups. The percentage of non-langerin\(^+\) cells that expressed IL-1RI was calculated. Data shown as mean ± SEM.

![Graph showing % IL1RI\(^+\) cells](image)

Interleukin-1 receptor II is expressed on the surface of keratinocytes and is also released in a soluble form (Groves et al., 1995). Epidermal cell suspensions from young and old volunteers (n=2 both groups) were dual stained with langerin and IL-
1RII. Both intra- and extracellular IL-1RII expression were determined. There was no detectable expression of extracellular IL-1RII on LCs from one young and both old volunteers, however, a small proportion of LCs (11.3%) from the other young volunteer expressed low levels of IL-1RII (Figure 4.27). Relatively few keratinocytes (langerin⁺ cells) from both the young and old volunteers expressed low levels of extracellular IL-1RII (Figures 4.27 and 4.28). There was no detectable intracellular IL-1RII expression on LCs from either young or old volunteers and there was minimal expression on keratinocytes (less than 1.1% of keratinocytes IL-1RII⁺) (Figure 4.29).

![Density plots of epidermal cell suspensions from a young and old individual stained for langerin and IL-1RII and analysed using flow cytometry. Small population of langerin⁺ cells expressing IL-1RII visible in upper right gate of plot in the right column in young individual only. Population of langerin⁻ cells expressing IL-1RII visible in left gate of both plots in the right column.](image-url)

**Figure 4.27** Density plots of epidermal cell suspensions from a young and old individual stained for langerin and IL-1RII and analysed using flow cytometry. Small population of langerin⁺ cells expressing IL-1RII visible in upper right gate of plot in the right column in young individual only. Population of langerin⁻ cells expressing IL-1RII visible in left gate of both plots in the right column.
Figure 4.28 Epidermal cell suspensions from young and old volunteers (n=2 both groups) were dual-stained with langerin and IL-1RII. The percentage of non-langerin$^+$ cells that expressed extracellular IL-1RII is shown.

Figure 4.29 Density plots of epidermal cell suspensions from a young and old individual stained for langerin and intracellular IL-1RII and analysed using flow cytometry.
4.5 Discussion

A series of experiments was performed firstly to determine the effect of age on the phenotype of LCs and dermal DCs, and secondly the effect of age on epidermal IL-1β content. In unstimulated skin no differences were found in the expression of the markers of LC maturation studied. Dermal DCs could not be readily characterised using flow cytometry, although preliminary experiments using immunofluorescence did not appear to show a reduction in CD1a+ dermal DCs in aged skin. Epidermal levels of IL-1β and caspase-1 (IL-1β converting enzyme) mRNA and protein were not altered with age in unstimulated skin. There was, however, a reduced secretion of IL-1Ra from epidermal explants prepared from aged individuals suggesting that there may be a change in IL-1β homeostasis in aged skin.

4.5.1 Volunteer Selection

All old volunteers recruited for these investigations were living independently and required no assistance with activities of daily living. Just over half the old volunteers were taking prescribed medication including antihypertensives and lipid lowering therapies. Statistical analyses of subgroups were performed where numbers permitted, and there were no significant differences in expression of phenotypic markers, epidermal IL-1β content, and secretion of IL-1Ra from epidermal explants between those taking medication and those who were not.

4.5.2 Langerin and HLA-DR Expression on Epidermal Cells

The yield of cells from epidermal cell suspensions varied between individuals, however, in general, sufficient cells were obtained from two 6mm skin biopsies to allow analysis of two markers and the appropriate isotype controls. For similar experiments, researchers have used surplus tissue from breast reduction surgery or abdominoplasty, which provides significantly larger amounts of tissue for study and allows enrichment for LCs, which is not feasible on smaller biopsies (Larregina et al., 1996; Peguet-Navarro et al., 1995). Since the current experiments were designed specifically to compare young and old individuals, surplus tissue from plastic surgery specimens were not available and comparatively small biopsy samples were used.
Previous studies using whole epidermal sheets or vertical sections to count LC numbers have identified reduced LC numbers in aged human skin (Bhushan et al., 2002; Gilchrest et al., 1982). As previously noted, in the current experiments different numbers of cells were analysed for each individual, therefore rather than calculating the number of LCs, the percentage of langerin+ cells was recorded. Consistent with published reports, LCs comprised approximately 1% of the epidermal population (Cumberbatch et al., 2006; Larregina et al., 1996). There was no difference between the young and old groups in terms of the percentage of LCs within the epidermal cell suspensions. This may be because even though LC numbers are reduced with age they still make up an equivalent proportion of the total epidermal cell population, or because any difference was too small to detect with the technique used. There is evidence to suggest that the number of keratinocytes is reduced in sun-protected skin of aged individuals due to both decreased proliferation and increased apoptosis (Gilhar et al., 2004; Marks, 1981). Interestingly, keratinocytes have been shown to express epidermal receptor activator of nuclear factor-κB ligand (RANKL) which can promote LC activation and survival, such that RANKL knockout mice have reduced LC numbers (Barbaroux et al., 2008). It is therefore possible that in aged individuals the reduction in keratinocytes is associated with a reduced availability of factors, such as RANKL, which are required to maintain the LC population. Bauer et al reported that the ratio of LCs to other epidermal cells is remarkably consistent in human skin (approximately one LC per 53 epidermal cells), again suggesting that a reduction in keratinocyte number could be mirrored by an equivalent reduction in LC number (Bauer et al., 2001).

To determine whether age affects the phenotype of LCs, which could in turn indicate a reduced propensity for migration, the expression of HLA-DR and CD86 on LCs from young and old individuals was compared. As expected, the majority of LCs from both young and old individuals expressed HLA-DR. Earlier studies using both immunohistochemistry and flow cytometry have shown that a significant proportion, if not all, LCs express HLA-DR (Angel et al., 2007; Ashworth et al., 1989; Harrist et al., 1983). Perhaps unexpectedly, there were more HLA-DR+ than langerin+ cells in epidermal cell suspensions from both age groups. Previous studies using flow cytometry have demonstrated that there are few or no langerin+/HLA-DR+ cells in the epidermis (Angel et al., 2007; Stutte et al., 2008). One possible explanation for this is that contaminating HLA-DR+ cells from the dermis were present in the epidermal cell
suspensions. Alternatively, it may be that some keratinocytes were induced to express HLA-DR during the preparation of the epidermal cell suspensions. It has been shown that IFN-λ can induce HLA-DR expression on cultured keratinocytes (Basham et al., 1984; Griffiths et al., 1989), furthermore Jacobs et al were able to demonstrate HLA-DR expression on keratinocytes from normal skin using immunohistochemical analysis of skin biopsies (Jacobs et al., 2001). It is also possible that HLA-DR expressing epidermal T cells were present in the langerin cell population (Seneschal et al., 2012). Importantly, in the current investigation the level of expression of HLA-DR was not significantly reduced in the old age group.

4.5.3 Langerin and CD86 Expression on Epidermal Cells

Consistent with previous reports using flow cytometric analysis of epidermal cell suspensions, a relatively small proportion of LCs expressed low levels of CD86 (Schuller et al., 2001; Yokozeki et al., 1998). There was no alteration in CD86 expression on the LCs from the old volunteers studied. Langerhans’ cells up-regulate CD86 expression upon stimulation or culture, therefore in future experiments it would be important to confirm that LCs from old individuals are able to up-regulate CD86 expression appropriately (Peiser et al., 2008; Rattis et al., 1996). Notably, there is some evidence that preparation of epidermal cell suspensions may lead to up-regulation of markers of activation on LCs. Using immunohistochemistry of skin sections, Yokozeki et al could not detect CD86 expression in the epidermis of fresh skin biopsies, yet in a subsequent study, the same group found that a small proportion (5%) of freshly prepared LCs in epidermal cell suspensions expressed CD86 (Yokozeki et al., 1996; Yokozeki et al., 1998). Furthermore, Angel et al reported that cell preparation for flow cytometry led to up-regulation of HLA-DR expression on LCs (Angel et al., 2007).

4.5.4 Analysis of Dermal Dendritic Cells using Dermal Cell Suspensions and Immunofluorescence

Preparation of dermal cell suspensions from two 6mm biopsies yielded a limited number of cells; however, in general there were sufficient cells to analyse two DC markers concurrently with the isotype controls. As discussed earlier, similar studies
have used significantly larger amounts of tissue, which was not possible for this age-specific research. Two methods using different types of collagenase were used to digest the dermis in an attempt to maximise the cell yield. Some studies have used a combination of dispase and collagenase to digest the dermis (Angel et al., 2007; Santegoets et al., 2008), which may possibly have improved the yield in this study.

The initial aim was to use flow cytometry to quantify the numbers of dermal DCs in biopsies from young and old individuals, however, a combination of factors made this difficult. Firstly, as mentioned, the cell yield was disappointing; secondly the frequency of cells expressing the DC markers of interest were low making it difficult to detect any difference between groups; thirdly processing with trypsin meant that CD11c, one of the key markers of dermal DCs, could not be studied.

Within the human dermis, at least three distinct DC populations may be identified: including CD1a+ DCs, migratory LCs, and CD14+ DCs (Angel et al., 2007; Santegoets et al., 2008; Segura et al., 2012). Most recently a population of steady state dermal DCs in human skin expressing 6-SulfoLacNac and CD11c, but not CD1a or CD14 has been described (Gunther et al., 2012). In the current investigations a small population of HLA-DR+ cells was identified within the dermis of samples from both young and old individuals, of which a relatively small proportion co-expressed CD1a. No significant differences were detected between the age groups, although the numbers were small. Using a combination of flow cytometry and immunohistochemistry, Angel et al identified HLA-DR+ cells that did not co-express DC markers within the dermis of normal skin. Further analysis revealed that these included T lymphocytes and vascular endothelial cells, which could help explain the discrepancy between the numbers of HLA-DR positive and CD1a positive cells observed in this study (Angel et al., 2007). Additional experiments using flow cytometry were not performed as with the tissue available the yield of dermal DCs was too low.

It was possible to identify a small number of CD1a+ dermal DCs using immunofluorescence. In keeping with earlier observations, the number of CD1a+ dermal DCs was significantly lower than the number of CD1a+ LCs within the epidermis (Gunther et al., 2012; Klechevsky et al., 2008). Again, and consistent with previous reports, the level of CD1a expression appeared lower in the dermal DCs than in the LCs (Angel et al., 2006; Santegoets et al., 2008). There was no apparent difference between the number of CD1a+ dermal DCs in the young and old individuals.
in the sections examined, however, in view of the small numbers of cells present it would be necessary to look at more sections before drawing any conclusions. It was noteworthy that the number of cells in the dermis expressing both langerin and CD1a was reduced in the aged. In the absence of a defined langerin+ dermal DC in humans (Eisenwort et al., 2011; Klechevsky et al., 2008) these cells were presumed to be migrating LCs. Under steady state conditions, semi-mature LCs migrate to skin draining lymph nodes to promote self-tolerance (Hemmi et al., 2001; Ohl et al., 2004). The findings of the current study suggest that LC migration may be impaired in aged individuals not only following stimulation, as has been reported, but also in the steady state (Bhushan et al., 2002; Cumberbatch et al., 2002).

There are limited data available on the effect of age on the dermal DC population. Quatresooz et al reported a non-significant reduction in the number of factor XIIIa+ dermal dendrocytes in healthy skin of males over 65 years compared to males under 30 years (Quatresooz and Pierard, 2009). It is thought that some of these cells are tissue resident macrophages rather than dermal DCs as they express the macrophage marker CD163 and do not express CD11c (Zaba et al., 2007). Therefore, further studies are required to address the question of whether ageing affects the dermal DC population in a similar way to the LC population.

