Skin sensitization: Langerhans’ cell mobilization, cytokine regulation and immunomodulation by lactoferrin.

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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**Abbreviations**

- **ACD**: allergic contact dermatitis
- **AhR**: aryl hydrocarbon receptor
- **AMP**: antimicrobial peptide
- **AOO**: acetone:olive oil
- **APC**: antigen presenting cells
- **ARE**: antioxidant response element
- **ASC**: apoptosis associated speck-like protein containing a CARD
- **ASK**: apoptosis-stimulated kinase 1
- **ATP**: adenosine triphosphate
- **bFGF**: basic fibroblast endothelial growth factor
- **BrdU**: bromodeoxyuridine
- **CD**: cluster of differentiation
- **cDNA**: complementary DNA
- **CDP**: common DC precursor
- **CDP**: common dendritic cell precursor
- **CHO**: chinese hamster ovary
- **CHS**: contact hypersensitivity
- **CLEC9**: C-type lectin domain family 9 member A
- **CMP**: common myeloid progenitor
- **DAMP**: damage associated molecular pattern
- **DAPI**: 4',6-diamidino-2-phenylindole
- **DC**: dendritic cell
- **DC-SIGN**: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
- **dDC**: dermal dendritic cell
- **DETC**: dendritic epidermal γδ T cell
- **DMF**: dimethylformamide
- **DMSO**: dimethyl sulfoxide
- **DNCB**: 2,4-dinitrochlorobenzene
- **DNFB**: 2,4-dinitrofluorobenzene
- **DNP**: dinitrophenol
- **DNTB**: dinitrothiocyanobenzene
- **DPC**: diphenylcyclopropenone
- **dpm**: disintegrations per min
- **DT**: diphtheria toxin
- **DTA**: diphtheria toxin subunit A
- **DTH**: delayed-type hypersensitivity
- **DTR**: diphtheria toxin receptor
- **ECM**: extracellular matrix
- **EDTA**: ethylenediaminetetraacetic acid
- **EGFP**: enhanced green fluorescent protein
- **ELISA**: enzyme-linked immunosorbent assay
- **EU**: endotoxin unit
- **FADD**: fas-associated death domain
- **FITC**: fluorescein isothiocyanate
- **GM-CSF**: granulocyte-macrophage colony-stimulating factor
- **GOI**: gene of interest
- **GSH**: glutathione
HA: hyaluronan
HSA: human serum albumin
HBSS: Hanks balanced salt solution
HIV: human immunodeficiency virus
HMW: high molecular weight
HPRT: hypoxanthine-guanine phosphoribosyltransferase
HSV: herpes simplex virus
HUVEC: human umbilical vein endothelial cell
ICAM-1: intercellular adhesion molecule 1
ICD: intracellular domain
ICD: irritant contact dermatitis
IFN: interferon
IGF-1: insulin-like growth factor 1
IHC: immunohistochemistry
IKK: IkB kinase
IL: interleukin
IL-1Ra: interleukin 1 receptor antagonist
JNK: c-Jun NH2-terminal kinase
K: keratin
KEAP 1: kelch-like ECH-associated protein 1
KO: knock out
LC: Langerhans’ cell
LF: lactoferrin
LFA-1: lymphocyte function-associated antigen 1
LFAmpin: lactoferrampin
LFcinB: bovine lactoferricin
LFcinH: human lactoferricin
LFR: lactoferrin receptor
LMW: low molecular weight
LN: lymph node
LNC: lymph node cell
LPS: lipopolysaccharide
LT: leukotriene
M-CSF: macrophage colony-stimulating factor
MCP-1: monocyte chemoattractant protein-1
MDP: macrophage/dendritic cell precursor
MHC: major histocompatibility complex
MIP-3α: macrophage inflammatory protein 3α
MMP: matrix metalloprotease
mRNA: messenger RNA
NGS: normal goat serum
NKT: natural killer T cell
NLR: nucleotide-binding domain leucine-rich repeat containing receptor
NO: nitric oxide
NRS: normal rabbit serum
OD: optical density
OVA: ovalbumin
Oxazolone: 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one
PAMP: pathogen associated molecular pattern
PBMC: peripheral blood mononuclear cell
Abstract

Aleksandra Metryka, University of Manchester
PhD in the Faculty of Life Sciences, 2014
Skin sensitization: Langerhans’ cell mobilization, cytokine regulation and immunomodulation by lactoferrin.

Allergic contact dermatitis is an important occupational health disease. It represents a useful experimental paradigm in which the mechanisms and characteristics of cutaneous immune responses can be investigated. This thesis has focused on the sensitization phase of contact allergy, including Langerhans’ cell (LC) migration, cytokine expression and the ability of the protein lactoferrin (LF) to modulate aspects of these processes. Lactoferrin was originally identified as an antimicrobial protein. However, it is being recognized increasingly to have immunomodulatory effects on the cells of the immune system. Migration of LC in mice and in humans is mediated via two independent cytokine signals delivered by tumour necrosis factor (TNF)-α and interleukin (IL)-1β, which were thought to derive from keratinocytes and LC, respectively. Further, topical application of LF was shown to inhibit LC migration in both man and mouse potentially through the inhibition of de novo TNF-α production.

The inhibitory effect of LF on LC mobilization induced by the contact allergen 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) has been confirmed in these investigations. Conversely, LF did not inhibit LC migration triggered by another contact allergen, 2,4-dinitrochlorobenzene (DNCB). That result prompted a comparison between oxazolone and DNCB with respect to their ability to induce LC migration and to provoke cutaneous cytokine production. It was discovered that DNCB induced LC mobilization in the absence of TNF-α signalling. Moreover, exposure to superoptimal doses of oxazolone resulted in TNF-α independent LC migration. Further experiments revealed that TNF-α independence might be mediated partially by the elevated concentration of IL-1β produced in the skin following exposure to DNCB and these superoptimal concentrations of oxazolone.

Investigations of the immunomodulatory mechanism of LF in vitro demonstrated that it did not inhibit TNF-α production by THP-1 macrophages. On the contrary, LF was shown to stimulate TNF-α and IL-8 release by THP-1 macrophages in a dose dependent manner, via endotoxin-independent and nucleolin-dependent mechanism. Subsequently, the role of LF in modulation of keratinocyte activation was investigated. Keratinocytes expressed high levels of inducible TNF-α mRNA, however, this was not modulated specifically by LF. Additional examination of the effects of LF in vivo revealed that it inhibited cutaneous IL-17 and CXCL1 mRNA expression, induced by IL-1β and IL-1α, respectively. Lactoferrin treatment did not affect oxazolone-induced lymph node (LN) cell proliferation. However, it was demonstrated to decrease IL-17 production by LN cells 24h following exposure to oxazolone, which may be important in driving the vigour and/or quality of response to the contact allergen.

Overall, these investigations have demonstrated a divergence within the family of contact allergens with regard to the requirement for TNF-α signalling for LC mobilization. It was established that when elevated concentrations of IL-1β are present LC migration can occur in the absence of TNF-α signalling. Moreover, a dual nature of LF, which can act in a stimulatory as well as inhibitory manner, was confirmed. These investigations have revealed a potential role for CXCL1 and IL-17 in the process of LC migration. Furthermore, it was shown that the inhibitory effect of LF on oxazolone induced LC migration might be mediated via its effect on IL-17.
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1 Introduction

1.1 General background

Dermatitis is inflammation of the skin, and contact dermatitis is caused by contact with a substance that causes the cutaneous inflammation. Further characterization of contact dermatitis distinguishes two forms: allergic and irritant contact dermatitis (ACD and ICD, respectively). In this work the particular focus will be on ACD. Allergic contact dermatitis is characterized by two distinct phases: symptomless sensitization and elicitation where clinical manifestations occur. During sensitization, the organism acquires the ability to respond to an allergic substance, whereas elicitation is characterized by a rapid inflammatory response to a second exposure to the same allergen (Peiser et al., 2012). Skin sensitization resulting in ACD is the most common form of immunotoxicity in humans and this thesis will focus on the mechanisms of sensitization to chemical contact allergens, its regulation by cytokines and on exploring the immunomodulatory potential of the protein lactoferrin (LF). Given that the skin is the focus of this thesis, the immunobiology of the skin and its cellular components will be considered next. Additionally, the hurdles which chemical allergens must overcome and their mechanism of action will be explored. Finally, more detailed information will be provided about LF, and its many versatile functions.

1.2 Anatomy of the skin

Human skin in adults has a total surface area of around 2m² and can constitute approximately 6% of total body weight, making it the largest organ in the body. Its role is to protect the organism from mechanical, chemical and microbial insults, prevent dehydration, maintain temperature, and to serve as a sensory organ (Tobin, 2006). The skin executes these tasks through its composition that combines durability and a finely tuned network of nervous and immune systems (Kanitakis, 2002). Mammalian skin has a stratified structure that is divided into epidermis, dermis and hypodermis. The epidermis is comprised of four layers (from the bottom): basal, spinous, granular and cornified or horny. The most abundant cells of the epidermis are keratinocytes. They originate from the basal layer. Basal keratinocytes divide, and undergo morphological
changes, in a continuous process called keratinization, while migrating through the spinous and granular layers until they reach the last epidermal sheet known also as stratum corneum (from Latin corneum=’horny’). At that point keratinocytes have lost all of their cytoplasmic organelles but are still biochemically active and are called corneocytes. They are flattened and mainly composed of keratin (K) and lipids. Keratin, a fibrous protein, from the intermediate filament family, is the main component of the keratinocyte cytoskeleton (Kanitakis, 2002). The mouse epidermis is home to two important immune cell types, Langerhans’ cells (LC) and dendritic γδ T cells (DETC) (Romani et al., 1985), whereas the human epidermis contains LC, αβ and γδ T cells (Bos et al., 1990; Dupuy et al., 1990). The non-cellular basal membrane divides the epidermis from the dermis. Fibroblasts are the predominate cell in the dermis. They produce and degrade components of extracellular matrix (ECM), namely collagen that comprises 90% of dermal proteins (Rook, 2010). Similarly, there are versatile immune cells present in the dermis during steady state, such as various dendritic cell (DC) populations, γδ T cells and mast cells. Thus, skin is the home to a myriad of immune cells that participate in both the innate and adaptive immune responses (Nestle et al., 2009). Their role in the allergic inflammation of the skin will be considered in subsequent sections.

1.3 Contact dermatitis

As described above, the skin is an important immune organ and is responsible for providing protection against infection and malignant disease. However, there are certain circumstances in which an inappropriate specific immune response develops in the skin to relatively benign materials, for instance chemicals. This is known as contact hypersensitivity (CHS) or allergy. The events induced by contact allergens are part of the normal response of the immune system, which is designed to eliminate invading pathogens. Chemical allergens are haptens with low molecular weight (LMW) (<500Da), which are not large enough to be recognized as foreign by the immune system, unless associated with a carrier protein, which subsequently triggers a chain of events in the skin and in the local lymph node (LN). This ultimately results in the acquisition of sensitization (Kaplan et al., 2012) and will be described in detail in the following sections.
Exposure to chemical allergens either in the home or at work (occupational exposure) can cause allergy or hypersensitivity reactions, either skin sensitization or sensitization of the respiratory tract. Inflammation of the skin is relatively prevalent and of particular importance as a work related health issue. In the UK in the period 2006-2012 there were an estimated 30,000 new diagnoses of contact dermatitis per year. The occupations with highest risk of occurrence of ACD are florists, hairdressers, cooks, beauticians and metal workers (HSE, 2013). It is estimated that 15-20 % of the European population suffers from hypersensitivity to at least one sensitizing chemical (Peiser et al., 2012). Clinical manifestations of ACD and ICD are very similar and include presence of rash, itchiness, erythema and oedema at the sight of exposure. Dermatitis can occur anywhere on the body but the hands are most often affected (emedicinehealth, 2014; Thyssen et al., 2007). Due to its high prevalence and the impact on the work force, it is important that the mechanisms involved in the initiation and orchestration of skin sensitization and ACD are understood.