### 4.5.5 Measurement of Epidermal Interleukin-1β and caspase-1

Both IL-1β and TNF-α are required for LC migration, and intradermal injection of either cytokine will induce LC migration (Cumberbatch et al., 1997b). In the elderly, LC migration is significantly reduced following intradermal TNF-α, but the response to intradermal IL-1β is maintained (Bhushan et al., 2002; Bhushan et al., 2004). One possible explanation for this observation is that there is a lack of IL-1β in the epidermis of older individuals, and thus when exogenous IL-1β is provided LC migration is restored. The inactive precursor of IL-1β is cleaved to the active form via the action of caspase-1; therefore the levels of both IL-1β and caspase-1 were studied in aged skin. In unstimulated skin, the levels of IL-1β and caspase-1 mRNA and protein were equivalent in the young and old age group. The RNA from each age group was pooled to try and reduce the effect of inter-individual variation and therefore determine whether age alone had an effect. In an attempt to ensure that the PCR results were reliable, three different housekeeping genes were used (GAPDH,
Several studies have identified \textit{GAPDH} as an appropriate endogenous control for human PCR experiments. One study reported that \textit{GAPDH} remained constant within different human tissues and was not affected by either age or gender (Barber \textit{et al}., 2005), whilst a second study found that \textit{GAPDH} was a suitable internal control for the study of ageing skeletal muscle (Touchberry \textit{et al}., 2006). Zampieri \textit{et al} also reported that \textit{GAPDH} was a valid internal control for ageing studies using human PBMCs (Zampieri \textit{et al}., 2010). Keratin 14 has been identified as a suitable internal control for studies of intrinsic skin ageing, and appears to be unchanged at both the mRNA and protein level (Oender \textit{et al}., 2008). Finally, one study demonstrated that \textit{HPRT} was a good internal control for PCR studies of human skin, particularly when the gene of interest was expressed at intermediate or low levels (de Kok \textit{et al}., 2005).

In human epidermis, both keratinocytes and LCs are a source of \textit{IL1B} mRNA (Feldmeyer \textit{et al}., 2007; Kupper \textit{et al}., 1986; Morhenn \textit{et al}., 1992; Zepter \textit{et al}., 1997). Furthermore, \textit{IL1B} mRNA in both cell types is increased upon stimulation \textit{in vivo} and \textit{in vitro}. Cultured human keratinocytes treated with a contact sensitiser expressed increased levels of \textit{IL1B} mRNA compared with untreated cells (Zepter \textit{et al}., 1997), and LCs were shown to express increased levels of \textit{IL1B} mRNA following exposure to UVB (Morhenn \textit{et al}., 1992). Wong \textit{et al} demonstrated an increase in \textit{IL1B} mRNA in shave biopsies taken from experimentally inflamed skin compared with control treated skin (Wong \textit{et al}., 2004). No previous studies have specifically addressed the effect of ageing on cutaneous IL-1β in humans, although an age related reduction in IL-1α in the epidermis of aged mice has been reported (Sauder \textit{et al}., 1989; Ye \textit{et al}., 2002).

In murine studies, LCs have been shown to express \textit{CASP1} mRNA (Ariizumi \textit{et al}., 1995), whilst in humans \textit{CASP1} mRNA has been demonstrated in both whole skin biopsies and cultured human keratinocytes (Feldmeyer \textit{et al}., 2007; Yamanaka \textit{et al}., 2006). As with \textit{IL1B}, \textit{CASP1} mRNA levels are increased upon stimulation (Zepter \textit{et al}., 1997). Human LCs and keratinocytes also express components of the inflammasome, an immune complex which leads to the formation of active caspase-1 (Feldmeyer \textit{et al}., 2007; Kummer \textit{et al}., 2007). In contrast to the findings of the current investigations, Lener \textit{et al} reported that the level of \textit{CASP1} mRNA was reduced (fold reduction 1.91) in aged skin (Lener \textit{et al}., 2006). In the latter study, foreskin samples were used for analysis, and the young group were significantly
younger than in the current study (aged 3-4 years). It is therefore possible that the difference in the young age groups could account for this discrepancy.

The levels of IL-1β and caspase-1 protein in unstimulated epidermis were also equivalent in the young and old age groups. In humans, IL-1β protein can be identified in both keratinocytes and LCs. Previous studies have shown that the inactive precursor of IL-1β is present in keratinocyte cultures and stratum corneum, however, active IL-1β is only detectable following stimulation (Feldmeyer et al., 2007; Mizutani et al., 1991; Nylander-Lundqvist et al., 1996; Zepter et al., 1997). Morhenn et al found IL-1β in lysates of both unstimulated and stimulated human LC cultures, although there was no differentiation between the pro- and active forms of the cytokine (Morhenn et al., 1992). In the current investigations, IL-1β was undetectable in epidermal cell lysates using western blot, and was measured using ELISA which did not allow differentiation between the pro- and active forms.

Earlier studies have also demonstrated that IL-1β is either undetectable or present only at very low levels in supernatants from skin explant cultures (Furio et al., 2010; Stoitzner et al., 1999; van der Zee et al., 2012); therefore, in the current study, it was unsurprising that IL-1β was not detected in epidermal explant culture supernatants. It would, however, be interesting to determine whether the amount of IL-1β present in the cell lysates of the explants differs between young and old.

Caspase-1 protein was present in the unprocessed form in epidermal lysates from both young and old individuals as measured by western blot. Beta-actin was used as a control for equal protein loading in these investigations, as it has been shown to be unchanged in ageing skin at the protein level (Laimer et al., 2010). Consistent with the current study, Raymond et al were also only able to detect unprocessed caspase-1 in both epidermal samples and stratum corneum extracts (Raymond et al., 2007).

Although no differences in IL-1β and caspase-1 mRNA and protein were apparent between the age groups in the current investigations, it could be that in aged individuals there is a relative inability to up-regulate expression of IL-1β upon stimulation, which could therefore affect LC migration. Interestingly, a recent study has shown that aged mice have a reduced response (and consequently an increased mortality) to a mouse adapted influenza strain due to impaired inflammasome activation in DCs, which in turn results in reduced caspase-1 and, therefore IL-1β protein production (Stout-Delgado et al., 2012). Notably the expression of (pro) IL1B
mRNA was preserved in cells from aged mice, however CASP1 mRNA was reduced during infection.

4.5.6 Interleukin-1 Receptor Antagonist Secretion from Epidermal Explants

Interleukin-1Ra is produced by any cell type that produces IL-1α or IL-1β. It binds to IL-1RI with a similar affinity to IL-1α and β, but does not induce signal transduction and therefore serves to regulate IL-1 mediated inflammation (Banda et al., 2005; Dripps et al., 1991a; Hannum et al., 1990). The antagonist can also bind to the decoy receptor, IL-1RII, albeit less avidly (Dripps et al., 1991b; Granowitz et al., 1991). Several isoforms of IL-1Ra have been identified including a secreted form and 3 intracellular forms (Banda et al., 2005; Eisenberg et al., 1990; Malyak et al., 1998; Muzio et al., 1995). Human LCs have been shown in one study to express ILIRN mRNA, although there was no differentiation between the isoforms (Burns et al., 2000). Cultured human keratinocytes express mainly intracellular IL-1Ra, such that cultured human keratinocyte cell lysates contain more IL-1Ra than culture supernatants (Bigler et al., 1992; Haskill et al., 1991). Proportionally 25x more keratinocyte IL-1Ra than recombinant IL-1β is required to produce a 50% inhibition of the biological effects of IL-1β (Bigler et al., 1992). Although an earlier study reported that IL-1Ra levels up to 100 fold higher than IL-1 levels were required to produce a 50% inhibition of the IL-1 mediated effects, depending on the cell type involved (Arend et al., 1990).

Using immunohistochemistry, Muzio et al showed that within the epidermis it is mainly suprabasal keratinocytes that express intracellular IL-1Ra. When keratinocytes are lysed the IL-1Ra is released and may therefore dampen the inflammatory response upon cell death (Muzio et al., 1999; Muzio et al., 1995). There is also evidence to suggest that intracellular IL-1Ra is biologically active and can bind to other proteins within the cell (Banda et al., 2005; Watson et al., 1995).

As mentioned earlier, it may be that the age associated decrement in LC migration is related to defective IL-1β signalling. One possible explanation for this is a change in the balance of IL-1β and IL-1Ra in the epidermis of old individuals. In the current investigations, IL-1Ra was detectable in epidermal explant culture supernatants from both the young and old groups. Presumably the IL-1Ra was mainly the secreted, rather than the intracellular, form as the cells were not lysed. The level of IL-1Ra was
significantly reduced in explant supernatants from old individuals. This finding is consistent with one previous report which found an age-related reduction in IL-1Ra protein in stratum corneum (Hirao et al., 1996). In the current study, the reduction in IL-1Ra may be a manifestation of a reduced level of IL-1β production and release in the old group during the explant culture. As mentioned earlier, IL-1β was not detectable in the explant supernatants by ELISA, which reflects the fact that the level of IL-1β is substantially lower than that of IL-1Ra. It is also possible that if the LCs within the explant were the principal source of IL-1Ra, the reduced levels observed in the old group could be related to the reduced number of LCs in the aged epidermis.

4.5.7 Interleukin-1 Receptor I and II Expression on Epidermal Cells

Interleukin-1β can bind to both IL-1RI and IL-1RII, although only IL-1RI is signal transducing. When IL-1β binds to IL-1RI, a complex is formed with the IL-1R accessory protein which then recruits the adapter protein MyD88 and various kinases which mediate IL-1 signalling (Dinarello, 2011b). Both keratinocytes and LCs express IL-1RI (Cumberbatch et al., 2006; Eller et al., 1995; Larregina et al., 1996). In the current study, approximately 60% of LCs expressed low levels of IL-1RI (as measured by flow cytometry). There were no differences between the young and old groups in terms of percentage positive cells and the relative level of IL-1RI expression, which suggests that changes in IL-1RI are not responsible for the proposed defect in IL-1β signalling in aged skin (Bhushan et al., 2004). The findings are consistent with those of Cumberbatch et al who, using flow cytometry, found that 63.3% of LCs from human epidermal cell suspensions expressed low levels of IL-1RI (Cumberbatch et al., 2006). Few keratinocytes expressed IL-1RI, which is not unexpected as a previous study in mice has shown that only a small percentage of keratinocytes in epidermal cell suspensions express IL-1RI (Cumberbatch et al., 1998). Furthermore, IL-1RI expression in human epidermis or unstimulated cultured keratinocytes is also low or undetectable when measured using immunohistochemistry or radio-labelled binding techniques (Groves et al., 1994). Interleukin-1RII has been shown to be a non-functioning decoy receptor (Colotta et al., 1993). It is expressed on the surface of LCs and keratinocytes and can also be released in a soluble form (Groves et al., 1995; Larregina et al., 1996). During skin explant culture, IL-1RII expression is increased, and stimulation of cultured
keratinocytes with PMA also leads to increased IL-1RII expression and secretion (Groves et al., 1995; Groves et al., 1994). In the current investigations, flow cytometry was used to measure intracellular and extracellular IL-1RII expression in LCs and keratinocytes. A small proportion of keratinocytes expressed detectable levels of extracellular IL-1RII in both age groups (6.0-9.6%); however, LCs expressed little or no extracellular or intracellular IL-1RII. In contrast, Larregina et al reported that 78% of CD1a+ cells in LC enriched epidermal cell suspensions expressed surface IL-1RII (using flow cytometry). It may be that the enrichment process led to stimulation of the LCs with an associated upregulation of IL-1RII expression. In addition, a different IL-1RII antibody was used in the current study which could have affected the results (Larregina et al., 1996). In a murine study, 39% of keratinocytes expressed low levels of extracellular IL-1RII which is significantly higher than in the current study (Cumberbatch et al., 1998). Further experiments, including measurement of IL-1RII in explant supernatants and PCR of whole epidermis, would be required to draw firm conclusions on the effect of age on epidermal IL-1RII expression. To date, only one study in mice has addressed the effect of age on IL-1R expression (Ye et al., 2002). Interestingly, IL1R2 and IL1RN mRNA were both reduced in the epidermis of aged mice, whereas IL-1RI was unchanged. There was also a non-significant reduction in IL1A mRNA (IL-1β was not measured). The authors concluded that overall defective IL-1 signalling may at least in part account for the age associated reduction in epidermal barrier function.