1.4 Allergens

The list of compounds capable of causing ACD in human comprises of at least 4300 chemicals (De Groot, 2008). These compounds can be from different product families, e.g. metals: nickel, chromium; ingredients of cosmetics: paraphenylenediamine (PPD) (present in hair dyes), balsam of Peru, fragrances; medications: antibiotics and steroids (Fonacier et al., 2010). Among these different groups of contact allergens there are a number of compounds that are routinely utilized in experimental investigations of CHS, these include 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone); 2,4-dinitrofluorobenzene (DNFB); 2,4-dinitrochlorobenzene (DNCB); 2,4,6-trinitrochlorobenzene (TNCB or picryl chloride); diphenylcyclopropenone (DPC). Out of these experimental allergens, data regarding human exposure are available only for DNCB and DPC. Indeed, a considerable body of data has been collected following exposure to DNCB, revealing that the proportion of individuals that will become sensitized is directly related to the application dose (Friedmann et al., 1983), depends on the area of exposure (when the area is below 1cm²) (Rees et al., 1990), and the sex of the subject, with females being more susceptible to sensitization than men (Rees et al., 1989). Human exposure data to the chemicals DNCB and DPC is available due to their use in the treatment of alopecia (El-Zawahry et al., 2010; Singh and Lavanya, 2010).
1.5 Allergic contact dermatitis

As discussed previously, the development of ACD occurs over two distinct phases: sensitization and elicitation. In the sensitization phase, a chemical with properties described before gains access to the viable skin for the first time and is sensed by skin resident DC and their broad pattern recognition receptors. Given that the appropriate inflammatory milieu is present DC then migrate from the skin to the draining LN where they present antigen to cluster of differentiation (CD)4+ and CD8+ T cells in the LN (Xu et al., 1996). Process of antigen presentation will be described in detail in the following section.

Subsequent exposure to the same contact allergen results in the elicitation phase where the clinical manifestations are observed. Effector T cells are recruited to the skin in response to hapten-mediated inflammation within 24h following exposure to contact sensitizer. The next step involves activation of hapten specific CD4+ and CD8+ T cells generated during the sensitization phase, which leads to production of interferon (IFN)-γ and interleukin (IL)-4 by infiltrating T cells. These cytokines have potent effects on keratinocytes, stimulating the expression of chemokines, such as CXCL9, CXCL10 and CXCL11, for further recruitment of T cells, neutrophils and macrophages, which contribute to the morphological changes to the skin, specific for ACD (Toebak et al., 2009; Vocanson et al., 2009).

Allergic contact dermatitis was once considered to be driven mainly by T helper (h) 1 (CD4+) cells as it is a delayed type hypersensitivity reaction (Black, 1999). T helper cells arise from CD4+ T cells upon recognition of antigen within the context of major histocompatibility complex (MHC) II molecules. Another subset of T cells, CD8+ cells, gives rise to T cytotoxic (c) cells following interaction with antigen bound to MHC I molecules. Additionally, Th or Tc cell subsets are further divided into type 1, 2 or 17 classes based on their cytokine production profile (Neefjes et al., 2011). This process will be described in more detail in the next section.

Recent studies have established that ACD reactions are in fact characterized by both CD4+ and CD8+ T cell subset activation, with the CD8+ population being the main effector cells and CD4+ subset acting as regulators of CHS. Thus, it was demonstrated
that depletion of CD4⁺ or CD8⁺ subsets prior to sensitization with DNFB or oxazolone resulted in exaggerated and reduced ear swelling following challenge, respectively (Xu et al., 1996). Indeed, sensitization with both allergens resulted in the production of IFN-γ but not IL-4 or IL-10 production by hapten specific CD8⁺ cells, while hapten specific CD4⁺ cells released IL-4 and IL-10, but little IFN-γ, suggesting a Tc1 and a Th2 phenotype of CD8⁺ and CD4⁺ populations, respectively (Xu et al., 1996). Similarly, MHC II knock out (KO) mice displayed an amplified allergic reaction (Bour et al., 1995) due to the absence of CD4⁺ subset. Conversely, when the contact allergen DNCB was utilized it was demonstrated that IFN-γ was produced by both CD4⁺ and CD8⁺ cell subsets, suggesting the presence of both Th1 and Tc1 cell populations (Dearman et al., 1996b). Therefore, it could be concluded that both CD4⁺ and CD8⁺ T cell subsets could be effectors of the ACD, depending on the inducing allergen; whilst regulation of the reaction is due to CD4⁺ T cells. Additionally, it has been shown that the cellular infiltrate during the elicitation phase was capable of IL-17 production. Subsequently, it was demonstrated that Th17 (Albanesi et al., 1999; Larsen et al., 2009) and Tc17 (Zhao et al., 2009) cells could be found in the skin of allergic individuals upon allergen exposure.

After reaching its peak, inflammation resolves via the production of IL-10, transforming growth factor (TGF)-β and prostaglandin (PG) E2. Thus, naturally occurring T cell derived regulatory (Treg) subset cells have been identified in blood of nickel sensitive individuals, as well as in healthy subjects. It was shown that secretion of IL-10 by Treg cells down regulated the nickel mediated immune response by blocking the maturation of DC, thus preventing further T cell activation (Cavani et al., 2000). In addition to Treg cells, keratinocytes and macrophages secrete IL-10, with keratinocytes also producing PGE2 and TGF-β that inhibit, respectively, cytokine production and adhesion molecule expression (Toebak et al., 2009). CD4⁺CD25⁺FoxP3⁺ T cells (Treg cells) originating from the thymus are also engaged in regulating the CHS response. Ring et al. (2006) found that injection of Treg cells prior to challenge with TNCB inhibited leukocyte adhesion and rolling via release of IL-10 (Ring et al., 2006). After resolving inflammation, the skin retains resident T cells (CD4⁺CCR10⁺) at the site of allergic reaction due to CCL27 (CCR10⁺ ligand) production by keratinocytes. This observation may explain the clinically relevant reactions of ‘flaring up’ when antigen is encountered again, e.g. via ingestion (Moed et al., 2004).
1.5.1 Mechanism of sensitization

This PhD project has focused only on the sensitization phase of the ACD in which the main events include access of the allergen to the viable epidermis and formation of the protein-hapten complexes that will interact with DC and provide them with danger signals that will facilitate their maturation and migration to the LN to present the antigen to T cells. Subsequently, specific T cells will proliferate and clonally expand thus providing memory and effector cells to cause the clinical manifestations when allergen is encountered the next time. The steps leading to efficient sensitization will be now described in detail and are illustrated in the Figure 1.1.
Figure 1.1 Schematic view of the events during the sensitization phase of ACD.

The hapten penetrates the stratum corneum, reaches the viable epidermis and triggers release of inflammatory mediators such as IL-18, IL-1β, tumour necrosis factor (TNF)-α, adenosine triphosphate (ATP), prostaglandin (PG) E2, leukotriene (LT) B4, reactive oxygen species (ROS), and histamine from keratinocytes and mast cells (step 1). The inflammatory milieu stimulates activation, hapten acquisition and maturation of DC, which subsequently migrate into the LN via the afferent lymphatics (step 2). In the LN, DC present antigen to naive T cells orchestrating their differentiation into effector subsets (step 3). Adapted from (Honda et al., 2013).
1.5.1.1 Formation of the protein-hapten complexes

To sensitize the organism haptens have to be bioavailable, that is, they have to cross the stratum corneum and create complexes with proteins via a process called haptenization (Figure 1.1). The ability of sensitizers and proteins to form adducts stems from their electrophilic and nucleophilic nature, respectively, whereby the nucleophile donates an electron pair to electrophile, thereby forming a chemical bond (Gerberick et al., 2008). There are instances when a nonallergic parent compounds can become activated to become sensitizing chemicals via oxidation or interaction with phase I or II enzymes present in the skin (so called prohapten). Phase I and II enzymes include, among others, cytochrome P450 mixed-function oxidase system and acyltransferases, glutathione (GSH) S-transferases, respectively (Karlberg et al., 2008). It is still not known, however, whether the hapten-protein complexes are created with proteins on the surface and/or inside keratinocytes and/or DC or maybe with extracellular proteins.

Certain contact sensitizers have been demonstrated to induce DC maturation both in vivo (Aiba and Katz, 1990) and in vitro (Aiba et al., 1997). However, despite much being known regarding the ability of haptens to cause ACD and activate DC, their exact fate in the skin still remains largely unknown. DNCB is thought to be able to enter the cell directly and to be processed with endogenous proteins and presented through the MHC I complex (Toebak et al., 2009). Studies by Hopkins et al. (2005) have shown that when incubated with cellular and serum proteins DNCB binds preferentially to cellular proteins, reinforcing the theory of MHC I presentation. Investigations of the mode of action of PPD demonstrated that human serum albumin (HSA) was modified by PPD in vitro. Adducts of HSA and PPD were capable of stimulating proliferation of lymphocytes from individuals sensitized to PPD (Jenkinson et al., 2010). In addition to modifying non cell bound proteins, allergens have been observed to interact with cell membrane markers, e.g CD54, CD86 that induce maturation of model DC cell lines (THP-1, U937) (Ashikaga et al., 2006; Python et al., 2007); as well as result in the production of cytokines, including tumour necrosis factor (TNF)-α, IL-1β, IL-1α, IL-8 and IL-18 among others (Teunis et al., 2013; Van Och et al., 2005). In a recent study that set out to identify targets of haptenation, bromobianes, fluorescent chemical compounds known for their strong reactivity with thiol groups, were utilized. It was
found, using microscopy, that in human skin exposed \textit{ex vivo}, bromobianes were bound preferentially to basal keratinocytes (Simonsson et al., 2011). Further investigations revealed that fluorescent hapten targeted specifically K5 and K14 (Simonsson et al., 2011). Overall, the receptors and direct modes of action of the majority of contact allergens remain unknown.

1.5.1.2 Danger signals

Creation of hapten-protein complexes is only the first step of allergen interactions within the skin and with cutaneous antigen presenting cells (APC), i.e. various DC populations. In order for sensitization to take place there has to be present an environment of inflammation to insure that the immune response is not mounted in the absence of danger. Therefore, it has been speculated that in addition to antigen signal, there is a requirement for a non-specific inflammatory signal to stimulate DC mobilization and lymph node cell (LNC) proliferation. Such non-specific inflammation was given the term “danger signal”. The concept was suggested by Matzinger (1994), who postulated that the immune system has the ability to detect and react to the damage of the organism (Matzinger, 1994). Consequently, danger signals can be divided into two broad ranges of stimuli: pathogen associated molecular patterns (PAMP) and damage associated molecular patterns (DAMP) that are capable of triggering immune responses and subsequently facilitating maturation of DC. In the context of ACD the presence of danger signals is crucial, as maturation and migration of DC is a pivotal step in ensuring sensitization to allergens, and this will be expanded on in the next section.

One group of receptors capable of recognizing danger signals are toll-like receptors (TLR). Indeed, it has been shown recently that in humans one of the common sensitizing metals, nickel, utilized the TLR4 receptor, which is best known for its ability to recognize a component of Gram negative bacterial cell walls, lipopolysaccharide (LPS) (Trinchieri and Sher, 2007). Schmidt et al. (2010) confirmed the interaction of nickel with histidine amino acids within TLR4 and showed that this contributes to the development of ACD in humans, but not in mice (as mice do not have corresponding histidine residues), supplying a direct inflammatory signal to DC and triggering their activation (Schmidt et al., 2010). Conversely, Klekotka et al. (2010) demonstrated that
the CHS reaction to DNFB was apparently independent of signals from TLR 2, 3, 4, 6, and 9 as Toll/IL-1R-containing adaptor-inducing interferon-β (TRIF) (TRIF is an accessory molecule TLR 2, 3, 4, 6 and 9) deficient mice exhibited normal levels of CHS (Klekotka et al., 2010).

Another important family of receptors are the nucleotide-binding domain leucine-rich repeat containing receptors (NLR). NLR participate in responding to PAMP such as LPS, flagellin, bacterial and viral nucleic acids, as well as DAMP, including adenosine triphosphate (ATP), uric acid and hyaluronan (HA). Following activation, the NLR forms a complex with apoptosis associated speck-like protein containing a CARD (ASC). This complex is called the inflammasome. The inflammasome plays a critical part in the activation of the protease, caspase-1, which participates in the creation of biologically active IL-1β and IL-18 from biologically inactive precursor forms (Jha and Ting, 2009).

One of the danger signals implicated in inflammasome signalling is ATP. It is present extracellularly only at low concentrations in resting conditions, which can increase dramatically when cells are stressed or dying. One of ATP receptors is P2X7, which is involved in the processing of biologically active IL-1β and IL-18 via activation of NLRP3 inflammasome. The role of P2X7 signalling in skin sensitization was revealed in the study by Weber et al. (2010). They found that mice deficient in P2X7 receptor exhibited impaired CHS responses, which were reversed by the delivery of exogenous IL-1β (Weber et al., 2010). Indeed, the importance of caspase-1 in the sensitization phase of CHS was explored and confirmed by Antonopoulos et al. (2001). They showed that DNFB and oxazolone induced CHS reactions were attenuated in caspase-1 KO mice in comparison with wild type (WT) counterparts. Similarly, topical application of caspase-1 inhibitor, YVAD, inhibited ear swelling in response to DNFB challenge. Further, no LC migration was observed in caspase-1 deficient mice in response to treatment with 0.5% DNFB or TNF-α. However, LC migration was observed when exogenous IL-1β was administered (Antonopoulos et al., 2001).

The importance of inflammasome activation is particularly apparent when considering dinitrothiocyanobenzene (DNTB), a chemical from the same family as DNFB and DNCB, that is, however, unable to initiate immune or inflammatory responses. DNTB and the dinitrohalobenzene allergens DNFB and DNCB share the same epitope
(dinitrophenol; DNP). Prior exposure to DNTB and subsequent sensitization and challenge with DNFB resulted in decreased ear swelling reactions when compared with the animals that were sensitized and challenged by treatment with DNFB alone. However, when IL-12 was supplied at the time of sensitization with DNTB, subsequent challenge with DNTB resulted in ear swelling comparable with that observed in mice that were sensitized and challenged with DNFB (Riemann et al., 2005). That phenomenon was further investigated by Watanabe and colleagues (2008) and they found that the main difference between DNTB and DNFB was their ability to induce inflammasome activation and trigger subsequent IL-1β production. Indeed, in the absence of IL-1β signalling (in IL-1 receptor (R) 1 KO mice) DNFB could provide tolerance via adoptive transfer of LN cells into WT mice. Conversely, when exogenous IL-1β was delivered during sensitization, DNTB effectively primed the immune response and maximal ear swelling was observed when animals were further challenged with DNFB (Watanabe et al., 2008). Therefore, Watanabe et al. (2008) elegantly demonstrated that a functioning inflammasome and the capacity to produce IL-1β are central to the sensitizing potential of chemicals. However, the ability to trigger IL-1β production is not sufficient to result in sensitization, as sodium lauryl sulphate (SLS) induced IL-1β production by primary keratinocytes to similar extent as DNFB, but it is known to be an irritant (Agner et al., 1989), and therefore unable to result in a specific immune response (Watanabe et al., 2008). Therefore, it is likely that in their study Riemann et al. (2008) by supplying exogenous IL-12 induced inflammasome activation and turned DNTB into an allergen (Riemann et al., 2005).

Treatment of the skin with contact sensitizers can result in damage to the skin, which in turn will yield DAMP and activate respective receptors. One of the events that can arise as a consequence of exposure to contact allergen is the creation of reactive oxygen species (ROS). Reactive oxygen species consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. Reactive oxygen species are produced in the cell under physiological conditions, as well as following interaction with xenobiotics. Cells possess the means of neutralizing ROS, however, in the instances where ROS production is greater than the rate of neutralization, oxidative stress occurs. Oxidative stress has been implicated in the mode of action of contact sensitizers and may represent one type of danger signal (Byamba et al., 2010). Thus, analysis of gene activation of allergen-stimulated keratinocytes and DC revealed the up-
regulation of genes in pathways that are in charge of oxidative stress management (Natsch and Emter, 2008). The Kelch-like ECH-associated protein 1 (Keap1)/Nrf2-signaling pathway is known to be important during oxidative stress. Keap1 is a sensor protein, rich in reactive cysteine residues. These cysteine moieties are modified via interaction with electrophilic compounds. In turn, this event releases Nrf2, which translocates to the nucleus, binds to antioxidant response element (ARE) and triggers expression of phase II detoxification enzymes (such as catalase, heme oxygenase-1, GSH S-transferase) (Dinkova-Kostova et al., 2005). A comprehensive analysis of the Nrf2 pathway was conducted by Natsch et al. (2008). They investigated a range of strong and moderate sensitizers for their ability to induce the ARE-responsive gene, quinine reductase, in a hepatic mouse cell line, Hepa1C1C7, or an activation of luciferase gene under the ARE-dependent promoter in the reporter cell line AREc32, a stable cell line derived from the human MCF7 breast carcinoma cell line. It was found that Nrf2 pathway was activated by sensitizers but not by non-sensitizing chemicals. This implies that sensitizers induce oxidative stress, and secondly that this feature could be utilized in the *in vitro* tests to distinguish between sensitizing and non-sensitizing chemicals (Natsch and Emter, 2008). Additionally, DNCB was demonstrated to increase Nrf2 accumulation via depletion of GSH and covalent modification of Keap1 in mouse liver cells (Chia et al., 2010). Moreover, the expression of Nfr2-dependent genes (*HMOX1* and *NQO1*) by both human peripheral blood mononuclear cell (PBMC) derived DC and the THP-1 cell line, has been shown to be induced specifically by treatment with a range of sensitizing chemicals, but not by non-sensitizers (Ade et al., 2009). Furthermore, prior incubation of THP-1 cells with N-acetylcysteine (a GSH precursor) inhibited DNCB induced expression of *HMOX1* and *NQO1* genes (Ade et al., 2009), suggesting a specific ability of contact sensitizers to induce oxidative stress.

Within the immune system, ROS have been shown to participate in the activation of NLRP3 inflammasome. Moreover, production of ROS was reported to be important during antigen specific interaction of DC and T cells. Thus, administration of a potent antioxidant (ebselen) to mice, either after sensitization or after challenge with oxazolone, significantly inhibited the development of CHS, measured as a function of challenge induced ear swelling responses, compared with untreated mice (Matsue et al., 2003).
Reactive oxygen species and TLR are required for the formation of, and response to, another important danger signalling molecule, HA. Hyaluronan is a component of ECM, produced mainly by dermal fibroblasts and keratinocytes. Hyaluronan is a high molecular weight glycosaminoglycan, composed of disaccharide repeats of d-glucuronic acid and d-N-acetylglucosamine, connected via alternating β-1,4 and β-1,3 glycosidic bonds (Mummert, 2005). A receptor for HA has been identified as CD44, which was demonstrated to mediate cell adhesion to ECM (Aruffo et al., 1990) and lymphocyte extravasation from the blood into tissues (DeGrendele et al., 1996). Hyaluronan under physiological conditions is present in the skin as a high molecular weight (HMW) molecule. It was found that when mice were exposed to HMW HA, it exerted anti-inflammatory properties, as manifested by reduced DNFB challenge-induced changes in ear thickness and decreased scarring and scaling recorded for CHS reactions provoked by DNFB (Kim et al., 2008). Conversely, LMW HA fragments were shown to induce inflammation and to mediate the production of inflammatory cytokines, such as TNF-α by human monocyte derived DC, to a similar extent to that observed for LPS. The immunostimulatory action of LMW HA has been reported to depend on TLR4 signalling, as bone marrow derived DC from mice with defective TLR4 receptors (C3H/HeJ strain mice and TLR4 KO on C57BL/10 background) failed to secrete TNF-α in response to LMW HA when compared with their WT counterparts (Termeer et al., 2002). Similarly, it has been found that in a human melanoma cell line exposure to LMW HA resulted in matrix metalloproteinase (MMP) 2 and IL-8 mRNA expression and protein production (Voelcker et al., 2008). Matrix metalloproteinase 2 and MMP9 enzymes are expressed on the surface of cutaneous DC and are important in facilitating DC migration from the skin to lymphatic vessels (Ratzinger et al., 2002). These changes in MMP2 and IL-8 production were also mediated via interaction with TLR4, as when TLR4 was silenced by transfection with small interfering (si) RNA, the stimulatory effect of LMW HA was no longer observed (Voelcker et al., 2008). Further involvement of TLR2 in HA signalling was observed by Scheibner et al. (2006). Degradation of HMW to LMW HA is mediated via enzymes called hyaluronidases; a family of endo-glycosidases that hydrolyze the β-1,4 linkages between N-acetyl-hexosamines and glucuronic acid (Petrey and de la Motte, 2014). Degradation of HA also has been observed via direct interaction with ROS, which could be inhibited by extracellular superoxide dismutase (Gao et al., 2008). Moreover, it was demonstrated that in addition
to direct degradation of HA, ROS were capable of indirectly affecting HA degradation via increasing the expression of hyaluronidase 2 (Monzon et al., 2010).

The roles of both ROS and products of HA degradation on the development of ACD were investigated by Esser et al. (2012). They found that TNCB-induced CHS was completely absent in double TLR2 and TLR4 KO mice. However, CHS reactions were induced successfully in germ free TLR2 and TLR4 competent mice, suggesting the involvement of endogenous TLR agonists in this process. In further experiments, Esser and colleagues confirmed that allergen induced the production of ROS in vitro in the PAM212 keratinocyte cell line, and in vivo in the ear (Esser et al., 2012). Allergen-induced degradation of HA in the skin was confirmed by electrophoresis of homogenized skin samples following 4h and 24h exposure to TNCB. It was shown that ROS mediated HA degradation was inhibited in the presence of antioxidants, N-acetylcysteine or plant derived antioxidant, RF-40. Additional evidence for the importance of ROS in the pathology of CHS came from experiments in which topical pre-treatment with a range of antioxidants prior to sensitization decreased the extent of ear swelling (Esser et al., 2012). Furthermore, it was reported that exposure to TNCB augmented hyaluronidase activity in a ROS dependent manner. Finally, treatment with the hyaluronidase inhibitor, aristolochic acid, before sensitization prevented the CHS reaction, confirming the involvement of ROS and degraded HA in contact allergic reactions (Esser et al., 2012).

In this section the participation of danger signals in the induction phase of contact sensitivity has been described. Danger signals arise in the skin following exposure to allergens and in response to other traumatic events or local cell or tissue damage. They can be versatile and stimulate divergent receptors. Thus far, the literature suggests that danger signals are uniformly required for successful sensitization. The necessity for a non-specific inflammation triggered by danger signals is a sophisticated mechanism of two-step control of the immune system that does not allow adaptive responses to take place in the absence of sufficient threat, like in the case of DNTB. It seems that one of the main purposes of danger signalling is the stimulation of production of biologically active IL-1β, which confers to DC the ability to transfer hapten responsiveness or stimulate T cell proliferation. Indeed, IL-1β plays a crucial role during the migration
and maturation of cutaneous DC and will be described in greater detail in the next section.

1.6 Dendritic cells

Antigen presentation is mediated by professional APC, DC. Dendritic cells are bone marrow derived cells of the innate immune system. They can reside in the lymphoid and non-lymphoid tissues. They act as sentinels of the immune system. Dendritic cell populations detect pathogens and tissue damage. They are located in tissues where the body comes into closest contact with the external environment, including in the gut, lung and skin. Additionally, DC are resident in the spleen or in the LN, where they effectively sample blood and lymph, respectively, in search of self and non-self antigens. The role of the DC is to sense danger and to carry antigens to the draining LN, where they can encounter other components of the immune system. Therefore they effectively act as a bridge between the innate and adaptive immune systems (Merad et al., 2013).
Figure 1.2 Subsets of dermal (d)DC in mouse and human skin.