4.5.8 Limitations of the Study

The current investigations were limited in several ways. Firstly, in general the sample sizes were low. In particular, the number of samples used to measure caspase-1 protein and IL-1R expression were small. It is possible that in these experiments a larger sample size would have yielded different results. Secondly, in the experiments to compare the phenotype of LCs between the young and old groups only 2 markers (HLA-DR and CD86) were studied. It may be that if different markers important in LC maturation and migration (such as the chemokine receptor CCR7) were used an age-related difference would be apparent. It is also possible that a difference in expression of phenotypic markers between the age groups only occurs upon stimulation: this was not addressed in the current investigations.
The study of dermal DCs using flow cytometry was limited by the low yield of cells, and no conclusions regarding the affect of age on the dermal DC population could be made from the experiments performed. It was possible to identify dermal DCs using immunofluorescence, however, sections were only stained for CD1a and langerin which would have only identified a proportion of dermal DCs as well as migrating LCs. The number of dermal DCs per hpf was low, therefore in future experiments it would be better to count more fields, perhaps within a set length of epidermis, and use a wider range of markers including CD11c and CD14.

Finally, although the levels of IL-1β and caspase-1 mRNA and protein were similar in both age groups, all experiments were performed using unstimulated skin. It may be that in old individuals there is a failure to up-regulate epidermal IL-1β upon stimulation. As discussed earlier, this has been recently demonstrated in DCs of aged mice following infection with influenza virus, where impaired activation of caspase-1 results in reduced IL-1β production (Stout-Delgado et al., 2012).

4.5.9 Future Work

Future experiments are needed to study the effect of stimulation upon the phenotype of LCs and epidermal IL-1β in aged skin. Cultured epidermal explants could be used to prepare samples for PCR, western blot and flow cytometry. In addition, samples could be used to study the functional activity of caspase-1.

It would also be interesting to study the expression of a wider range of markers on LCs in the age groups, including the chemokine receptors CXCR4 and CCR7 which are important in LC migration (Ouwehand et al., 2008). In the current investigations there was limited IL-1RII expression on both keratinocytes and LCs. Further experiments with a different IL-1RII antibody are warranted, perhaps in combination with measurement of secreted IL-1RII in explant supernatants.

As detailed above, a more extensive study of dermal DCs in aged skin using immunofluorescence is also required. Other markers including CD14 and CD11c, as well as CD1a and langerin, could be used to identify different dermal DC populations.
5. An Epidermal Explant Model to Study Langerhans’ Cell Function in Young and Aged Skin

In both man and mouse ageing is associated with a reduction in epidermal LC number and an impaired migratory response (Bhushan et al., 2002; Choi and Sauder, 1987; Cumberbatch et al., 2002; Gilchrest et al., 1982; Thiers et al., 1984). Langerhans’ cells migrate spontaneously from epidermal explants (Prignano et al., 1998), and explant models have been used to study cutaneous DC populations particularly for experiments (such as exposure to contact sensitisers or multiple cytokines) that could not be easily performed in vivo (Bond et al., 2009; Flacher et al., 2010; Jacobs et al., 2006; Prignano et al., 2001). There have been no previous studies to address the effect of age on LC migration from epidermal explants. Experiments were performed to determine whether, with age, LC migration is impaired from epidermal explants, and thereafter to study the effect of the cytokines IL-1β and TNF-α on LC migration in the explant model.

5.1 Langerhans’ Cell Counts in Sun Protected Skin

Epidermal sheets were prepared from 6mm biopsies taken from sun-protected buttock skin of 16 young (mean age 22.5 ± 0.8 years, 4 males and 12 females) and 14 aged volunteers (mean age 73.6 ± 3.2 years, 4 males and 10 females). The sheets were stained with CD1a and LC counts performed using fluorescence microscopy. There were no obvious differences in the morphology or distribution of LCs within the epidermal sheets between the young and the old groups (Figure 5.1). However, there was a significant reduction in LC number in the old age group (Figure 5.2, mean 641 ± 14 LC/mm² compared to 732 ± 17 LC/mm² in the young group, p<0.001). Within each age group there was no significant difference between the sexes in terms of LC number (data not shown). Some old volunteers were taking prescribed medication: mainly antihypertensive and lipid lowering therapies. Comparison of the old volunteers taking medication with those who were not did not reveal any significant differences in terms of LC number (data not shown).
Figure 5.1 Representative photomicrographs of epidermal sheets prepared from sun protected skin and stained with CD1a from a young (779 Langerhans’ cells/mm²) and an old volunteer (571 Langerhans’cells/mm²). Scale bar = 50μm.

Figure 5.2 Langerhans’ cell (LC) counts in epidermal sheets prepared from sun-protected skin of young (n=16) and old (n=14) volunteers. Bar to demonstrate mean, *** p<0.001.
5.2 Emigration of Langerhans’ Cells from Epidermal Explants

Langerhans’ cells migrate from epidermal explants in culture (Bond et al., 2009; Flacher et al., 2010; Prignano et al., 2001), therefore the percentage of LCs that migrated from epidermal explants prepared from young and aged individuals after 24 hours in culture was compared (calculated by dividing the difference between the baseline (T0) LC count and the LC count at 24 hours (T24) by the baseline count). As shown in Figure 5.3, spontaneous LC migration occurred from epidermal sheets prepared from all young individuals (n=12, mean 16.6 ± 1.7% migration, range 8.6 to 26.2%), whereas there was little or no LC migration from sheets prepared from the aged individuals (n=13, mean 2.1 ± 0.8% migration, range 0 to 10.2%, p<0.0001). Again, there were no consistent differences in the morphology of the LCs after 24 hours in culture in either age group (Figure 5.4). There was also little correlation between the baseline LC count in the young individuals and the percentage of LCs that had migrated from the explants at T24 (Figure 5.5). Within the old group there was no significant difference in LC migration between those taking prescribed medication and those who were not (data not shown).

Figure 5.3 Percentage of Langerhans’ cells that migrated from epidermal sheets prepared from the skin of young (n=12) and old (n=13) volunteers after 24 hours in culture. Bar to demonstrate mean, *** p<0.001.
Figure 5.4 Representative photomicrographs of epidermal sheets from a young volunteer stained with CD1a, at (A) time 0 (T0, 779 Langerhans’ cells/mm²) and (B) after 24 hours in culture (T24, 596 Langerhans’ cells/mm²). Scale bar = 50μm.

Figure 5.5 Comparison of baseline Langerhans’ cell (LC) counts and % migration from epidermal explants prepared from the skin of young volunteers (n=12) after 24 hours in culture.
5.3 Comparison of CD1a and Langerin Staining for Langerhans’ Cell Enumeration

There is some evidence to suggest that LC maturation is associated with down-regulation of CD1a expression (Prignano et al., 2001). To determine whether the reduction in LC counts observed in explants prepared from young individuals was due to emigration rather than a change in phenotype, epidermal sheets were prepared from a young and an old volunteer and stained with CD1a or langerin at T0 and T24 and LC counts performed. The counts at both time points were compared for each antibody and were consistent in both the young and the old volunteer (Figure 5.6). The percentage of LC migration in the young individual at T24 was 12.6% with CD1a and 13.4% with langerin, compared to 4.2% with CD1a and 1.1% with langerin in the old volunteer. Langerin only stained the LC bodies which facilitated enumeration, but as a consequence it was not possible to comment on the appearance of the dendrites (Figure 5.7).

![Figure 5.6](image)

**Figure 5.6** Comparison of Langerhans’ cell (LC) counts in epidermal explants prepared from a young and an old volunteer and stained with CD1a or langerin at time 0 (T0) and after 24 hours in culture (T24).
5.4 Kinetics of Langerhans’ Cell Migration from Epidermal Explants

Previous research has shown that LC migration from murine skin explants starts within 5 hours (Stoitzner et al., 2002). To investigate the kinetics of LC migration, epidermal explants from 2 young volunteers were placed in culture for 2, 6 and 24 hours. In addition explants from a further 3 young volunteers were cultured for 2 hours as part of a different experiment. In the time course experiment, after 2 hours in culture (T2) there was already a reduction in the LC count compared with baseline (T0), and by 6 hours (T6) more than two thirds of the migration observed by 24 hours had occurred (Figure 5.8). There was also a reduction in LC count at 2 hours in the explants prepared from the other 3 young volunteers, although there was significant inter-individual variation (4.0%, 13.5% and 18.6% migration, Figure 5.9).

**Figure 5.7** Photomicrographs of epidermal sheets from a young volunteer stained with CD1a and langerin at time 0 (T0) and after 24 hours in culture (T24). Scale bar = 50μm.
Figure 5.8 Time course of Langerhans’ cell migration from epidermal explants prepared from 2 young volunteers (each represented by a separate line).

Figure 5.9 Percentage Langerhans’ cell migration from epidermal explants prepared from 3 young individuals (Y1, Y2 and Y3) following 2 hours in culture.

5.5 The Effect of Interleukin-1β and Tumour Necrosis Factor-α on Langerhans’ Cell Migration from Epidermal Explants

Previous work has indicated that local LC migration in aged individuals can be restored in vivo by intradermal injection of IL-1β, but not TNF-α (Bhushan et al.,
To determine whether addition of IL-1β and TNF-α to the explant culture medium had any effect on LC migration, epidermal explants were prepared from 5 young and 5 old individuals and cultured for 24 hours in media alone or media supplemented with 2000u of IL-1β or 5000u of TNF-α. Addition of cytokine to the culture media did not enhance LC migration in the young group (Figure 5.10). In the old group, addition of either cytokine enhanced migration compared with medium alone (mean percentage migration T24 media alone 4.1 ± 1.5%, IL-1β 13.0 ± 1.8%, TNF-α 7.1 ± 1.5%, p<0.01 analysed using one way ANOVA, Figure 5.10), although only the difference between T24 and IL-1β was significant after the Bonferroni post test was applied (p<0.01). The morphology of LCs within the epidermal sheets following culture in cytokine supplemented media did not change (Figure 5.11).

**Figure 5.10** The effect of addition of IL-1β or TNF-α to epidermal explant culture media on Langerhans’ cell migration at 24 hours (T24) in young and old volunteers. Each symbol and line represents a separate volunteer, n=5 both groups, ** p<0.01.
Figure 5.11 Photomicrographs of epidermal sheets from a representative old volunteer at time 0 (T0), and after 24 hours in culture in media alone (T24), or with IL-1β (T24 IL-1β), or TNF-α (T24 TNF-α). Scale bar = 50μm.

To investigate the possibility that IL-1β or TNF-α could increase the migration of LCs from explants prepared from young volunteers at an earlier time point in culture, explants were cultured in the presence of the cytokines for 2 hours (Figure 5.12, n=3). In 2 of the 3 individuals there was an increased migratory response to IL-1β after 2 hours in culture. In the remaining individual the cytokines had no additional effect upon percentage migration; however, it is noteworthy that in this individual there was already a significant degree of spontaneous migration (18.6%).
Figure 5.12 Effect of cytokines on Langerhans’ cell migration from epidermal explants prepared from young volunteers (n=3) and cultured for 2 hours in media alone (T2) or with IL-1β (T2 IL-1β) or TNF-α (T2 TNF-α). Each line represents a separate individual.