Mouse skin contains LC in the epidermis and three dDC subsets in the dermis, which are: migratory LC on their way to the lymphatic vessels, langerin$^+$ dDC and langerin$^-$ dDC (majority population). Human skin also contains LC in the epidermis. There are four dDC populations in the human dermis, which include: migratory LC, CD141$^+$ dDC considered to be the equivalent of mouse langerin$^+$ dDC population; CD14$^+$ dDC and CD1c$^+$ dDC. It is hypothesized that there might be diversity within the mouse langerin$^-$ dDC subset to accommodate the two populations of human dDC without mouse counterparts. Contrary to dDC found in the mouse, only migratory LC found in the human dermis express langerin.
1.6.1 Cutaneous dendritic cell subsets

Skin DC are heterogeneous and comprise of LC and dermal DC (dDC); the breadth of DC populations in mouse and human skin are illustrated in Figure 1.2. The role of DC in the skin is to maintain tolerance to commensal bacteria found on the surface of the skin and to self-antigens, as well as to initiate immune responses to invading pathogens and assaulting chemical compounds. The function of skin resident DC depends on their ability to migrate to the LN, where they can orchestrate the type and the extent of adaptive immune responses. As such, they are involved in sensing whether a particular cutaneous insult is potentially dangerous, taking up that signal and transporting it (Helft et al., 2010). As discussed previously, contact allergens are misread by the immune system as potential pathogens, thus stimulating an inflammatory and allergic response. Within the mouse dDC subset, 2 main populations are distinguished: langerin\(^+\) dDC and langerin\(^-\) dDC (Merad et al., 2008) (Table 1.1; Figure 1.2). In humans, 3 cell subsets can be found in the dermis: CD14\(^+\) dDC and DC1c\(^+\) dDC (Klechevsky et al., 2008) and CD141\(^+\) dDC (Haniffa et al., 2012) (Table 1.2; Figure 1.2). Functional similarities can be found between mouse and human dDC subsets, namely mouse langerin\(^+\) dDC are thought to be an equivalent of CD141\(^+\) human dDC (Haniffa et al., 2013; Haniffa et al., 2012; Heath and Carbone, 2013). Similarly, it is considered that there is a diversity within the mouse langerin\(^-\) dDC population to accommodate human populations of CD14\(^+\) dDC and CD1c\(^+\) dDC (Haniffa et al., 2012; Heath and Carbone, 2013) (Figure 1.2).
Table 1.1 Marker expression by mouse dDC subsets.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Langerin⁺dDC</th>
<th>Langerin⁻dDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MHC II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11c</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Langerin (CD207)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD11b</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>SIRPα (CD172a)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F4/80</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CD103</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CLEC9A</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Adapted from (Helft et al., 2010). ++, highly expressed; +, expressed; -, absent. SIRPα, signal regulatory protein α; CLEC9, C-type lectin domain family 9 member A.

Table 1.2 Marker expression by human dDC subsets.

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD14⁺ dDC</th>
<th>CD1c⁺ dDC</th>
<th>CD141⁺ dDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>+</td>
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<td>low</td>
</tr>
<tr>
<td>CD1a</td>
<td>low/variable</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD1c</td>
<td>low/variable</td>
<td>+</td>
<td>low</td>
</tr>
<tr>
<td>CD14</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Langerin (CD207)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DC-SIGN (CD209)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CLEC9A</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Adapted from (Boltjes and van Wijk, 2014).+, expressed; -, absent; low, low but present expression; variable, population comprises of cells that express the marker and cells where marker is absent.
### 1.6.1.1 Ontogeny of Langerhans’ cells and marker expression

Langerhans’ cells comprise 2-5% of all epidermal cells in the adult mouse skin, with keratinocytes being the other major cell type (Romani et al., 2010). Langerhans’ cells were discovered in 1868 by Paul Langerhans and were originally thought to be a part of the nervous system due to their dendritic appearance (Ginhoux and Merad, 2010). Further studies showed, however, that LC derive from the bone marrow (Katz et al., 1979) and have the ability to mature *in vitro* into potent stimulators of T cell proliferation (Schuler and Steinman, 1985). Mouse LC are characterized by expression of MHC II, CD45, langerin (CD207) (type II calcium dependent lectin receptor binding mannose) and E-cadherin (the molecule responsible for the adhesion of LC to keratinocytes) among others. A unique feature of LC is the possession of the cytoplasmic organelle, called the Birbeck granule, involved in antigen processing (Merad et al., 2008). Comparison of cell markers expressed by mouse and human LC is presented in the Table 1.3.

**Table 1.3 Comparison of marker expression by mouse and human LC.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mouse LC</th>
<th>Human LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MHC II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD11b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SIRPα (CD172a)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Langerin (CD207)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EpCam</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CCR7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD1c</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CD1a</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>F4/80</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD205</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Adapted from (Haniffa et al., 2013). ++, highly expressed; +, expressed; -, absent.
Langerhans’ cells are the only DC present in the epidermis. They appear in the epidermis of mice from 18 day of embryonic development (Chorro et al., 2009). Crucial for the development of LC is the presence of TGF-β. The presence of the TGF-β is required for in vitro differentiation of human CD34+ precursors into langerin expressing LC (Caux et al., 1999; Guironnet et al., 2002). Indeed, it had been observed that TGF-β KO mice do not have MHC II+ cells present in the epidermis (Borkowski et al., 1996; Borkowski et al., 1997). Mice deficient in TGF-β presented with inflammation and an early death. To exclude the possibility that the persistent inflammation was responsible for the absence of LC, mice were treated with rapamycin that inhibited the inflammation and prolonged the life of KO animals. However, rapamycin did not reestablish LC in the epidermis of these animals, suggesting that their absence was not caused by cutaneous inflammation, but rather was a result of a defective LC development, which is dependent upon TGF-β (Borkowski et al., 1996).

Langerhans’ cells turnover in the epidermis in the steady state is independent of bone marrow precursors and is maintained in situ by a population of skin resident cells. Langerhans’ cells are resistant to ionizing radiation and remain of host origin after bone marrow transplant or in congenic parabiotic (with shared circulation) mice. However, following ultraviolet (UV) light radiation (inflammatory conditions) LC can be derived from precursors in the blood. Indeed, in a severely inflamed skin, where LC and their progenitors are lost and skin integrity is compromised, LC are repopulated by circulating monocytes that respond to chemokines secreted by endothelial cells, namely monocyte chemoattractant protein-1 (MCP-1). MCP-1 is the ligand for CCR2 that drives monocytes to the dermis. Another signal from keratinocyte-derived macrophage inflammatory protein (MIP) 3α, a CCR6 ligand, controls their entry into the epidermis (Ginhoux and Merad, 2010). The half-life of LC is much longer than other DC (7-8 days) and is thought to be between 53-78 days. Moreover, between 1 and 2% of all LC were demonstrated to be able to divide at any given time compared with 5% for other DC subsets (Merad et al., 2002).
1.6.1.2 Ontogeny of dermal dendritic cells and marker expression

In contrast to LC, tissue DC have a shorter life span and have to be replenished by circulating precursors from the blood (Liu et al., 2007; Merad et al., 2002). Turnover of DC in non-lymphoid tissues was assessed in mice following bromodeoxyuridine (BrdU) injection. Bromodeoxyuridine is a synthetic analogue of thymidine, used to label dividing cells. It was demonstrated that 12h following BrdU injection 5–10% of DC in the dermis and lung, and 10–20% of DC in the liver and kidney were labelled with BrdU (Ginhoux et al., 2009).

Dendritic cell-restricted precursors that give rise to tissue resident DC have been identified in the bone marrow. Multipotent precursor cells in the bone marrow divide into two distinct lineages lymphoid and myeloid, which give rise to lymphocytes and granulocytes, erythrocytes, megakaryocytes, monocytes, respectively. Dendritic cells can differentiate from macrophage/DC precursors (MDP) which differentiate into monocytes and common DC precursors (CDP) (illustrated in the Figure 1.3). The common DC precursor subsequently becomes a DC restricted precursor, called pre-DC (Liu et al., 2007). Pre-DC leave the bone marrow, circulate in the blood and home to lymphoid organs, where they become lymphoid tissue-resident DC or settle in the non-lymphoid tissues, such as liver, skin, kidneys or lungs (Ginhoux et al., 2009; Helft et al., 2010). In addition to DC derived from pre-DC precursors, DC can arise from monocytes. Indeed, it was found that out of two monocyte populations present in mouse blood, Ly6Chigh CCR2+CX3CR1lowCD62L+ and Ly6C− CCR2− CX3CR1highCD62L−, the former is recruited to inflamed tissues, whereas the latter is recruited into non-inflamed tissues via a CX3CR1-dependent mechanism (Geissmann et al., 2003). Subsequently, it was discovered that Ly6Chigh and Ly6C− monocytes gave rise to CD103+ DC and CD103− DC, respectively, in the lung (Jakubzick et al., 2008). Mouse monocytes Ly6Chigh and Ly6C− subsets were found to correspond with human monocytes CD14+CD16− and CD14−CD16+ populations, respectively (León et al., 2005). The ability of monocytes to give rise to DC the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 has greatly facilitated DC investigation of in vitro derived DC (Sallusto and Lanzavecchia, 1994).
Skin DC are a heterogeneous population. Langerhans’ cells are derived mainly from hematopoietic precursors that seed into the skin before birth, mainly from yolk sac progenitors and later during embryogenesis from fetal liver monocytes. Ontogeny of dDC begins in the bone marrow, where common myeloid progenitors (CMP) differentiate into macrophage/DC precursors (MDP) which can give rise to monocytes, macrophages and DC. Subsequently, MDP becomes common DC precursors (CDP) which produces pre-DC cells that leave the bone marrow, circulate in the blood stream and settle in the dermis, where they give rise to CD11b+ and CD103+ DC. Additionally, blood derived monocytes can differentiate into CD11b+ dDC during inflammatory conditions. Adapted from (Helft et al., 2010).

Differentiation and homeostasis of DC are driven by several cytokines. Among them the cytokine Flt3L and its receptor Flt3 are crucial. It has been found that mice lacking Flt3L and Flt3 have a reduced number of DC in lymphoid organs (McKenna et al., 2000), whilst the absence of Flt3L results in diminished numbers of DC in the lung, liver and kidney (Ginhoux et al., 2009). Conversely, injection of Flt3L resulted in DC proliferation in non lymphoid tissues (lung, liver and kidneys) (Ginhoux et al., 2009). Another cytokine implicated in DC differentiation is GM-CSF, which has been shown

Figure 1.3 Ontogeny of cutaneous DC.
to play a role in the development of CD11b⁺, but not CD11b⁻ DC in the dermis (Kingston et al., 2009). Macrophage colony-stimulating factor (M-CSF) was originally considered to be required for the development of macrophages and it has been demonstrated recently to facilitate the development of LC, such that mice lacking M-CSF receptor did not develop LC (Ginhoux et al., 2006). Additionally, as previously mentioned, TGF-β has been found to be required for the formation of LC (Borkowski et al., 1996).

1.6.2 Roles of skin DC: sensing danger, taking up antigen and transporting antigen

The main function of DC is to bridge the gap between innate and adaptive immune responses by sensing danger, as described in the section about danger signals, and triggering specific responses by T cells. Dendritic cells are superbly equipped to fulfil these roles. Firstly, their name stems from their morphologic appearance of characteristic long or veil-like protrusions, which serve to maximize the surface area of the cell on which the antigens might be presented (high surface area to volume ratio) (Banchereau and Steinman, 1998). Immature DC are proficient in sensing danger, antigen capture and processing. They lack expression of T cell co-stimulatory molecules CD40, CD54, CD80 and CD86. Conversely, they possess receptors required for antigen acquisition. Dendritic cells utilize phagocytosis and macropinocytosis to capture microbes and the soluble antigens in the extracellular environment, respectively. In addition to these two methods of antigen sampling, DC utilize receptor mediated endocytosis via receptors recognizing Fc portions of antibodies (eg. Fcγ and Fcε), heat shock protein receptors and scavenger receptors, C-type lectin receptors, such as the mannose receptor, CD205 receptor (Guermounrez et al., 2002), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; CD209) (Geijtenbeek et al., 2000) and langerin (CD207) (Valladeau et al., 2000). These methods of antigen acquisition are effective even at picomolar and nanomolar levels (Sallusto et al., 1995). Captured antigen is then loaded onto MHC to form antigen-MHC complexes that are presented on the DC surface for subsequent recognition by T cell receptors (TCR) (Guermounrez et al., 2002). Cells down-regulate their expression of antigen acquisition receptors and begin to undergo changes known collectively as maturation. Mature DC migrate via efferent lymphatics to the LN where they localize in the paracortex region (Randolph et al., 2005).
The sequence of events in DC migration is triggered by variety of factors of biological (Haley et al., 2012; Shankar et al., 1996), chemical (Cumberbatch et al., 1992a) and physical (Ghaznawie et al., 1999) nature. Langerhans’ cells have a complicated path to follow during migration to the LN. It has been described as a two step model (Villablanca and Mora, 2008), where the first leg of LC journey is from the epidermis to dermis (Ouwehand et al., 2008), and the second part entails mobilization from the dermis to the lymphatic vessels. Langerhans’ cell migration is dependent on two compulsory signals: IL-1β and TNF-α. Upon recognition of danger signals LC secrete IL-1β that in turn stimulates LC via IL-1R1 and triggers the release of TNF-α, presumably from surrounding keratinocytes. TNF-α acts on LC through type 2 receptor (TNFRII) delivering a second stimulus necessary for LC migration (Cumberbatch et al., 1997a, b, 1999c).

Cumberbatch et al. (1997) investigated the influence of antibodies that neutralize TNF-α and IL-1β on LC migration and LN DC accumulation caused by topical administration of oxazolone or by intra-dermal delivery of TNF-α or IL-1β. Thus, systemic (intraperitoneal) delivery of anti-IL-1β or anti-TNF-α neutralizing antibody reduced the DC influx to the LN normally observed after exposure to oxazolone. The reduction in LC frequency in the murine ear epidermis usually observed following TNF-α intra-dermal administration was also inhibited by anti-IL-1β antibodies. Correspondingly, anti-TNF-α antibody prevented IL-1β triggered LC migration and LN DC accumulation (Cumberbatch et al., 1997b).

Others have also demonstrated the requirements for IL-1β and TNF-α cytokine signals in the process of LC mobilization. Wang et al. (1997) found that following exposure to fluorescein isothiocyanate (FITC) in mice deficient in TNFRII, the only TNF-α receptor present on LC (Peschon et al., 1998), there was a decreased number of antigen bearing cells in the LN in comparison with WT mice. Furthermore, it was reported that TNFRII KO mice were less responsive to oxazolone induced CHS, as measured by reduced challenge-induced ear swelling (Wang et al., 1996).

It was found that DC migration in humans was also dependent on TNF-α and IL-1β signalling. Stoitzner et al. (1999) reported that inhibition of TNF-α via exposure to anti-TNF-α antibody inhibited spontaneous LC migration in human skin explants. Moreover, it was established that delivery of exogenous IL-1α, IL-1β and TNF-α enhanced DC
migration from skin explants in comparison with spontaneous migration (Stoitzner et al., 1999).

Further investigations of cytokine requirements for LC migration were performed by Cumberbatch and colleagues. A role for IL-18 was observed, in addition to requirement for TNF-α and IL-1β signalling. Intradermal delivery of exogenous IL-18 resulted in LC mobilisation that was significantly inhibited in the presence of anti-TNF-α, anti-IL-1R1 antibody and in caspase-1 KO mice. Moreover, oxazolone induced decrease in LC frequency was inhibited by pre-treatment with anti-IL-18 antibody. These data suggested that IL-18 signals upstream from TNF-α and IL-1β (Cumberbatch et al., 2001).

Once in the LN, mature DC cannot capture antigens; however, they possess the unique ability to induce the activation of naïve T cells. In the LN, when antigen carried by the DC is recognized by a T cell, two cells interact, creating an immunological synapse (Dustin et al., 2010). In the LN, circulating T cells sample antigens on DC via interaction between lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), present on T cells and DC, respectively. Recognition of antigen-MHC complex on DC by TCR drives conformational change in the LFA-1 which stabilizes interaction between T cells and the DC (Hogg et al., 2002). Recognition of antigen-MHC complex by TCR delivers a first signal to naïve T cells. A second signal is delivered by interaction of the DC co-stimulatory molecules CD80 and CD86 with T cell receptor, CD28 (Jenkins et al., 1991). Activation of CD28 receptor specifically stimulates production of IL-2, which further drives its own production (Jain et al., 1995). Interleukin 2 is crucial for T cell growth, proliferation and differentiation (Smith, 1988). Additionally, interaction between CD40 and CD40L on DC and T cells, respectively, has been demonstrated to up-regulate expression of MHC II, CD80 and CD86 (Caux et al., 1994), and stimulate IL-12 release by DC (Cella et al., 1996).

1.6.3 Polarization of immune responses by DC

A third signal is delivered by the DC, which induces T cell polarization and development into either type 1 or type 2 cells (Gutcher and Becher, 2007) (Figure 1.4). The paradigm of Th1 and Th2 responses was proposed in 1986 by Mosmann et al. (1986) and argued that CD4⁺ T cells could develop into functionally distinctive
subpopulations that display differential cytokine profiles and diverse roles in adaptive immunity (Mosmann et al., 1986). It was further discovered that Th1 subset could be characterized by production of IL-2, IL-3, TNF-α, and most notably IFN-γ and participation in a cell-mediated immunity, whereas the Th2 population secreted mainly IL-4, IL-5, and IL-13 and aided humoral immunity (Coffman, 2006; Zhou et al., 2009). The polarization of T cell response is dependent on the cytokines secreted by DC, which in turn are determined by the type of antigen encountered by the DC. The presence of intracellular pathogens causes IL-12 release by the DC and subsequent polarization of CD4+ cells into Th1 populations. Conversely, helminths and other extracellular pathogens trigger production of IL-4 and subsequent polarization of CD4+ cells into Th2 cells (Walsh and Mills, 2013; Zhou et al., 2009).

Similarly to CD4+ cell populations, type 1 and type 2 cells are also present among CD8+ cytotoxic T cells (Tc1 and Tc2, respectively), and are characterized by similar cytokine secretion profiles to those displayed by Th cell subsets (Vukmanovic-Stejic et al., 2000). Generation of Tc1 and Tc2 subsets is also regulated by IL-12 and IL-4 release by DC, respectively (Woodland and Dutton, 2003). These and other Th cell subsets are displayed in the Figure 1.4.

Recently, another Th subset has been described, namely Th17 (Figure 1.4). Th17 cells are involved in responding to bacterial and fungal infections (Yamane and Paul, 2013). They release IL-17A, IL-17F, IL-21 and IL-22 cytokines. Their polarization is controlled by TGF-β, IL-1, IL-6 and IL-23 (Korn et al., 2009; Mangan et al., 2006). The Th17 cell subset was identified in the cellular infiltrate of human skin undergoing the elicitation phase of ACD. Furthermore, enhanced expression of mRNA for signature Th17 cytokines IL-17A, IL-17F and IL-23 was detected in the skin during the elicitation phase of ACD. Increased levels of with IFN-γ and IL-4 mRNA also were observed in allergen challenged skin, which were characteristic of Th1 and Th2 response, respectively (Zhao et al., 2009). Further clues as to the importance of IL-17 in the skin came from IL-17 KO mice, which exhibited impaired CHS to DNFβ, as measured by diminished ear swelling. There were no differences in the TNF-α, IL-1α and IL-1β cytokine levels in the ear following the challenge, suggesting that the reduced CHS reaction was due to the absence of IL-17, and not because of the secondary effects on IL-1 or TNF-α (Nakae et al., 2002).
Figure 1.4 Induction of T helper cell subsets.

In the LN naive T cells are activated into antigen-specific T cells via interaction with APC. Within the immunological synapse antigen bound with MHC complex is recognized by specific TCR and comprises the first activation signal. The second signal is delivered by CD28 receptor upon binding co-stimulatory molecules CD80/CD86. The final, third signal is delivered by cytokines secreted by APC and determines T cell effector phenotype. Release of IL-12 and IL-4 results in differentiation into Th1 and Th2 subsets, respectively. The combination of signals from IL-1, IL-6 and IL-23 trigger the activation of a Th17 phenotype. Production of IL-10 and TGF-β by APC generates Treg cell population. Various Th cell subsets release a wide repertoire of cytokines which are shown on the far left hand side.

Dendritic cells can activate another Th subset of Treg cells (Figure 1.4). Two subsets of Treg cells can be distinguished natural and adaptive (inducible) regulatory T cells (Bluestone and Abbas, 2003). Natural Treg cells originate in the thymus, express
CD4^+CD25^+Foxp3^+ markers and are involved in maintaining central and peripheral tolerance to autoantigens (Bach and François Bach, 2003). Adaptive regulatory T cells are created in the periphery from naive T cells by interaction with immature or partially matured DC in the absence of sufficient co-stimulatory molecule expression (Walsh and Mills, 2013). A major role in the development of adaptive regulatory T cells is played by TGF-β and IL-10. Interleukin 10 interferes with DC function and inhibits their full maturation; it inhibits T cell CD28 signalling and in turn promotes differentiation into IL-10 producing T cells (Joss et al., 2000; Sato et al., 2003; Steinbrink et al., 1997). Similarly, TGF-β prevents maturation of DC (Steinman et al., 2003) and inhibits activation of T cells (Coffman et al., 1989; Kehrl et al., 1986). Adaptive regulatory T cells are associated with mucosal tolerance towards non-pathogenic microflora and are thought to aid regulation of autoimmunity (de Jong et al., 2005).

1.6.3.1 Allergen dependent polarization of immune response

As mentioned previously, the immune system can misread allergens as pathogens, therefore, allergens are capable of polarizing the immune response in the same way as just has been described for biological insults. Indeed, exposure to different classes of allergens was demonstrated to result in distinct polarization profiles. Work with reference contact allergen DNCB and respiratory allergen trimellitic anhydride (TMA) in mice has shown that these two allergens are capable of inducing opposing polarization of immune response into Th1/Tc1 and Th2, respectively. It has been known that inhalation of certain acid anhydrides and aromatic isocyanates can result in airway hypersensitivity in humans (Bernstein et al., 1982; Danks et al., 1981; Moller et al., 1985). It was shown that inhalation of TMA, but not of DNCB, induced IgE antibody production in BALB/c strain mice (Dearman et al., 1991). Further work revealed that topical exposure of BALB/c strain mice to doses of DNCB and TMA was capable of inducing equivalent levels of immune response, as measured by the ability to induce LNC proliferation, resulted in IgE antibody production only in mice treated with TMA (Dearman and Kimber, 1991). Production of IgE is associated with Th2 response and is mediated by IL-4 and strongly inhibited by IFN-γ (Coffman and Carty, 1986; Coffman et al., 1986; Snapper and Paul, 1987). Indeed, further investigations have revealed that chronic exposure to oxazolone or DNFB (contact allergens) and TMA or toluene diisocyanate (respiratory allergens) resulted in production of IFN-γ and IL-4,
respectively, by LNC (Dearman et al., 1995, 1996a). These results confirmed that contact and respiratory allergens have a differential ability to skew immune response predominantly towards type 1 and type 2, respectively. Interestingly, topical exposure to FITC, a fluorescent chemical capable of inducing skin sensitization, was found to result in Th2 type response (Dearman and Kimber, 2000), highlighting diversity within mechanisms of skin sensitization to chemicals (Figure 1.5).

![Figure 1.5 Divergent polarization cytokine profiles triggered by DNCB and TMA.](image)

Prolonged exposure to the reference contact allergen, DNCB, and the reference respiratory allergen, TMA, results in differential polarization of immune response into Tc1/Th1 and Th2 phenotype, respectively.

It has been mentioned previously that there are several DC populations in the skin (Valladeau and Saeland, 2005) and that DC are capable of shaping the adaptive immune response via the third signal supplied to T cells in the LN (Walsh and Mills, 2013). It also has been explained that allergens have the capacity of inducing differential immune responses (Dearman et al., 1996a). Therefore, it was crucial to establish the roles of cutaneous DC populations during the sensitization phase of ACD.
1.7 Langerhans’ cell migration

Langerhans’ cell migration has been one of the main endpoints of interest in the current investigations. Additionally, LC migration is a more complex process than dDC migration due to the fact that LC have a two step path to follow, that is from the epidermis to dermis, and from the dermis to lymphatic vessels. Therefore, it is important to understand the process of LC migration in greater detail.