5.6 Attempts to Characterise Emigrated Langerhans’ Cells

Several studies have been able to characterise and culture LCs that have migrated from epidermal and whole skin explants (Bond et al., 2009; de Gruijl et al., 2006; Flacher et al., 2010; Jacobs et al., 2006; Prignano et al., 2001). In the current study, several attempts were made to characterise cells that had migrated from epidermal explants prepared from young volunteers. Based on 20% LC migration from a 6mm epidermal sheet containing 730 LC/mm² there would only be approximately 4100 émigrés (not including non-LC cells). The low number of cells available meant that there was limited success in characterisation. Cells were visible by light microscopy of media following 24 hours in culture (Figure 5.13). However, when the cells were stained with langerin and DAPI and visualised using fluorescence microscopy very few LCs were apparent (Figure 5.14).

Émigrés were also collected and stained with CD1a and analysed using flow cytometry: again, due to the small number of cells present, few cells were acquired on the flow cytometer, however, a number of CD1a⁺ cells were present (11.34% of viable cells CD1a⁺, Figure 5.15).
Figure 5.13 Cells within culture media following a 24 hour culture of an epidermal explant from a young volunteer. Scale bar = 50μm.

Figure 5.14 Émigrés from explant culture media stained with langerin and 4',6-diamidino-2-phenylindole (DAPI) and visualised using fluorescence microscopy, examples highlighted by arrows. Scale bar = 50μm.
Figure 5.15 Émigrés from explant culture media were stained with CD1a or isotype control (IgG2a) and analysed via flow cytometry. Non-viable cells were gated out. The percentage of CD1a\(^+\) cells labelled in lower right quadrant.

5.7 Discussion

An epidermal explant model was used to study the migration of LCs \textit{ex vivo} in young and aged skin. The explant model clearly demonstrated both a reduction in LC number and impaired LC migration in photoprotected skin from aged individuals. Interestingly, the findings closely mirrored changes that have been reported previously \textit{in vivo} (Bhushan \textit{et al.}, 2002; Bhushan \textit{et al.}, 2004), with LC migration in the aged being at least partially restored by the addition of IL-1\(\beta\). This suggests a possible defect in the availability of IL-1\(\beta\) in the epidermis of aged individuals.

5.7.1 Volunteer Selection

All old individuals recruited for this research were living independently and were able to travel to the hospital unaided. Some elderly individuals were taking prescribed medication including lipid-lowering and antihypertensive drugs. Subgroup analyses revealed there were no differences in LC numbers or percentage migration in those
volunteers taking prescribed medication compared with those who were not. The age groups were also sex-matched.

5.7.2 Langerhans’ Cell Counts in Young and Aged Skin

A variety of methods have been used to enumerate LCs within the epidermis including immunohistochemistry or immunofluorescence of either vertical cryosections of skin biopsies, or epidermal sheets. Furthermore, the number of LCs may also be expressed in a number of different ways which makes it difficult to compare results of studies. Early studies used the ATPase staining method to detect LCs within the epidermis. One such study reported LC counts of between 406 and 480 LC/mm² in formalin fixed epidermal sheets from forearm and buttock skin prepared from males aged between 25 and 44 years. The biopsies were taken immediately after irradiation with UVA or UVB, but the counts were similar to a non-irradiated control at 478 LC/mm² (Aberer et al., 1981). These counts are somewhat lower than those reported by Friedmann who used the ATPase method to stain LCs from epidermal sheets prepared from suction blisters of the upper arm (ages of volunteers not stated). The mean LC frequency was 730 LC/mm² which is similar to the results of the LC frequency in the young volunteers in the current study (Friedmann, 1981). Consistent with this, Chen et al reported a mean LC frequency of 640 LC/mm² on skin biopsies taken from the extremities of individuals aged between 18 and 60 years. The mean LC count in skin biopsies taken from the trunk was 740 LC/mm² (Chen et al., 1985). Interestingly, the LC counts in this study were similar when the ATP-ase method was compared with staining for CD1a. As has been previously documented, there were no differences between males and females (Chen et al., 1985; Scheibner et al., 1983).

It is difficult to explain the lower counts observed in the study by Aberer et al, since studies of LC densities in different body sites do not show large variations between the trunk and limbs unless older individuals (aged >65 years) are included, in which case sun exposure is an important factor (Aberer et al., 1981; Ashworth and Mackie, 1986; Chen et al., 1985; Thiers et al., 1984). One possible explanation is differences in the methods used, for example in the study by Chen et al the epidermis was separated from the dermis following a 2 hour incubation in EDTA and the sheet was fixed in a formalin solution for 20 minutes, compared with the use of sodium bromide.
to remove the epidermis and an overnight incubation in formalin solution in the study by Aberer et al. There is evidence to suggest that the use of EDTA leads to better visualisation of the LCs and this may account for the increased counts (Mackenzie and Squier, 1975).

More recent studies have also reported similar LC counts to those presented earlier in this chapter. One study reported a mean LC count of 755 LC/mm² in epidermal sheets from sun-protected skin from the upper inner arm of 13 Caucasian volunteers aged less than 30 years (Hatao et al., 1996), whilst a second study reported counts between 800 and 1025 LC/mm² in skin from breast or abdominal surgery, although the age of the subjects was not stated (Lukas et al., 1996).

There was a significant reduction in LC number in the old group in the current study. Previous reports have also shown that LC numbers are reduced in both sun-protected and sun-exposed skin in older individuals (Gilchrest et al., 1982; Gilhar et al., 1991; Scheibner et al., 1983; Thiers et al., 1984). In sun-protected buttock skin, Thiers et al reported a reduction of approximately 14% in LC counts in old males (≥ 65 years) compared with young males (≤ 24 years), which is comparable to the 12% reduction observed in this study (Thiers et al., 1984). They also described a difference in the morphology of the LCs in the old individuals in that they had fewer and shorter dendrites. These changes were not noted in this study, although different staining techniques were used.

Interestingly, Gilhar et al documented a more profound age-related reduction (over 50%) in LC number in epidermal sheets prepared from biopsies taken from the thigh (Gilhar et al., 1991). One possible explanation for this is that the subjects were older (mean age 82.3 years) than in the current study and that of Thiers et al, particularly since some authors have hypothesised that LC counts may be maintained until approximately 70 years of age (Hatao et al., 1996). The LC counts reported by Gilhar et al were also in general lower than other quoted studies. This may be because they used HLA-DR to identify epidermal LCs which has been shown in one study to produce lower counts than CD1a (Hatao et al., 1996).

The reason for the reduced LC count observed in aged individuals has not yet been elucidated. Possible explanations include: a reduction in local, unknown precursors; impaired homing of local or circulating precursors; or a reduction in the ability of LCs from aged individuals to undergo mitosis. Although the results in chapter 3 suggest no reduction in circulating monocyte precursors, it is thought that under homeostatic
conditions LCs may be replenished from a local source (Merad et al., 2002). It is also not known whether the reduced number of LCs in aged skin, is of clinical relevance. Recent work has shown that LCs can promote proliferation of skin resident T cells, and that in vivo, LCs are found in close contact with Tregs (Seneschal et al., 2012). It is, therefore, possible that a reduction in LC number could result in reduced T cell interaction and a consequent impaired immune response.

5.7.3 Langerhans’ Cell Migration from Epidermal Explants

In the current investigations the reduction in LC numbers following the 24 hour culture period was presumed to correlate to the percentage of migrated cells. Jacobs et al have previously shown that reduction in CD1a+ LCs counted in frozen sections following culture of human skin explants (epidermis and dermis) correlated closely with the number of CD1a+émigrés as measured by flow cytometry (Jacobs et al., 2006).

Spontaneous LC migration occurred from all epidermal explants prepared from young volunteers after 24 hours in culture. This is in keeping with published reports that have shown that LCs migrate from explants comprising epidermis alone or epidermis and dermis. Studies in mice of cultured dorsal ear halves demonstrated spontaneous migration of LCs from explants whether or not the epidermis had been separated from the dermis (Larsen et al., 1990; Stoitzner et al., 2002; Weinlich et al., 1998). Changes in LCs were visible within 4 hours, and by 5 hours of culture some LCs had migrated into the dermis: suggesting that a reduction in epidermal LC count after culture could be attributed to migration (Larsen et al., 1990; Stoitzner et al., 2002). Larsen et al demonstrated a 17.4% reduction in LC counts after 24 hours in culture which is similar to the findings of the current study. Interestingly by 48 hours in culture the LC count was reduced by more than 60% of the baseline (Larsen et al., 1990). In the current study epidermal sheets were not cultured beyond 24 hours. Enumeration of LCs in sheets cultured for 48 and 72 hours did not reveal a consistent increase in migration and was technically difficult due to irregular staining (unpublished observations). Other studies of human epidermal explants suggest that migration is complete between 24 and 48 hours (Bond et al., 2009; de Gruijl et al., 2006).

Prignano et al demonstrated a mean reduction in LC counts of 26% after 48 hour culture of epidermal sheets prepared from skin of 3 individuals undergoing plastic
surgery (Prignano et al., 2001). As in the current study, LC counts were performed on epidermal sheets stained with CD1a. In this study the authors noted a reduction in LC surface area and perimeter, as well as the number of dendrites. The cell morphology was characterised using computerised image analysis of tracings of LCs that were taken from photomicrographs of the epidermal sheets at x400 magnification (it was not stated how many cells were compared for each experiment). Although in the current study there were no obvious differences between LC morphology in young and old before and after culture, it is possible that subtle differences in LC morphology would be apparent on more detailed analysis. Such analysis could be the subject of future experiments.

A similar study of epidermal explants (from 6mm biopsies of skin removed during plastic surgery) also demonstrated spontaneous LC emigration (de Gruijl et al., 2006). The emigrated cells were counted using a haemocytometer or flow cytometer, and the mean number (4454) was similar to that calculated using the percentage reduction in LCs observed in the young group after 24 hours in culture in the current study. There were significant inter-individual variations in terms of the numbers of émigrés (875 to 14500 cells); an observation consistent with the current study as there was a relatively wide range in the percentage LC migration in the young. Importantly de Gruijl et al demonstrated that the émigrés expressed CD1a.

Lukas et al reported a greater reduction in LCs (46-66%) from explants comprised of human epidermis and dermis after 48 hours in culture (Lukas et al., 1996). One possible explanation is that dermal factors could increase the migratory response, as addition of fibroblasts to an explant culture system can enhance LC migration (Ouwehand et al., 2008; Ouwehand et al., 2010).

Only a proportion of LCs will migrate following a stimulus. For example studies in man have shown that exposure to a contact allergen, intradermal injection of IL-1β or TNF-α or mechanical trauma causes approximately 20-30% of the “resting” LC population to migrate (Bhushan et al., 2002; Bhushan et al., 2004; Dearman et al., 2004; Griffiths et al., 2001), although following UV exposure a larger proportion of LCs may be lost from the epidermis (Gilchrest et al., 1982). It is likely that the trauma of both the biopsy procedure and explant preparation is sufficient to provoke LC migration in the explant model via the release of pro-inflammatory cytokines, such as TNF-α, from keratinocytes. The mean percentage migration in the young group in the current study was slightly lower than that reported in the aforementioned studies, and
this may be because the other studies were performed *in vivo* where dermal factors could have an additive effect.