Upon exposure to inflammatory stimuli, LC migrate towards the draining LN which they reach after approximately 72h, where they present antigen to T cells (Kissenpfennig et al., 2005). In order to reach the lymphatic vessels the LC have to dissociate from the tightly packed network of keratinocytes. Interaction between keratinocytes and LC is mediated by the adhesion molecule E-cadherin, which is expressed by all keratinocytes, with approximately half of the cells exhibiting very high levels of this molecule. E-cadherin mediates homophilic interactions between different adjacent cell types in both mice (Tang et al., 1993) and in humans (Blauvelt et al., 1995). Upon activation, LC are induced to decrease their expression of E-cadherin to disengage from surrounding keratinocytes. Indeed, it was demonstrated that in clusters of fetal skin dermal DC, the triggers that induce LC migration in vivo (IL-1α and IL-1β, TNF-α and LPS), also resulted in a rapid (after 4h) down-regulation of E-cadherin expression and increased levels of MHC II, CD40 and CD86 expression (Jakob and Udey, 1998). Recently, it was reported that in addition to keeping LC ‘in place’, E-cadherin expression by keratinocytes was required for LC differentiation from immature precursors, which takes place under inflammatory conditions, when LC have to be recruited to the epidermis from blood precursors (Mayumi et al., 2013).

The first step of LC migration was investigated by Ouwehand et al. (2008). They found that human LC migrated ex vivo following exposure of the skin to NiSO₄. Furthermore, when human epidermal sheets were exposed to non-toxic concentrations of NiSO₄ in a transwell system, there was a decrease in the number of LC. When fibroblasts were placed in the lower chamber of the transwell system, the extent of LC mobilization was even higher. Later it was discovered that the LC migration towards fibroblasts could be reversed by addition of the anti-CXCL12 antibody; suggesting that fibroblasts released CXCL12 that attracted LC out of the epidermis. Subsequent experiments with the
MUTZ-3 LC, human LC-like cell line, demonstrated that exposure to NiSO$_4$ resulted in up-regulation of CXCR4, a receptor for CXCL12 (Ouwehand et al., 2008). Additionally, migration of mature MUTZ-3 cells towards fibroblasts in the transwell system was inhibited by the addition of anti-CXCR4 antibody to the cells, suggesting that CXCR4-CXCL12 axis is directly involved in the LC migration towards the dermis (Ouwehand et al., 2008). Further evidence pointing to the involvement of CXCR4-CXCL12 axis in LC migration in mice came from the study by Kabashima and colleagues (2007) that found the presence of CXCR4 on LN resident, as well as migratory, cutaneous DC. Analysis of *in vitro* migrated cutaneous DC showed that they exhibited dose dependent chemotaxis towards CXCL12. A specific role for this CXCR4-CXCL12 axis in allergic responses to chemical allergens other than nickel has also been shown. Thus, CXCL12 was detected in the skin, at the levels of both message and protein, following treatment with DNFB. Inhibition of CXCR4 with a specific antagonist (4-F-Benzoyl-TN14003) during the sensitization, but not the elicitation phase, significantly inhibited the clinical manifestations of DNFB-dependent CHS (Kabashima et al., 2007).

Once LC reach the dermis, they pass through the basement membrane. This step is facilitated by MMP enzymes. Matrix metalloproteinase 9 and changes in adhesion molecule expression aid the process of migration of LC through the basement membrane (Kobayashi, 1997; Ratzinger et al., 2002). Increased production of adhesion molecules, α6 and β1 integrins, further facilitates progress through the epidermal-dermal junction. Down regulation of skin homing chemokine receptors (CCR1, CCR2, CCR5 and CCR6) takes place with simultaneous up-regulation of LN specific receptors (CCR4, CXCR4 and CCR7) (Sallusto et al., 1999).

Following LC mobilization from the epidermis, it was found that lymphatic vessels in the dermis released secondary lymphoid tissue chemokine, which was later described as CCL21, a chemokine that signals via CCR7 present on activated (by TNF-α) but not resting LC (Saeki et al., 1999). Entry of DC into lymphatic vessels is thought to occur via the gaps between endothelial cells (Stoitzner et al., 2002). *In vitro* generated human LC were shown to up-regulate expression of CCR7 mRNA, as well as the production of CCR7 protein following treatment with DNCB, nickel and TNF-α. Inhibition of IL-1β signalling (with IL-1R antagonist [IL-1Ra] treatment) did not inhibit DNCB induced CCR7 up-regulation, whereas inhibition of TNF-α with anti-TNF-α antibody inhibited
the induction of CCR7 (Boislève et al., 2004). The switching on and off of certain chemokine receptors appears to be crucial for DC responsiveness. It has been shown by Sallusto et al. (1998) that during maturation (following treatment with LPS) of human DC derived from PBMC, CCR1 and CCR5 were rapidly (within 3h) down-regulated. Conversely, expression of CXCR4 and CCR7 was augmented (Sallusto et al., 1998), further confirming the role of CXCR4-CXCL12 axis in LC migration from the epidermis to dermis and subsequent CCR7-CCL21 axis mediating LC chemotaxis towards the LN. Another CCR7 ligand, CCL19, is produced by lymphatic vessels, as well as by mature DC in the LN. Chemokine CCL19 attracts DC and naive T cells that also express CCR7, to facilitate the encounter of naive T cells with antigen bearing DC (Gunn et al., 1998; Willimann et al., 1998).

1.8 Role of cutaneous DC populations in the development of CHS

The skin represents a particularly rich source of different DC populations. As the existence of these different populations has become apparent, there has been a considerable interest in examining the roles of these different subsets in the development of cutaneous immune responses, including skin allergic responses. Indeed, contact allergens represent a relatively simple system in which the role of these DC in skin immunity could be probed. Use of conditional and constitutive KO mouse models allows for a selective depletion of specific DC populations and investigation of their functions (Kaplan, 2010; Kaplan et al., 2008; Romani et al., 2010). There have been many attempts to achieve this and as a result there were often conflicting results regarding the role of LC and dDC in the CHS response. One such model utilized langerin-DTR mice which had a knock in of human diphtheria toxin (DT) receptor (DTR) under the control of the langerin promotor. Upon delivery of DT to these mice depletion of all langerin-expressing cells took place within 24h of toxin injection. Epidermal LC did not repopulate the epidermis until 4 weeks after toxin injection (Bennett et al., 2005) leaving a window where langerin-DTR mice were deficient in langerin+ cells. It was confirmed that depletion of langerin+ cells per se did not result in inflammation and that skin homeostasis was maintained by visualization of undisturbed DETC in the epidermis. Following sensitization with 1% TNCB and challenge with 0.25% TNCB, CHS reactions in langerin-DTR mice were significantly diminished, compared with WT mice, although still detectable. These results suggested that
langerin-expressing cells were sufficient to induce CHS reaction (Bennett et al., 2005). However, a study by Kissenpfennig et al. (2005) also utilized the same langerin-DTR mice and found, in contrast to a report by Bennett et al. (2005), that the CHS reaction in mice deficient in langerin-expressing cells was the same as in control, WT animals (Kissenpfennig et al., 2005). Another available model of LC ablation was described by Kaplan et al. (2005). In that model a bacterial artificial chromosome containing the DT subunit A (DTA) under the control of human langerin was constitutively expressed, making these transgenic mice lack langerin+ cells from birth. Interestingly, in these mice (langerin-DTA) there were langerin+ cells present in the LN, however, that population of cells was missing in the mouse model described by Bennett et al. (2005). When CHS responses were examined in langerin-DTA mice that are deficient in LC from birth, it was found that the response to oxazolone and DNFB was exaggerated in comparison with WT counterparts. Moreover, it was found that the enhanced CHS response from langerin-DTA mice could have been adoptively transferred into WT mice. These data suggested that LC play a regulatory role.

What Bennett et al. and Kissenpfennig and colleagues (2005) were not aware of at the time, was that upon delivery of DT two populations of cells were depleted: LC and langerin+ dDC; the latter were identified in 2007 and described in a publication by Bursch et al. (2007). Therefore, in retrospect, only the ablation model described by Kaplan et al. (2005) was indeed the one that could investigate the role of LC in the CHS, as those were the only cells that were depleted in the model that he described; while in models used by Kissenpfennig et al. (2005) and Bennett et al. (2005) two cell populations were depleted: LC and langerin+ dDC. A novel population of dermis resident, radiosensitive langerin+ dDC was identified by crossing the two depletion models, langerin-DTR and langerin-DTA (Bursch et al., 2007). It was shown that this novel subset of cells could be distinguished from epidermal LC by the absence of CD11b and the presence of CD103. In addition to the dermis, langerin+ DC were detected in the lungs and in the liver. Investigations of the role of langerin+ dDC in the CHS reaction demonstrated that the complete ablation of langerin+ cells resulted in decreased challenge responses, whereas in the situation when only LC were absent CHS reaction to DNFB was not different from that observed in mice with intact populations of cutaneous DC (Bursch et al., 2007).
Further investigation of the roles of langerin$^+$ cells in the skin was conducted by Noordegraaf et al. (2010). In this study langerin-DTR mice that were injected with DT 2 days and 10 days prior to sensitization, and were shown to be depleted of LC and langerin$^+$ dDC subsets, or only LC, respectively, due to differential repopulation times of langerin$^+$ cell subsets. The langerin$^+$ dDC population reappeared as soon as 3 days following delivery of DT. Conversely, LC were not detected until 7 days after DT injection (Noordegraaf et al., 2010). It has been shown that both populations of langerin$^+$ cells were required for CHS reaction when low concentrations of allergen were employed. Therefore, when either DNFB or oxazolone at concentrations of 0.5% and 0.25% for sensitization and challenge, respectively, were utilized, it was observed that in mice injected with DT either 2 days and 10 days prior to sensitization, challenge-induced ear swelling was diminished. However, when higher concentrations of allergen were administered (1% and 0.5% for sensitization and challenge, respectively) ear swelling was diminished in animals depleted of both LC and langerin$^+$ dDC, while the mice without LC exhibited the same level of CHS as WT animals (Noordegraaf et al., 2010).

Another model in which the role of cutaneous DC was investigated were Baft3 KO mice in which populations of conventional CD8α$^+$ DC were missing from lymphoid tissues, as well as langerin$^+$CD103$^+$CD11b$^-$ DCs in the lung, intestine, dermis, and skin-draining LN. In Baft3 KO mice population of LC was intact, thus providing a suitable model to examine the role of LC in the absence of langerin$^+$ dDC. It was found that DNFB challenge-induced ear swelling in the absence of langerin$^+$ dDC was no different from WT animals, suggesting that in the absence of langerin$^+$ dDC, LC were sufficient to induce CHS reaction (Edelson et al., 2010).

Despite these more sophisticated models for investigating the roles of cutaneous DC subsets, there is still uncertainty regarding exact roles of skin resident DC, mainly due to discrepancies between studies. For instance studies by Bennett et al. and Kissenpfennig et al. (2005) have both utilized the same model of langerin$^+$ cell ablation system, but their results were not in agreement. Their protocols of DT administration were similar, with Bennett et al. delivering DT 3 days prior to sensitization, while Kissenpfennig et al. administered DT injection also 1 day before sensitization. Given that langerin$^+$ dDC were shown to reappear in the skin 3 days after DT administration, it can be speculated that in the model used by Bennett et al. there could have been some
langerin$^+$ dDC returning to the dermis at the time of sensitization. However, that still does not explain divergent results, as Bennett et al. observed decreased CHS, which is in line with the belief that langerin$^+$ dDC are the main DC population responsible for antigen delivery to the LN (which suggests that the langerin$^+$ dDC were not there). One other possibility for the differences between the two studies was the allergens that were used. TNCB and DNFB were utilized by Bennett et al. (2005) and Kissenpfennig et al. (2005), respectively, and it is possible that different chemical allergens may display divergent needs for classes of skin DC.

Taken together, these data demonstrate that there has been somewhat of a paradigm shift in the understanding of the role of LC. Some years ago, LC were considered to be the chief APC responsible for antigen presentation to naive T cells in the skin draining LN. Recently, it has become clear that in common with other aspects of the immune system, there is some redundancy with respect to cutaneous DC subsets and antigen presentation. Indeed, under some circumstances LC may not be the most efficient skin APC, although when allergen is limiting, for example, they may play a more major role. Furthermore, there is increasing evidence that LC may have other functions, including immunoregulatory activities.

1.9 Tolerogenic role of Langerhans’ cells

The evidence obtained from langerin-DTA mice suggested that LC played a regulatory role in the CHS response, whereby in their absence the symptoms in response to both FITC and DNFB were more pronounced than in the WT mice. Further investigation of this system revealed that the immunomodulatory effects of LC required the presence of MHC II on the surface of the LC. Thus, increased ear swelling was observed in animals with selective depletion of MHC II in the LC population, suggesting the role for MHC II in LC-mediated immunosuppression. Furthermore, it has been shown that the lack of LC-derived IL-10 in langerin-DTA mice contributed also to the augmented clinical manifestation of CHS (Igyarto et al., 2009).

More clues about the additional roles of LC in the immune response came from the study by Gomez de Agüero and colleagues (2012). They used DNFB and the chemically related molecule DNTB. DNTB has been shown to be tolerizing (see section 1.5.1.2), with respect to DNFB, that is, prior topical exposure of mice to DNTB renders
the mice less sensitive to challenge with DNFB (Riemann et al., 2005). Following treatment with DNTB, LC migrated to the LN and presented antigen to CD8+ T cells, suggesting their ability to cross-present antigen. It was found that by interacting with naive CD8+ T cells LC were able to render them unresponsive to subsequent exposure to DNFB. To exert their tolerogenic effect, LC required the presence of CD4+CD25+Foxp3+ cells, such that in the mice where that population of cells was absent, the tolerizing effect of DNTB was no longer observed (Gomez de Agüero et al., 2012).

To add further support to a potential immunoregulatory function of LC in contact sensitization, there are other reports suggesting that LC play a role as mediators of tolerance in other types of skin immune response, for example non-responsiveness towards commensal, skin resident bacteria (van der Aar et al., 2013). Van der Aar and colleagues demonstrated that human LC mediated tolerance towards bacterial flora via inefficient bacteria engulfment and subsequent, ineffective antigen presentation. As a consequence, LC induced bacteria specific Treg cells, instead of activating antibacterial effector T cells (van der Aar et al., 2013).

Similarly, investigations of human LC revealed that LC, but not dDC, have the ability to induce proliferation of skin resident memory T cells, the majority (~84%) of which expressed markers specific for Treg cells. Further experiments demonstrated that LC mediated induction of memory T cell proliferation depended on direct cell to cell contact of the T cells with LC. However, when a pathogenic stimulus of heat inactivated Candida albicans (C.albicans) was added to the culture, LC induced the proliferation of effector memory T cells, which produced IFN-γ and IL-17 (Seneschal et al., 2012), which was in accordance with data reported by Igyarto et al. (2011). Overall, the data presented by Seneschal et al. (2012) suggested that under physiological conditions LC exhibited a tolerogenic function by mediating the proliferation of Treg cells, but in the presence of danger signal, i.e. C. albicans, LC had the ability to induce expansion of effector memory T cells.

It is appears that LC can act as professional APC, however, under certain circumstances they can play a tolerogenic role. Additionally, it has been recently suggested that LC might mediate Th17 responses, which have been implicated in skin inflammation and ACD (Peiser et al., 2012). Moreover, there are reports that suggest that LC are the main
subset responsible for antigen presentation to CD8\(^+\) T cells via the process of cross-presentation.

1.10 Langerhans’ cells functions: cross-presentation and activation of Th17 cells

Langerhans’ cells are the outermost antigen presenting cells that are the first line of contact of the skin with biological, chemical and physical insults as well self antigens (Igyártó and Kaplan, 2013). In addition to their conventional role as APC, whereby LC present antigens in the context of MHC II molecules to CD4\(^+\) T cells, LC have been shown to possess cross-presentation abilities. Cross-presentation refers to the presentation of antigens in the context of MHC I molecules. It is responsible for the development of Tc mediated immunity directed against parasites and tumours that do not infect APC, thus allowing presentation through the classical endogenous MHC class I pathway, as well as maintaining tolerance (Joffre et al., 2012). There are conflicting reports regarding the role of LC as the most efficient inducers of CD8\(^+\) T cell responses. In 2008 Klechevsky and colleagues showed that cutaneous human DC have distinct functions with regard to efficient antigen cross-presentation and the generation of an effective Tc response. Langerhans’ cells (both isolated from the human skin, as well as created in vitro) were superior at inducing CD8\(^+\) T cell proliferation and the induction of antigen-specific CD8\(^+\) T cell responses, than were CD14\(^+\) or CD1a\(^+\) dDC (Klechevsky et al., 2008), which are equivalents of mouse dDC populations (Boltjes and van Wijk, 2014; Guilliams et al., 2010). Moreover, Polak et al. (2012) demonstrated that human LC were more efficient than dDC at presenting Epstein Barr virus to CD8\(^+\) T cells, as measured by their ability to proliferate and secrete IFN-\(\gamma\) (Polak et al., 2012). Similar results were obtained by Stoitzner et al. (2006) regarding mouse LC (Stoitzner et al., 2006).

However, there are data suggesting that LC might not be the main DC subset responsible for the antigen cross-presentation in cutaneous immune responses. In a study that employed an in vivo C. albicans infection model, it was found that LC promoted the development of Th17 cell subsets, whereas langerin\(^+\) dDC induced Tc and Th1 responses and were capable of inhibiting the LC-triggered Th17 response. Mice that lacked specific subsets of cutaneous DC were utilized to investigate the distinct roles of these subsets in T cell activation (Igyártó et al., 2011). Expression of Th17
derived cytokines (IL-17, IL-21 and IL-22) has been detected in ACD patients following exposure to contact allergen (Larsen et al., 2009; Peiser et al., 2012; Simon et al., 2014). Therefore, LC could have a role in the orchestration of ACD via its impact on Th17 mediated responses.

In the study by Igyarto et al. (2011) mice were injected initially with OT I cells, a MHC I restricted, ovalbumin (OVA)-specific CD8+ T cell line (Clarke et al., 2000). Subsequently, mice were infected with a transgenic C. albicans strain that was engineered to express the OVA-derived peptide to which the OT I cells respond. It was found that the level of proliferation of OT I cells in mice in which only LC were absent (Langerin-DTA) was not different from that observed in WT mice. In contrast, mice that lacked both LC and langerin+ dDC subsets (Langerin-DTR) exhibited decreased levels of OT I cell proliferation, suggesting that the langerin+ dDC population was responsible for cross-presentation of the OVA antigen (Igyártó et al., 2011). The results of Igyarto et al. (2011) were in accordance with the study by Bedoui and colleagues (2009) who showed that migratory langerin+ dDC isolated from mice that expressed OVA under the K5 gene constitutive promoter (hence OVA was expressed by keratinocytes), preferentially induced proliferation of OT I cells. Conversely, LC or langerin− dDC did not have the capacity to trigger division of OT I cells (Bedoui et al., 2009). Similarly, migratory langerin+ dDC, but not LC, from animals infected with herpes simplex virus (HSV) were the most potent at inducing proliferation of gBT-I cells (Bedoui et al., 2009), a MHC I restricted CD8+ T cell line specific for HSV derived antigen (Mueller et al., 2002). Lastly, studies of human cutaneous DC subsets revealed that CD141hi DC cells pulsed with Hepatitis B surface antigen were more effective in triggering Tc responses (measured by IFN-γ production) by CD8+ T cells restricted to Hepatitis B surface antigen. Moreover, CD141hi dDC [a subtype of CD1a dDC (Boltjes and van Wijk, 2014)] from human skin were shown, via transcriptional analysis, to be the equivalent of langerin+ dDC in mouse (Haniffa et al., 2012), that were also demonstrated to cross-present antigens (Bedoui et al., 2009; Igyártó et al., 2011).

However, there is still some controversy regarding the main cell subtype responsible for antigen cross-presentation in the skin. Recent approaches utilizing langerin-DTR mice revealed that expansion of OVA specific OT I cells was differentially regulated
depending on the site of exposure to OVA. Therefore, delivery of DT both 4 days and 1 day before immunization (conditions that deplete of both LC and langerin+ dDC); caused either no effect or decreased OT I expansion, when OVA was applied to ears and flank, respectively. Differential access to antigen at disparate sites was thought to be responsible for these observations, with LC having a more pronounced role in the flank as assessed by a higher proportion of tetramethylrhodamine (TRITC)+ LC arriving in the LN after exposure of flank skin to TRITC in comparison with exposure to the ear skin. However, when the role of LC was further investigated in the flank skin, it was found that LC were dispensable for activation of OT I cells, as their proliferation was only diminished when LC and langerin+ dDC were absent (Wang et al., 2008). The question of antigen cross-presentation abilities of various cutaneous DC is in fact the one that will determine which cell population is responsible for driving the adaptive immune response during CHS, as CD8+ cells activated via interaction with MHC I molecule are important effector cells during elicitation phase (Dearman et al., 1996b; Xu et al., 1996).

Skin is an active immune organ and is a home to several different populations of cutaneous DC. Some of those populations, including langerin+ dDC, have been described recently and are currently explored with regards to their role during ACD sensitization phase. It is possible that this newly discovered subset of cells might be the most efficient inducer of both CD4+ and CD8+ response. However, there is a certain level of redundancy in the immune system and while LC can perform this function, they might be more important depending on the type of allergen (Nakajima et al., 2012) or on the dose and site of exposure (Noordegraaf et al., 2010; Wang et al., 2008).

1.11 Keratinocytes

Langerhans’ cells are clearly the first immune cells that come into contact with the external environment and contact allergens. However, the most common cell type of the epidermis is a keratinocyte. Although keratinocytes are not immune cells as such, they are a rich source of cytokines and chemokines (expressed both constitutively and inducibly) and thus are likely to be a major contributor to the cutaneous immunological microenvironment that can interact with exogenous danger signals and provide endogenous danger signals to which LC may respond.
The main building blocks of epidermis are keratinocytes and for a long time these cells were considered to be merely a scaffold for specialized cells, including melanocytes, LC and γδ T cells. However, it is increasingly obvious that keratinocytes are active participants in skin immunity (Gutowska-Owsiak and Ogg, 2012). Thus, keratinocytes have been shown to sense and respond to PAMP like nucleic acids, peptidoglycan (PGN), flagellin and LPS via pattern recognition receptors, such as membrane and intracellular TLR. Human keratinocytes and the human keratinocyte cell line HaCaT have been demonstrated to express TLR 1, 2, 3, 5, 6, 9 and 10 by qPCR and immunohistochemistry (IHC) (Köllisch et al., 2005; Miller et al., 2005; Nestle et al., 2009). The expression of TLR4 by keratinocytes has been controversial (Mempel et al., 2003), but it has been detected in both human primary keratinocytes and the HaCaT cell line (Pivarcsi et al., 2003). Additionally, keratinocytes detect and react to endogenous DAMP deriving from damaged tissues, like ATP or uric acid crystals through NLR receptors (Nestle et al., 2009).

Keratinocytes produce antimicrobial peptides (AMP), such as β-defensins and cathelicidins. These cationic peptides are responsible for inhibiting bacterial growth on skin surfaces by binding anionic microbial elements leading to membrane permeabilization. In addition to their bactericidal properties, AMP are able to modulate inflammation. β-defensins can recruit immature DC and memory T cells via CCR6 binding (Howell, 2007). The only known human cathelicidin, LL37, is constitutively expressed in leukocytes but is not present in healthy keratinocytes. Cathelicidin LL37 is produced by keratinocytes following skin injury and it has been shown to facilitate wound healing with its levels decreasing upon wound closure (Heilborn et al., 2003).

Upon exposure to hapten, during the sensitization phase, keratinocytes release inflammatory cytokines which contribute to the inflammatory environment required for DC migration (Albanesi, 2010; Bonneville et al., 2007). Throughout the elicitation phase keratinocytes can serve as non-professional APC because of their ability to constitutively express MHC II molecules which can be further induced by stimulation with IFN-γ (Albanesi et al., 1998; Nickoloff and Turka, 1994). At first it was thought that keratinocytes could only generate anergic T cells due to low levels of co-stimulatory molecules present on these cells. This is true for naive T cells. However,
Black et al., (2007) demonstrated that keratinocytes were capable of eliciting responses from antigen-experienced CD4+ and CD8+ T cells. Keratinocytes are an abundant source of cytokines and chemokines that act in an autocrine and paracrine manner and recruit T cells and neutrophils. Keratinocytes have been shown to express IL-1, TNF-α, IL-6, IL-8, IL-7, IL-15, IL-10, IL-12 and IL-18, as well as following cytokine receptors: IL-1RI and II, TNF-R, IL-6R, IL-18R, IL-10R, IL-4R, IL-13R, IL-17R, IL-12R (Gröne, 2002).