There have been no studies to date comparing the migration of LCs from explants prepared from the skin of young and old individuals. The majority of previous studies have been performed using surplus skin from plastic surgery procedures, and although the ages of the patients are often not stated, evidence in the literature suggests that the mean age for abdominoplasty and breast reduction is approximately 40 years (Momeni *et al.*, 2009; Shermak *et al.*, 2011). It was therefore of great interest that LCs failed to migrate from the explants in the old age group. Studies have established that *in vivo* LC migration is impaired in aged mice in response to exposure to either a contact allergen, or intradermal injection of TNF-α (Cumberbatch *et al.*, 2002). Similarly, in aged humans LC migration is impaired in response to TNF-α (Bhushan *et al.*, 2002), and although specific studies of LC migration following exposure to contact allergens have not been performed in the aged, there is a reduced incidence of contact hypersensitivity with increasing age (Kwangsukstith and Maibach, 1995). One possible explanation for the reduced migratory response observed in the old could be that the reduction in baseline LC number correlates with the proportion of LCs that would normally migrate in response to a stimulus. However, in the young group there was no correlation between baseline LC number and percentage LC migration, and subsequent experiments revealed that migration in the old could be at least in part be restored by addition of IL-1β to the culture medium.

5.7.4 Langerhans’ Cell Counts: a Comparison of Staining with CD1a and Langerin

In these investigations the majority of LC counts were performed using antibody staining against CD1a. The decision to use CD1a was based on previously published work using this antibody as a method of identifying and counting LCs within epidermal sheets, and the ability to visualise the appearance of the LC dendrites when using this antibody (Ashworth and Mackie, 1986; Bhushan *et al.*, 2002; Bhushan *et al.*, 2004; Cumberbatch *et al.*, 2006; Prignano *et al.*, 2001).

It was, however, important to verify that the reduction in LC counts observed in the explants prepared from young individuals was not simply due to down-regulation of CD1a expression. One previous study reported reduced expression of CD1a on
émigrés from epidermal explants, although LCs within the epidermal sheets could still be identified with CD1a staining (Prignano et al., 2001). There was a close correlation between LC counts performed in both a young and an old volunteer using CD1a and langerin antibodies to stain epidermal sheets. Langerhans’ cells within epidermal sheets stained with langerin were easier to count than those stained with CD1a, since the langerin antibody stained only the cell bodies and each cell could be easily defined. When counting LCs stained with CD1a, it was more difficult to ensure accurate counting, particularly if the LCs were in close proximity to one another and had long prominent dendrites.

An earlier study compared the use of HLA-DR, CD1a and lag (an antibody which stains Birbeck granules and therefore stains predominantly the cell body rather than dendrites, and has been subsequently superseded by langerin) to identify LCs in human epidermal sheets before and after culture (Lukas et al., 1996). The authors reported that reduction in LC numbers following culture was most evident using lag. Jacobs et al also reported that LC counting was easier and more reproducible when lag rather than CD1a was used to identify LCs, although LC counts were performed on frozen skin sections rather than epidermal sheets (Jacobs et al., 2001). Importantly, the authors also found that the use of image analysis software reduced intra- and inter-individual variation in LC counts compared with manual counts when CD1a or lag were used to stain LCs. Inter-individual variation in counts was higher than intra-individual variation for both manual and software aided counting.

Stoitzner et al used langerin to identify LCs within epidermal sheets prepared from murine dorsal ear halves before and after culture (Stoitzner et al., 2003). Langerin expression was maintained in the remaining LCs within the sheets even after 4 days of culture; furthermore the émigrés also expressed langerin. A similar study using human skin also demonstrated that LCs within epidermal sheets maintained langerin expression following a 2-3 day culture period. Again émigrés were harvested, and using flow cytometry, a significant proportion of the cells were found to express langerin (Ebner et al., 2004).

5.7.5 Timing of Langerhans’ Cell Migration from Epidermal Explants

The results of the current investigations suggest that LC migration starts to occur within hours of epidermal explant preparation. This is in keeping with published
reports in murine explant studies which suggest that some LC migration is evident within 4-8 hours of culture (Larsen et al., 1990; Stoitzner et al., 2002). Cumberbatch et al were able to demonstrate in vivo that intradermal injection of either TNF-α or IL-1β into mouse ears led to a reduction in LCs within 4 hours as evidenced by enumeration of LCs in epidermal sheets in untreated compared with treated mice (Cumberbatch et al., 1997a). This corresponded with an increase in LCs within draining lymph nodes suggesting migration had occurred. Interestingly, a reduction in LCs in epidermal sheets could be observed as early as 30 minutes after TNF-α injection. Subsequent studies in humans confirmed that loss of LCs from epidermal sheets occurred within 2 hours of either intradermal IL-1β or TNF-α injection (compared with injection of saline) (Cumberbatch et al., 2003; Cumberbatch et al., 1999b). Based on these findings, if the spontaneous LC migration observed from the epidermal explants is indeed due to cytokine release triggered by the trauma of the biopsy procedure, it is not surprising that some migration was evident within hours of the explant preparation.

5.7.6 The Effect of Cytokines on Langerhans’ Cell Migration from Epidermal Explants

Consistent with in vivo studies, the current investigations have shown that exogenous IL-1β can at least partially restore LC migration in aged skin (Bhushan et al., 2004; Cumberbatch et al., 2002). All old volunteers also showed an increased level of LC migration following the addition of TNF-α to the culture medium, although this was not statistically significant. This is again consistent with a previous in vivo study that showed that the LC migratory response to intradermal injection of TNF-α was reduced in old compared with young individuals (Bhushan et al., 2002). Both in vivo studies did not determine whether LC migration could occur in older individuals in response to skin trauma (i.e. biopsy and epidermal sheet preparation) alone, as no “baseline” LC count was performed. Instead, the effect of cytokine injection was compared with a saline injection. It is particularly noteworthy, however, that the findings of the current investigations in terms of the response to IL-1β and TNF-α exactly mirror the findings of the in vivo studies, suggesting that the explant model could be used as a way of studying therapies to restore LC migration in the aged.
The effect of IL-1β on LC migration in the elderly again suggests that there may be some alteration in IL-1β signalling in the aged epidermis. As shown earlier in these investigations, the levels of either IL-1β mRNA or protein in unstimulated epidermis did not appear reduced, although the levels of IL-1β were uniformly low. The levels of IL-1β in the explant supernatants were below the limit of detection of ELISA. It would be important in future investigations to compare the levels of IL-1β in stimulated skin, since as shown earlier IL-1Ra secretion was reduced from cultured explants prepared from aged volunteers, and this could be a reflection of reduced IL-1β release following stimulation.

There was no additional effect of IL-1β and TNF-α in terms of percentage LC migration from explants of young volunteers. This is presumably because the LCs which migrated spontaneously were the proportion that were already primed to migrate. The factors that govern this phenomenon are unknown. When explants from young volunteers were cultured for 2 hours in the presence of IL-1β or TNF-α, there was some suggestion that IL-1β could increase the migratory response. Again it may be that by this time point cytokine release triggered by the biopsy procedure was sufficient to initiate migration.

Stoitzner et al investigated the effects of IL-1β and TNF-α on LC migration from epidermal explants prepared from murine and human skin (Stoitzner et al., 1999). The cytokines were added to the culture media and the explants cultured for 48 hours. Low doses of TNF-α increased LC migration from murine explants after 48 hours in culture as did IL-1β. Higher doses of TNF-α actually inhibited migration: the authors hypothesised that this was due to an increased expression of CD54 on lymphatic endothelium which caused the LCs to “stick” to the endothelium and thus not migrate. In the current investigations there was a small non-significant decrease in LC migration from explants treated with TNF-α in the young volunteers. It is doubtful that this could be explained by a change in CD54 expression, as the epidermis was cultured without the dermis. Furthermore, the increased (non-significant) migration observed in the old group suggests that it is unlikely to be due to a toxic effect of the cytokine. It would be necessary to increase the number of young individuals in the current study to determine whether the decrease in LC migration with TNF-α is a true effect. One study using human explants revealed that addition of TNF-α to the culture medium did not affect LC migration, which is more in keeping with the current results (Prignano et al., 2001). In addition, an in vivo study of LC migration following
preparation of suction blisters showed that the trauma of the blistering process was sufficient to induce LC migration, which was not augmented by prior intradermal injection with IL-1β (Dearman et al., 2004).

Interestingly, Stoitzner et al also showed that addition of neutralising anti-TNF-α and IL-1β antibodies to the culture system could inhibit migration (although to show a significant effect anti-IL-1β had to be administered systemically prior to explant preparation in the mouse) (Stoitzner et al., 1999). This demonstrates that LCs within explant cultures are capable of internalising larger molecules. Consistent with this, Flacher et al reported that LCs could internalise labelled antibodies (150kDa) that were injected intradermally ex vivo in human skin explants (Flacher et al., 2010).

The current investigations did not reveal any notable differences in LC morphology in explants cultured in cytokine supplemented medium. In accordance with these findings, previous work has shown that intradermal injection of TNF-α followed after 2 hours by epidermal sheet preparation, did not cause any difference in LC morphology compared to a control saline injection (Cumberbatch et al., 1999b). However, a similar study using IL-1β did demonstrate an increase in cell size and length of dendrites when biopsies were taken 4 hours after injection (Cumberbatch et al., 2003). It may be, as discussed earlier, that in the current investigations more detailed analyses of LC morphology would reveal differences between the different culture conditions used in the explant model.

### 5.7.7 Characterisation of Emigrated Cells

In these investigations the characterisation of emigrated cells in the young group was hampered by the small numbers of cells available. Studies which have used larger pieces of skin, or have pooled samples, have used either flow cytometry or immunostaining of cytopsins to confirm that CD1a⁺ or langerin⁺ LCs migrate from epidermal explants (de Gruijl et al., 2006; Ebner et al., 2004; Jacobs et al., 2006; Lukas et al., 1996; Prignano et al., 2001; Prignano et al., 1998; Stoitzner et al., 2003). It was possible to identify a population of CD1a positive cells within the explant culture medium using flow cytometry, however, there were too few cells to conduct experiments using cytopsin. In the literature there is a significant degree of variation in the proportion of émigrés that express CD1a. Prignano et al reported that approximately 40% of émigrés from epidermal explant culture were CD1a⁺, however
the LC population had been enriched using lymphoprep prior to cytospin (Prignano et al., 1998). A more recent study using human epidermal explants demonstrated a similar proportion of CD1a+ cells in the emigrant population via flow cytometry (without enrichment) (de Gruijl et al., 2006). In contrast Jacobs et al reported that only 6% of the émigrés from full thickness skin explants expressed CD1a (measured via flow cytometry). In this latter study the presence of the dermis may have significantly altered the results (Jacobs et al., 2006). The non-DC population is thought to represent cellular debris, and keratinocytes that have not actively migrated from explants (Bond et al., 2009).

When langerin is used to identify LCs within the emigrant population, the cells are fixed and permeabilised therefore it is not possible to gate out non-viable cells using PI. However, Stoitzner et al, using flow cytometry, showed that a high percentage (>70%) of the cells that migrated from murine epidermal explants expressed langerin (Stoitzner et al., 2003). It may be that differences in the techniques used to harvest and identify émigrés (for example use of EDTA to detach adherent cells from plastic culture dishes, and numbers of cells acquired and positions of gates on the flow cytometer) could account for the different results observed.

5.7.8 Conclusions

These investigations have demonstrated that LC mobilisation is impaired in aged skin. The absence of spontaneous LC migration from epidermal explants prepared from old individuals has not been reported previously. The effect of IL-1β on LC migration from explants in the elderly suggests that local factors governing IL-1β availability within aged epidermis may at least in part explain this phenomenon.

5.7.9 Limitations of the Study

There are several limitations associated with this study. Firstly, the LC counts were performed manually and although the epidermal sheets were always blinded in terms of whether the sheet represented a baseline (T0) or cultured sample, it was not always possible to blind between young and old volunteers. There is evidence to suggest that the use of image analysis software to enumerate LCs gives more reproducible results, and this should be considered for future experiments (Jacobs et al., 2001).
alternative would be to have the counts verified independently by a second blinded assessor.

It is possible that following separation of epidermis from dermis using EDTA some dermal cells (including dermal DCs) remained. Although this is unlikely to have altered the overall results of the study in terms of differences between the young and the old age groups, a small number of émigrés may have represented dermal DCs. The low yield of émigrés from the 6mm explants was also a limiting factor in these investigations. It was presumed that the reduction in LCs in epidermal explants following culture represented the percentage of LCs that had migrated, however it was not possible to corroborate this in terms of exact numbers of CD1a\(^+\) or langerin\(^+\) émigrés. As mentioned earlier, previous studies have used larger pieces of skin or have pooled samples, however this is not feasible when targeting particular age groups for study.

Finally, in some experiments only a small number of volunteers were recruited: in particular the investigation of the kinetics of migration from explants prepared from young volunteers, and the effect of cytokines on LC migration at 2 hours. In these experiments it is possible that an increase in the number of volunteers would have yielded different results.

### 5.7.10 Future Work

It would be of interest to perform similar experiments in whole skin explants to observe whether the difference in LC migration between the young and old groups is maintained in the presence of dermal factors. However, based on \textit{in vivo} observations it seems likely that LC migration would be still be impaired in the old group. The results of the current investigations point towards epidermal environmental factors as playing a major role in the age related observations. To investigate this further keratinocyte cultures could be prepared using skin from young and old individuals, and the culture supernatant harvested and used to culture explants. It would then be possible to determine whether the factors secreted from keratinocyte cultures of young individuals could restore LC migration from explants of old individuals. Conversely the supernatants from keratinocyte cultures derived from old skin could be used to culture epidermal explants from young volunteers to see if there was any inhibition of LC migration. If the keratinocyte media did have an effect, different
experiments including mass spectrometry and cytokine bead array, could be performed to try and identify possible mediators. It would also be interesting to measure the levels of IL-1β mRNA and protein in the epidermal explants following the 24 hour culture, as although there are no differences between the age groups in unstimulated skin (as discussed in chapter 4), it may be that in old skin there is an inability to up-regulate IL-1β production upon stimulation.

A recent study by Zhao et al has shown that respiratory DC migration is impaired in aged mice due to increased levels of PGD2, and that migration could be restored using a PGD2 antagonist (Zhao et al., 2011). Intriguingly this prostaglandin can inhibit LC migration (Angeli et al., 2001); furthermore an agonist for one of the PGD2 receptors (D prostanoid receptor) has been shown to inhibit production of IL-1β by DCs in vitro (Yamamoto et al., 2011). Future experiments could therefore be performed to ascertain whether addition of a PGD2 antagonist to the explant culture media could restore LC migration in the aged.
6. The Effect of Topical All-trans Retinoic Acid on Langerhans’ Cell Migration in an Epidermal Explant Model

There is evidence to suggest that all-trans RA can affect both LC function and IL-1β production in the skin (Elder et al., 1991; Gruaz et al., 1990; Meunier et al., 1994). Furthermore, it has been proposed that there is a relative reduction in the availability of retinoids in the skin of aged individuals (Watson et al., 2004). Experiments were therefore performed to assess whether topical application of all-trans RA under occlusion for 4 days could restore LC migration from epidermal explants prepared from aged individuals.

6.1 Baseline Langerhans’ Cell Counts Following Application of All-trans Retinoic Acid Under Occlusion

To determine whether application of all-trans RA, (0.025% Retin-A cream) affected LC counts in young and aged skin, epidermal sheets were prepared from 4 young (mean age 22.3 ± 1.6 years, 1 male and 3 female) and 7 old individuals (mean age 75.9 ± 0.8 years, 2 male and 5 female) following 4 days of RA or control cream under occlusion (Figure 6.1). No erythema or skin changes were noted in any individual following either topical treatment. There were no significant differences between the RA treated and control cream treated skin in terms of LC number in both age groups. The baseline LC counts (T0) were significantly lower in the old age group (p<0.01, analysed using 2-way ANOVA). Due to the limited number of biopsies available an untreated/ unoccluded baseline LC count could not be performed, however, the mean counts were comparable with the baseline LC (T0) counts from young and old individuals shown in chapter 5 (Figure 6.2). No differences in the size or morphology of the LCs were apparent following treatment with RA (Figure 6.3). Five of the seven old volunteers were taking prescribed medication including lipid lowering therapies and antihypertensive agents. No obvious differences were detected between the volunteers taking medication and those who were not, however separate subgroup analyses could not be performed due to the small numbers.
Figure 6.1 Langerhans’ cell (LC) counts in epidermal sheets prepared following 4 days of either all-trans topical retinoic acid (RA T0) or control cream (control T0) under occlusion (n=4 young group, n=7 old group). Data shown as mean ± SEM, ** p<0.01.

Figure 6.2 Langerhans’ cell (LC) counts in epidermal sheets prepared from young and old individuals at baseline (T0, n=16 young group, n=14 old group) and following 4 days of either all-trans topical retinoic acid (RA T0, n=4 young group, n=7 old group) or control cream (control T0, n=4 young group, n=7 old group) under occlusion.
Figure 6.3 Representative photomicrographs of epidermal sheets from a young and an old volunteer stained with CD1a, following 4 days of either all-trans topical retinoic acid (RA) or control cream under occlusion. Scale bar = 50μm.

6.2 Langerhans’ Cell Migration from Epidermal Explants Prepared Following Application of All-trans Retinoic Acid under Occlusion

To investigate whether topical RA could enhance LC migration, epidermal explants were prepared from the skin of young and old volunteers following 4 days of topical exposure to RA or control cream under occlusion. The explants were then cultured for 24 hours and the percentage LC migration calculated as before. Langerhans’ cell migration occurred from explants prepared from young volunteers following both RA and control cream under occlusion. There was no difference between the two treatments in terms of percentage LC migration (Figure 6.4). Furthermore, the level of migration was similar to the spontaneous migration recorded in untreated explants as presented in the previous chapter (16.6%). There was significantly less migration observed from the explants prepared from old individuals following treatment with
RA or control cream (topical RA mean 5.7 ± 2.3 % LC migration compared with control cream 4.4 ± 1.5%, p<0.05 analysed using 2-way ANOVA, Figure 6.4). Although the mean percentage LC migration in the old group following either treatment was slightly higher than the spontaneous migration presented previously (2.1%), the difference was not significant, and there was no difference between the effects of the RA and the control cream in the old group. Again, no changes in the size or morphology of the LCs were apparent in cultured explants following treatment with RA compared with control cream (Figure 6.5).

![Graph showing Langerhans' cell migration from epidermal sheets prepared from young (n=4) and old individuals (n=7) following 4 days of either all-trans topical retinoic acid (RA) or control cream under occlusion. Data shown as mean ± SEM, * p<0.05, ** p<0.01.](image)

**Figure 6.4** Langerhans' cell migration from epidermal sheets prepared from young (n=4) and old individuals (n=7) following 4 days of either all-trans topical retinoic acid (RA) or control cream under occlusion. Data shown as mean ± SEM, * p<0.05, ** p<0.01.
Figure 6.5 Representative photomicrographs of epidermal sheets from a young and an old volunteer stained with CD1a, after 4 days of either all-trans topical retinoic acid (RA) or control cream under occlusion followed by explant preparation and 24 hour culture. Scale bar = 50μm.

6.3 Measurement of Interleukin-1 Receptor Antagonist Secretion in Explant Culture Supernatants

As described earlier, the levels of IL-1β in the explant culture supernatants were below the limit of detection of the ELISA used. However, it was possible to detect IL-1Ra in the culture supernatants using ELISA. The levels of IL-1Ra secreted from explants prepared from young volunteers (n=4) were significantly higher than those secreted from explants prepared from old volunteers (n=5) following both application of RA and control cream (p<0.05 analysis performed using 2-way ANOVA, Figure 6.6). There was no difference in IL-1Ra secretion following 4 days of RA compared with the control cream in either age group. The levels of IL-1Ra were also similar in both age groups to that secreted from explants prepared from untreated skin (described in chapter 4).
**Figure 6.6** Levels of IL-1Ra in epidermal explant culture supernatants as measured by enzyme linked immunosorbent assay. Epidermal explants were prepared from young and old individuals following 4 days of either all-trans topical retinoic acid (RA) or control cream under occlusion. Data shown as mean ± SEM, * p<0.05.

### 6.4 Discussion

All-trans RA has been shown previously to increase both keratinocyte production of IL-1β, and LC function in terms of antigen presentation and subsequent T cell proliferation (Baron et al., 2005; Geissmann et al., 2003; Meunier et al., 1994). On this basis the epidermal explant model was used to study the effects of topical all-trans RA on LC migration in aged skin. Application of all-trans RA under occlusion for 4 days did not affect LC number compared with a control cream, nor did it restore LC migration from explants prepared from old individuals.

#### 6.4.1 Volunteer Selection

As discussed in earlier chapters, the old volunteers were all living independently and were able to travel to and from the hospital. There were no apparent differences in terms of LC counts and migration between those who were taking regular prescribed medication and those who were not, although subgroup analyses were not performed due to the small numbers involved.
6.4.2 The effect of All-trans Retinoic Acid under Occlusion on Baseline Langerhans’ Cell Counts

As shown previously the LC counts were significantly reduced in epidermal sheets prepared from old compared with young volunteers. Due to the limited numbers of biopsies that could be taken from each volunteer there was no unoccluded control, and therefore the baseline LC counts were either from all-trans RA or control cream treated skin. In these investigations aqueous cream was used as a control cream rather than a vehicle cream, since a specific vehicle cream was not available. It should be noted that aqueous cream can affect epidermal barrier function (Mohammed et al., 2011), however, it is unlikely that the aqueous cream affected LC numbers in the current study, as the LC counts in the aqueous and RA cream treated sites in both age groups did not differ from those reported in untreated skin in chapter 5.

There is evidence to suggest that mechanical stimulation alone can induce LC migration (Holzmann et al., 2004; Ruutu et al., 2011). In one study, application of a flat metal plate to murine ear skin provoked LC migration (Ruutu et al., 2011). It is possible, therefore, that in the current investigations the pressure of the Finn chamber which was used to occlude the topical preparations could have provoked LC migration. Again, the similarity between baseline LC numbers presented previously and those in the cream treated T0 epidermal sheets suggests that this did not occur.

Previous reports have shown that RA can affect LC numbers, although this appears to be dependent upon the method of application and the duration of treatment. Fisher et al reported that a single 4 day application of 0.1% all-trans RA under occlusion caused a slight reduction in the number of LCs compared to a vehicle cream (Fisher et al., 1991). Of note all subjects had visible erythema at the site of RA application compared with no reaction in the control treated skin. It is recognised that exposure to topical irritants can provoke LC migration (Brand et al., 1993; Ouwehand et al., 2011), and it may be in the aforementioned study that RA had an irritant effect which was sufficient to induce LC migration. Consistent with the findings of the current investigation, Meunier et al found no change in LC numbers following 4 applications of 0.1% all-trans RA on consecutive days under a semi-occlusive wrap (Meunier et al., 1994). As in the current study there were no visible reactions to either the RA or control cream, although LCs from RA treated skin had an increased ability to induce
proliferation of alloreactive T cells, suggesting that topical RA can have a direct effect upon LCs even without the presence of a visible cutaneous reaction. It is important to note that the concentration of RA in the topical preparation in the current study (0.025%) was lower than that used in these other reports. Previous research has shown that 0.025% all-trans RA can induce changes within the epidermis, including an increase in epidermal thickness, following 4 days of treatment under occlusion (Griffiths et al., 1993). In the current study there were also no apparent differences in LC morphology following treatment with RA in either age group. Studies of short-term application of topical RA have not commented on the appearance of LCs pre- and post-treatment, and although not directly comparable, Koulu and Jansen reported that after 4-5 weeks of systemic retinoid therapy for various skin diseases, including psoriasis, there was no change in LC morphology (Koulu and Jansen, 1982). However, in a more recent study, 6-12 months of systemic retinoid therapy in renal transplant recipients was associated with an increased length of LC dendrites within the epidermis of both photo-exposed and photo-protected skin, suggesting that prolonged treatment with RA can affect LC morphology (Carneiro et al., 2005).

6.4.3 The effect of All-trans Retinoic Acid under Occlusion on Langerhans’ Cell Migration

A 4-day application of all-trans RA under occlusion did not enhance LC migration from cultured epidermal explants prepared from young or old volunteers. As discussed in the previous chapter, spontaneous LC migration occurred from the explants of all young volunteers. The percentage of spontaneous LC migration was equivalent to that observed following either RA or control cream suggesting that neither the RA nor occlusion process had any additional effect. Studies in mice have shown that all-trans RA can increase the migratory response of in vitro matured bone marrow derived DCs and promote LC migration (when applied topically), although the latter observation could have been due to an irritant effect (Darmanin et al., 2007; Manickasingham et al., 1996). As discussed in the previous chapter, it is likely that the spontaneous migration that occurred following the explant preparation in the young group represented the proportion of LCs primed to migrate, and therefore further stimulation with RA did not enhance LC migration.
Apart from improving the clinical signs of photo- and intrinsically aged skin (Griffiths et al., 1995; Kafi et al., 2007), RA has also been shown to increase levels of IL1B mRNA in cultured keratinocytes and to increase IL-1 release from cultured gingival explants (Baron et al., 2005; Walsh et al., 1985). Since LC migration can be at least partially restored in aged individuals by exogenous IL-1β, it was hypothesised that topical all-trans RA could potentially affect LC migration in this age group. There are several possible explanations for the lack of response observed in the current study. Firstly, the RA was applied topically rather than added to culture media: in one previous study daily application of topical all-trans RA (0.1% compared with 0.025% in the current study) under occlusion over a 4-day period led to an increase in epidermal IL-1β levels. It should be noted that there was also a increase in IL-1β levels within the skin treated with the vehicle under occlusion, which was not significantly different from that observed with RA (Gruaz et al., 1990). Secondly, it may be that there is a general failure of IL-1β up-regulation in the epidermis of older individuals in response to stimulation.

6.4.4 Interleukin-1 Receptor Antagonist Levels in Explant Culture Supernatants

Since IL-1β was not detectable in the explant supernatants, it was not possible to conclude whether the RA induced IL-1β production which could theoretically have promoted LC migration within the old age group. It would be interesting to measure IL-1β mRNA and protein levels in RA treated epidermis following the 24 hour culture in both the young and old age groups to identify whether the topical treatment has any effect compared to the control.

IL-1Ra levels were reduced in the explant supernatants from aged volunteers. There was no significant difference in either age group between the levels of IL-1Ra following treatment with all-trans RA or control cream. There was a trend towards an increased level of IL-1Ra secretion from explants from young volunteers following treatment with RA: particularly when compared with IL-1Ra secretion from untreated or cytokine treated explants as described in chapter 4. Further experiments within the same individuals would be required to confirm whether this is a true effect. As discussed earlier, the levels of IL-1Ra in the explant supernatants may be a surrogate marker of a reduced level of active IL-1β in the aged epidermis, although if explants
from aged volunteers have relatively fewer LCs and keratinocytes it may be that IL-1Ra production is equivalent on a per cell basis.

Some studies have proposed that the effects of RA on DCs in terms of enhanced antigen presentation could be exploited to boost responses to vaccinations or cancer treatments (Darmanin et al., 2007; Geissmann et al., 2003); however if DCs from older individuals fail to migrate an alternative or additional adjuvant would be required for RA to exert this beneficial effect.

6.4.5 Limitations

The main limitation of the current investigation was that, based on the experiments performed, it was not possible to confirm that the all-trans RA had any biological effect upon the epidermis and more specifically the LCs. Although previous studies using the same formulation of RA and the same 4-day protocol have shown that the RA induces changes within the epidermis (Griffiths et al., 1993), it would have been preferable to replicate these findings within the current study.

A further drawback was that due to the limited number of biopsies available from each subject, it was not possible to compare the baseline (untreated) LC numbers with those in the aqueous cream and RA treated skin. Whilst the LC counts were similar to those presented in the previous chapter, it is possible that the occlusion process could have had an effect. Furthermore, aqueous cream was used as a control cream rather than a specifically formulated vehicle cream which would have been optimal.

As mentioned earlier, previous studies have used topical preparations containing higher concentrations of all-trans RA. It is possible that a higher concentration of RA or prolonged application would have affected LC migration; however at higher concentrations it is likely that the irritancy of the preparation would have confounded the results. Finally the number of subjects in each age group was relatively low and it is possible that a larger sample size would have yielded different results.

6.4.6 Future Work

It would be interesting to ascertain whether addition of all-trans RA to the explant culture media could affect LC migration, since as discussed earlier RA can affect IL-1β synthesis and release from cultured keratinocytes and gingival explants (Baron et
al., 2005; Walsh et al., 1985). As well as using epidermal explants to compare LC counts before and after explant culture in the presence of RA, epidermal cell suspensions could be prepared, and markers of LC maturation and activation measured using flow cytometry. Epidermal IL-1β levels could also be measured after the culture period using PCR and ELISA.
7. General Discussion

7.1 Summary of Research Findings

Initial experiments were performed to determine whether there was any age related defect in the ability of circulating monocyte precursors to differentiate into functional LCs in vitro. The culture yield was equivalent in both age groups suggesting that monocytes from aged donors could differentiate into LCs if the necessary growth factors and cytokines were available. Monocyte derived LCs from old individuals were equivalent to those from young individuals in terms of expression of markers of maturation, and cytokine production upon stimulation with LPS, TNF-α and IL-1β. However, experiments to study the migratory response of stimulated MoLCs, revealed that stimulation with TNF-α promoted migration towards a chemokine ligand in the young, but not the old, group. These findings were consistent with those reported in vivo in both man and mouse, where intradermal injection of IL-1β, but not TNF-α, stimulated LC migration in the aged (Bhushan et al., 2002; Bhushan et al., 2004; Cumberbatch et al., 2002).

These data suggest that the defect observed in LC migration in the aged was not due to abnormalities in LCs themselves, but rather as a result of local environmental factors, such as the availability of cytokines required for migration. It was, however, important to confirm whether there were any differences in the phenotype of the LCs within the epidermis of older individuals, particularly since cultured cells are not necessarily equivalent to cells in vivo. Expression of MHC-class II and CD86 was equivalent in the young and old, again suggesting that the LCs have a similar phenotype, although it is possible that examination of further markers would have demonstrated a difference.

As discussed earlier, one key environmental factor involved in LC mobilisation is IL-1β, and based on previous work identifying restoration of LC migration in the aged with IL-1β, investigations were carried out to identify any differences in IL-1β availability or IL-1 receptor expression in the epidermis of aged individuals. In unstimulated skin, levels of IL-1β mRNA and protein were equivalent in the young and old. There were also no differences in the levels of caspase-1 mRNA or protein (the enzyme responsible for the conversion of pro-IL-1β into the active form). Interestingly, there is evidence available from the investigations of others that in aged mice reduced caspase-1, and consequently IL-1β, production in DCs is responsible for
a reduced response to mouse adapted influenza (Stout-Delgado et al., 2012). Interleukin-1β mRNA expression was preserved in cells from aged mice, however upon infection the aged mice could not up-regulate IL-1β protein production. Further experiments are required to assess whether this is also the case in aged human skin, possibly using cultured epidermal explants as a source of stimulated skin. There were no age-associated alterations in epidermal expression of IL-1RI or IL-1RII to account for a potential defect in IL-1β signalling in aged skin. Notably, there was a reduction in the levels of IL-1Ra in the epidermal explant culture supernatants from the old group. This may be a reflection of a relative reduction in IL-1β production and release during the culture process in the old group, however, this could not be confirmed as the levels of IL-1β in the culture supernatants were below the limit of detection of the ELISA used.

An epidermal explant model was used to study ex vivo the effect of age on LC migration. Consistent with previous studies, numbers of LCs were significantly reduced in photoprotected skin of aged individuals. Langerhans’ cells migrated from explants prepared from skin of all young individuals; in contrast there was little or no migration from those of aged individuals. Addition of TNF-α to the explant culture medium failed to promote migration in the elderly, however, there was at least a partial restoration of migration following addition of IL-1β. This was in keeping with in vivo studies which identified impaired LC migration in aged individuals following intradermal injection of TNF-α, but not IL-1β (Bhushan et al., 2002; Bhushan et al., 2004). The correlation between experiments using explants and those performed in vivo, suggest that the explant model could be exploited further in future studies; in particular for assessing ways of restoring migration in the aged, which may confer health benefits.

Preliminary experiments were performed to see whether application of topical all-trans RA for 96 hours under occlusion could enhance LC migration from epidermal explants. The treatment did not affect LC numbers or migration in either age group. In the future, it may however, be possible to exploit the same methods to determine whether other topical therapies, for example TLR agonists, could restore LC migration in the aged.

Consistent with the reduced number of LCs in epidermal sheets prepared from aged individuals, immunofluorescence analyses of sections prepared from unstimulated skin also revealed a reduced number of cells co-expressing CD1a and langerin in the
dermis. Although a population of langerin\(^+\) dermal DCs has been identified in mice (Bursch et al., 2007), to date no human equivalent has been found (Eisenwort et al., 2011; Klechevsky et al., 2008). Presumably, therefore, the CD1a\(^+\)/langerin\(^+\) cells seen within the dermis were migrating LCs. This suggests that in the elderly, LC migration is impaired both in the steady state, and following stimulation, which has consequences for the development of appropriate self-tolerance, in addition to responses to infectious challenge. To confirm this finding a larger sample number would be required.

Initial experiments were also performed to identify CD1a\(^+\) dermal DCs in skin sections from both young and old individuals. There were relatively few CD1a\(^+\) dermal DCs, and in order to compare the numbers between the age groups more accurately, multiple sections would need to be examined using a standardised counting method, such as the number of dermal DCs per unit epidermal length.

### 7.2 Future Directions

#### 7.2.1 Langerhans’ Cell Numbers and Exposure to Ultraviolet Radiation

One of the key areas for future investigation is to determine why there is a reduction in LC numbers with age, and whether it is possible to restore LC numbers. It is possible that the decline in LC number compounds the functional decrement, particularly in terms of interaction with epidermal resident T cells (Seneschal et al., 2012), and thus restoration of number may provide additional health benefit. The findings of the current research suggest that the reduction is not due to excess migration from the skin, as even in unstimulated skin there appeared to be fewer LCs present in the dermis in the old individuals. There is evidence to suggest that LCs are derived from local precursors under steady state conditions (Merad et al., 2002). Larregina et al identified a population of CD14\(^+\) dermal DCs that migrated from human skin explants and co-expressed langerin. These cells were CD1la\(^-\), but differentiated into CD1a\(^+\) LCs containing Birbeck granules after culture with TGF-\(\beta\) (Larregina et al., 2001). More research is needed to compare the numbers of these potential precursor cells in the dermis of young and old individuals. Furthermore, factors within the epidermal environment are likely to be important. For example, the availability of TGF-\(\beta\), or keratinocyte expression of CCL20, the latter having been
shown to attract LC precursors derived from circulating CD34+ cells (Dieu-Nosjean et al., 2000). Keratinocytes also produce RANKL which has been shown to promote LC survival (Barbaroux et al., 2008), and since the number of keratinocytes is also reduced in aged skin (Gilhar et al., 2004; Marks, 1981), this may have a direct impact on the LC number. Future experiments may be conducted to compare the expression of key factors such as CCL20 and RANKL in young and old skin. Another important consideration is that LCs can undergo mitosis (Czernielewski et al., 1985; Hemmerling et al., 2011), and it would be interesting to compare LC mitosis in young and old skin by dual staining epidermal sheets with langerin or CD1a and Ki67.

In the current study all experiments were performed on sun protected skin, so as to determine the effect of age alone (rather than UV radiation) on LCs. Previous work has shown that the age-associated reduction in LC number is more marked in sun exposed skin. Notably in young individuals the density of LCs appears to be consistent across sun protected and sun exposed skin (Thiers et al., 1984). Ultraviolet radiation has an immunosuppressive effect, such that exposure to UVB prior to topical application of a contact allergen reduces the contact hypersensitivity response (Cooper et al., 1992). Recent studies have shown that LCs play a key role in UV induced immunosuppression. Fukunaga et al showed that UVB exposure promoted LC migration in mice, which corresponded with a reduced ear swelling response to a contact allergen. Depletion of LCs prior to UVB exposure resulted in a normal contact hypersensitivity response (Fukunaga et al., 2010). Following UVB exposure, LCs appeared to co-localize with NKT cells in the lymph node. Subsequent experiments demonstrated that induction of NKT cells was required for the immunosuppressive effect. Similarly, Schwarz et al showed that LCs were required for UVB induced immunosuppression (Schwarz et al., 2010). When splenocytes and lymph node cells from UVB exposed mice were injected into naive mice, the contact hypersensitivity response was suppressed. The authors proposed that this was due to LC dependent production of a subset of UV induced Tregs. The effect of age on this process has not been studied, however Gilchrest et al showed that LC migration occurred in both young and old subjects following exposure of sun protected buttck skin to UV radiation. The migratory response was slower in the old group (Gilchrest et al., 1982). If LCs from old individuals are able to migrate in response to UV, as suggested above, the mechanism by which this occurs may provide an insight into potential mechanisms to restore LC migration in the aged under other physiological conditions.
7.2.2 Cytokines Involved in Langerhans’ Cell Migration

As mentioned earlier, the experiments performed did not address the effect of stimulation (for example, exposure to an infectious microorganism), upon IL-1β levels in aged skin. It may be that with age there is a failure to up-regulate IL-1β production and/or release when required, which could have important consequences for clinical interventions that target cutaneous DCs. Further experiments are therefore required, perhaps using cultured explants as a source of stimulated skin.

It is important to consider that other cytokines have a role in regulating LC migration and function, and could potentially be altered with age. Interleukin-10 and TGF-β have both been identified as negative regulators of inflammation. In a recent study in man both cytokines were shown to reduce LC induced CD8+ T cell proliferation (Banchereau et al., 2012). Furthermore, a population of CD14+/CD141+ dermal DCs has been identified in human skin that constitutively secretes IL-10 and expands Tregs that promote self tolerance (Chu et al., 2012). Stary et al have shown that a short course of oral corticosteroids in nickel allergic patients resulted in a reduced clinical response to a nickel patch test due to increased TGF-β production which resulted in expansion of Tregs (Stary et al., 2011). Notably, a combination of neutralising TGF-β and IL-10 antibodies inhibited both the increase in Treg numbers and their inhibitory function. In murine epidermis IL-10 has been shown to reduce IL-1β and TNF-α production and suppress the contact hypersensitivity response (Enk et al., 1994; Wang et al., 1999).

Interleukin-18 can induce LC migration in mice, and interestingly this effect can be blocked by neutralising anti-TNF-α or IL-1RI antibodies (Cumberbatch et al., 2001). Furthermore, IL-18 knockout mice have been shown to have a reduced clinical response to a contact allergen, although LC migration to a contact irritant, or administration of IL-1β or TNF-α, appears intact suggesting that LC migration can occur in the absence of IL-18 (Antonopoulos et al., 2008). In humans, cultured keratinocytes derived from neonatal foreskin have been shown to express IL18 mRNA (Mee et al., 2000), and keratinocytes cultured from healthy adult skin express the IL-18 receptor (Wittmann et al., 2005). Interestingly, Wittmann et al also showed that IL-18 induced keratinocyte production of CXCL10, which has been shown to have a role in chemoattraction of T cells to sites of inflammation. It would be interesting to study the effect of age on levels of cytokines, such as IL-18 and IL-10,
in both unstimulated and stimulated skin. The epidermal explant model could also be exploited to assess whether addition of IL-18, or neutralisation of IL-10, could promote LC migration in aged skin.

7.2.3 Restoration of Langerhans’ Cell Migration

Future investigations should include attempts at restoring LC migration in the aged. The results of the current study suggest that environmental factors, such as availability of key cytokines, are important. Aside from IL-1β, another potential candidate is PGD2, which has recently been found to cause impaired respiratory DC migration in aged mice (Zhao et al., 2011). Prostaglandin D2 was present at higher levels in aged mice, and DC migration could be restored with a PGD2 antagonist. It would be interesting to ascertain whether the PGD2 antagonist has any effect on LC migration in aged skin, particularly since there is evidence to suggest PGD2 can prevent LC migration, and affect IL-1β production by DCs in vitro (Angeli et al., 2001; Yamamoto et al., 2011).

As discussed earlier, keratinocytes produce a number of factors important for both chemotaxis of LC precursors, and LC mobilisation and migration. It would be useful to ascertain whether factors produced by keratinocytes from young individuals could restore LC migration in the aged. Supernatants from keratinocyte cultures prepared from skin biopsies taken from young individuals could be used to culture epidermal explants from aged individuals. If LC migration was then restored in the aged, experiments could be performed to compare supernatants from keratinocyte cultures derived from young and aged individuals to identify possible mediators.

7.2.4 Functional Ability

There is an increasing body of research on the functional role of LCs. In humans, LCs have been identified as key players in the development of the T cell response, and there is evidence that LCs are more potent stimulators of cytotoxic T lymphocytes than dermal DCs (Banchereau et al., 2012; Polak et al., 2012). The current investigations have not addressed whether there are any differences between LCs from young and old individuals in terms of their immunostimulatory and immunoregulatory actions upon T cells. It cannot be assumed that LC migration
equates to the development of robust T cell responses (Haley et al., 2012), and therefore it would be useful to compare both MoLCs and skin derived LCs from young and old individuals in this respect.

### 7.2.5 Dermal Dendritic Cells

Preliminary experiments were performed to compare the dermal DC population in the young and old. A more detailed study of dermal DCs using different markers, including CD1a, langerin and CD14 is warranted. In the first instance immunofluorescence of skin sections could be pursued, especially since large tissue specimens that could be used for dermal explant preparation and harvesting of émigrés are not readily available for the older age group. If whole punch biopsy specimens were cultured, it may also be possible to study dermal DC migration in the aged.

### 7.3 Clinical Relevance of Langerhans’ Cells

There has been increasing interest in the development of DC based therapies for the treatment of human disease, in particular cancer. Dendritic cells exposed to tumour antigens can be used to promote the production of tumour specific T cells. Langerhans’ cells generated from CD34+ progenitor cells have been shown to elicit anti-tumour immunity comparable with MoDCs in a trial of vaccine therapy for patients with advanced melanoma (Romano et al., 2011). Interleukin-15 was an important factor in the development of melanoma specific CD8+ T cell reactivity. Cultured LCs produced IL-15, whereas MoDCs required exogenous IL-15 in order to induce a comparable immune response. A recent study comparing the ability of human skin explant derived LCs and dermal DCs to promote proliferation of CD8+ T cells, found that LCs induced stronger cytotoxic T lymphocyte responses due to the production of IL-15, which was not expressed by CD14+ dermal DCs (Banchereau et al., 2012).

There is also evidence to suggest that intradermal or transcutaneous vaccination strategies are at least as effective as intramuscular vaccination. Kenney et al demonstrated that intradermal injection of influenza vaccine required a lower dose of vaccine (one fifth) compared with intramuscular injection, yet provided comparable
or better immunity (Kenney et al., 2004). In addition, intradermally administered nanoparticles have been shown to cause LC migration in human skin explants (Liard et al., 2012). Langerhans’ cells were important for development of antigen specific CD8+ T cells, but were not essential for CD4+ T cell or humoral responses. Combadière et al showed that LCs were also required for the development of CD8+ antigen specific T cells in a study of transcutaneous influenza vaccination, where droplets of vaccine were applied directly to skin after cyanoacrylate skin stripping (Combadiere et al., 2010). Different cutaneous immunisation strategies may in the future allow the development of either: cytotoxic responses to treat infection or cancer, or tolerogenic responses to treat autoimmune diseases. For example, disruption of the skin barrier, followed by immunisation containing an adjuvant, such as a TLR ligand, could be used to promote a tumour specific cytotoxic T lymphocyte response (Stoitzner et al., 2010).

Another area of interest is the use of topical immunomodulatory agents to treat certain skin cancers and skin infections. Imiquimod, a TLR 7 ligand, is used in a topical formulation to treat basal cell carcinoma and viral warts. It has been shown to induce LC migration when applied to murine skin (Suzuki et al., 2000), and to increase LC mediated production and release of IL-1β, TNF-α and IL-6 (Miao et al., 2012), suggesting that LC maturation and migration may at least play a part in the therapeutic response. This would be in keeping with the findings of Fujita et al who compared LCs from cutaneous squamous cell carcinomas with those from adjacent normal skin. They found that LCs from involved skin were more mature and stimulated increased allogeneic CD4+ and CD8+ T cell proliferation compared with those from perilesional skin (Fujita et al., 2012). Langerhans’ cells cultured from CD34+ progenitors were more effective than MoDCs at inducing allogeneic T cell proliferation, when exposed to the squamous cell carcinoma supernatant.

A study in mice has demonstrated that LCs also have an important role in the prevention of allergic contact dermatitis by inducing proliferation of Tregs and promoting deletion or anergy of allergen specific CD8+ T cells (Gomez de Aguero et al., 2012). In the future these properties may be harnessed in treatments that can treat or reduce the incidence of allergic contact dermatitis; a condition which can have both a profound impact on an individual’s quality of life, and a significant socioeconomic cost. Studies of DC based therapies have not addressed the effect of age on DC function: in particular whether DCs of elderly individuals, who often have the highest
incidence of the diseases studied, can mount a comparable response to those of younger patients.

7.4 Concluding Remarks

The findings of this research confirm that LC number and mobilisation are reduced in aged skin. It remains to be seen whether LC based therapies will be successful in the elderly. Based on the findings of the current investigations, it is possible that LCs cultured from peripheral blood precursors of elderly individuals would be equivalent to their young counterparts in terms of phenotype and cytokine production. It may be, however, that cultured LCs, topical immunomodulatory therapies, or intra dermal vaccinations, would require an adjuvant to promote LC mobilisation in the aged. One obvious candidate would be IL-1β, which has been shown previously, and in the current investigations, to at least partially restore LC mobilisation (Bhushan et al., 2004). Future studies should focus on restoring LC number and function in the aged, such that this age group will be able to benefit from therapeutic advances which are based on LC activation.
8. References


measurements in tumor tissues: comparison of 13 endogenous control genes. 


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Sharma, S., A.L. Dominguez, and J. Lustgarten. 2006. Aging affect the anti-tumor potential of dendritic cell vaccination, but it can be overcome by co-stimulation with anti-OX40 or anti-4-1BB. Exp Gerontol. 41:78-84.


Appendix 1

Publication

Published Abstracts


Ogden. S., R.J. Dearman, I. Kimber, and C.E. Griffiths. 2010. Phenotype and function of monocyte derived dendritic cells in aged individuals. *Immunology.* 131;S1:67.

Oral Presentations
An Investigation of Langerhans’ Cell Function in Aged Skin. Manchester Medical Society, Manchester, April 2012.

**Poster Presentations**


**Prizes**

Manchester Medical Society Clinical Research Prize, 2012.