1.12 Role of innate immune cells in CHS reactions

As described above, ACD is a reaction which is mediated by members of adaptive immune system, CD4+ and CD8+ T cells in particular. However, cells of an innate immune response, such as natural killer T cells (NKT) cells, mast cells, neutrophils and γδ T cells also contribute to cutaneous immune and allergic responses, and are likely to be particularly important in the early events of contact sensitization, before the adaptive immune response has developed. Their roles are considered in subsequent sections.

1.12.1 NKT cells

In addition to priming naive T cells in local LN, another key event during skin sensitization is the production of hapten-specific IgM antibodies by the B1 B cell subset (Tsuji et al., 2002). The B1 subset is stimulated by IL-4 derived from CD1d restricted NKT. Natural killer T cells have been shown to be crucial for initiating ACD reactions. For example, depletion of NKT cells or cells expressing the CD1d molecule resulted in inhibition of the CHS reaction (Nieuwenhuis et al., 2005). It was demonstrated that sensitization with TNCB resulted in rapid (within 2h) expansion of NKT cells in the liver. No elicitation of CHS was observed in NKT deficient mice or mice reconstituted with NKT cells from IL-4 KO mice (Campos et al., 2003).

1.12.2 Mast cells

Mast cells reside in connective tissues in the body and in the mucosa. In the skin, they mainly localize around the perivascular area and in the proximity of nerve endings. Mast cells are important in mediating the IgE (immediate type) allergic response by releasing contents of their abundant cytoplasmic granules upon crosslinking of the high
affinity receptor for IgE (FceRI). These granules contain plethora of mediators, namely histamine, serotonin, proteases, cytokines and chemokines (Debenedictis et al., 2001). Mast cells also play a key role in initiating a rapid (antibody-independent) response to pathogens like bacteria and fungi, and recently it was found that they secrete histamine and serotonin after encountering ROS, independently of FceRI (Chodaczek et al., 2009), so they are able to respond to exogenous danger signals (PAMP) with the production of endogenous danger signals (DAMP).

Mouse and human LC have been shown to express histamine receptor H4, thus are able to respond to mast cell derived DAMP. Furthermore, LC migration was observed following exposure to H4 agonist, 4-methylhistamine (Gschwandtner et al., 2010), suggesting that there is an interplay between LC and mast cells. Indirect involvement of mast cells in the CHS was observed by Bryce et al. (2004). In IgE deficient mice, challenge with either oxazolone or DNFB resulted in diminished challenge-induced oedema in comparison with WT mice. Adoptive transfer experiments demonstrated that the presence of IgE was required during the sensitization phase. Normal ear swelling was restored by the delivery of exogenous IgE prior to sensitization; it was not necessary for the IgE to be specific for the hapten. For instance, in the study by Bryce and colleagues, IgE specific for trinitrophenol, DNP and OVA (all unrelated allergens) were all equally effective in restoring CHS in response to oxazolone. Additionally, in the absence of IgE, no LC migration was observed 18h following exposure to oxazolone. The ability of LC to mobilize was restored by a passive transfer of IgE. Further investigations demonstrated that a deficiency of mast cells or of FceRI also resulted in diminished ear swelling following challenge with oxazolone (Bryce et al., 2004). The requirement for the presence of mast cells during the sensitization phase of CHS was confirmed in transgenic mice where mast cells were ablated by exposure to DT (MaS TRECK Tg mice). Migration of DC to the LN following exposure to DNFB was diminished in MaS TRECK Tg mice compared with WT animals. Furthermore, the ability of DC from these transgenic mice to induce T cell proliferation was significantly diminished. In vitro investigations of the interactions between mast cells and DC have shown that the presence of mast cells and direct cell contact was required for optimal expression of co-stimulatory molecules CD40, CD80, CD86 and CCR7 receptor by DC. Finally, two factors were identified to be required for the effects of mast cells on DC: mast cell-bound TNF-α and ICAM-1 receptor expression by DC (Otsuka et al., 2011).
Indeed, there have been many reports implicating mast cells, and their release of inflammatory cytokines, in particular TNF-α, in the development of CHS reactions. The release of mast cell-derived inflammatory mediators, TNF-α and MIP-2, was shown to be mediated via ligation of FceRI. It was observed that in mice deficient in FcεRI β-chain (FcRβ), the CHS reaction to oxazolone was significantly decreased (Kobayashi et al., 2010). Through the use of mast cell deficient mice and adoptive transfer of mast cells from WT animals, the involvement of mast cell-derived TNF-α and MIP-2 in neutrophil recruitment during the CHS reaction to TNCB was established (Biedermann et al., 2000). The absence of either of these two cytokines from mast cells resulted in decreased ear swelling. Additionally, their study confirmed the requirement for the presence of neutrophils during the elicitation phase of CHS (Biedermann et al., 2000) (see section 1.12.3). In other studies with an alternative allergen, FITC, challenge induced CHS reactions were significantly decreased in both mast cell and TNF-α deficient mice (Suto et al., 2006). When mice lacking mast cells were reconstituted with bone marrow derived mast cells from WT mice, challenge-induced ear swelling was restored to levels observed in WT mice. However, if mast cell deficient animals were injected with mast cells from TNF-α KO mice, ear swelling was higher than that observed in mast cell KO, but lower than that recorded for WT mice, suggesting that mast cell-derived TNF-α is important, but is not the only mediator released by mast cells that affected the course of CHS reaction to FITC (Suto et al., 2006).

Interestingly, an immunosuppressive role for mast cells, and more specifically for mast cell-derived IL-10, has been proposed. Grimbaldeston et al. (2007) demonstrated that in mast cell KO mice, ear swelling following challenge with DNFB or urushiol (the active chemical in the plant poison ivy) was increased in comparison with WT animals. Ear swelling was diminished when mast cell KO mice were reconstituted with WT mast cells but not from IL-10 KO animals (Grimbaldeston et al., 2007).

Thus, it would appear that mast cells are an important cell type for the manifestation of CHS. The majority of reports suggest that their role is mainly to facilitate the induction phase of contact sensitization via the release of TNF-α and contact with cutaneous DC. However, there may be circumstances whereby mast cells can exert suppressive functions.
1.12.3 Neutrophils

Neutrophils are polymorphonuclear leukocytes that are key players in the control of infection and the acute immune response. They are present in the bloodstream and are the first cell type to be recruited to the site of inflammation. They have the ability to kill pathogens via several mechanisms, including phagocytosis and degranulation with subsequent release of molecules such as myeloperoxidase, LF and MMP-9, among others. Neutrophils are considered to be crucial for resolution of inflammation. Indeed, it has been shown that neutrophil deficiency in humans leads to severe immunodeficiency (Kolaczkowska and Kubes, 2013).

Neutrophils have been shown to play a role in the elicitation of CHS, with application of allergens inducing neutrophil recruiting chemokines, and the loss of neutrophils or neutrophil derived factors resulting in suboptimal CHS reactions. Thus, the neutrophil recruiting chemokine, Gro-α (CXCL1), or its mouse equivalent, KC, has been detected in the skin of both primed with DNFB and non-primed mice as soon as 30 minutes following exposure to DNFB, which was consistent with the rapid recruitment of neutrophils. The administration of anti-KC serum or anti-neutrophil antibody to mice prior to DNFB challenge decreased the ear swelling response (Dilulio et al., 1999). A diminished CHS response also was observed after depletion of neutrophils, however, that depletion was rescued by injection of CD8⁺ cells from sensitized mice (Dilulio et al., 1999). Leukotriene (LT) B4 is a lipid mediator present at the sites of inflammation and released by mast cells, neutrophils and macrophages, that acts upon myeloid cells via the BLT1 receptor. It was established that LTB4 was produced by neutrophils in the skin following mechanical injury (tape stripping). Additionally, the accumulation of neutrophils that was observed after tape stripping was dependent on their expression of both LTB4 and BLT1. Moreover, allergic skin reactions to OVA were diminished in BLT1 KO mice, but could be restored by transfer of neutrophils from WT mice (Oyoshi et al., 2012). Overall, this study demonstrated that neutrophils and the signalling pathway LTB4-BLT1 are essential for full presentation of allergic skin inflammation to the protein allergen OVA (Oyoshi et al., 2012).
1.12.4 Skin resident γδ T cells

Another, innate immune cell population that may play an important role in the induction of skin sensitization are the γδ T cells, which are found in both the epidermis and the dermis of mice.

It was long thought that in the epidermis the only cells of hematopoetic origin were LC. In 1985 a novel population of cells was described in the epidermis (Romani et al., 1985). These cells were later named DETC. They are characterized by expression of γδ receptor that distinguishes them from ‘conventional’ αβ T cells (Steiner et al., 1988). DETC, which almost exclusively express Vγ3/Vδ1 variant of γδ receptor, were identified as producing IL-2, expressing IL-2R and exhibiting cytotoxic potential (Kaminski et al., 1993). It was shown that DETC stably interact with neighbouring KC through γδ TCR. Their dendrites were mostly directed towards stratum granulosum, with only about 30% of cellular protrusions being motile (Chodaczek et al., 2012). Aryl hydrocarbon receptor (AhR) is a xenobiotic-regulated transcription factor which upon ligation affects many of the cells of the immune system. It has been recently demonstrated that AhR plays a crucial role in the development of DETC. DETC are seeded to the epidermis during embryogenesis and are not repopulated from the peripheral precursors and AhR KO mice display a marked reduction in the number of DETC (Kadow et al., 2011).

DETC are not motile cells, hence they are not found in the LN (Gray et al., 2011), however, this view was recently challenged by Nielsen at al. (2014) who demonstrated the presence of DETC in the LN (Nielsen et al., 2014). DETC express TLR and recognize self antigens from neighbouring keratinocytes, as well as invading pathogens (Gray et al., 2011). However, despite their identification 30 years ago exact ligands for γδ receptors of DETC are not well characterized. It was recently discovered that DETC ligands are expressed in the fetal thymus prior to DETC distribution to the skin. Furthermore, recombinant γδ receptor was rapidly bound to keratinocytes on the edge of wounded skin. Despite the identification of the presence of the ligand its specific nature was not identified (Komori et al., 2012). DETC have been shown to be an important constituent of the cutaneous immune response. DETC recognize antigens presented by injured, stressed or transformed keratinocytes (Havran et al., 1991). Their maintenance
in the epidermis is sustained by keratinocyte-derived cytokines, IL-7 and IL-15. DETC in turn release fibroblast growth factor 7, which participates in maintaining keratinocyte homeostasis, IL-2, IL-3, GM-CSF, IFN-γ and TNF-α, which interact with keratinocytes, as well as act upon DETC themselves (Jameson and Havran, 2007). Interestingly, TNF-α transcripts are present in DETC in the steady state, but are only released upon activation (Macleod and Havran, 2011).

Similarly, γδ T cells have been identified in the human epidermis, in addition to a population of conventional (αβ) T cells, that are absent from mouse epidermis under physiological conditions (Bos et al., 1990; Dupuy et al., 1990; Ebert et al., 2006). Despite the presence of γδ T cells in the human epidermis, these cells were not deemed to be equivalent to DETC, based on their differential numbers and localization in comparison to mouse epidermis (Bos et al., 1990). However, more recent studies have demonstrated the role of both conventional (αβ) and un-conventional (γδ) T cells in the human epidermis, in the wound healing process via production of insulin-like growth factor 1 (IGF-1) (Toulon et al., 2009). Correspondingly, DETC also have been demonstrated to produce IGF-1 during steady state and wound healing suggesting similarities between both T cells populations from human epidermis and the DETC subset in mouse epidermis (Sharp et al., 2005).

There are some indications that DETC may play a role in contact allergic responses. As mentioned previously, AhR KO mice have a reduced frequency of DETC and in these KO mice, ear swelling in response to challenge with FITC was reduced in comparison with WT counterparts. This was shown to be due to decreased GM-CSF production in the epidermis of AhR KO mice, as DETC are the source of this cytokine, and subsequent impairment in LC maturation (Jux et al., 2009). The role of DETC in the DNFB-induced CHS was analyzed by Nielsen et al. (2014). They found that ear swelling following challenge of sensitized mice was lower in γδ and IL-17 KO animals. Furthermore, they demonstrated that DETC became activated and produced IL-17A following challenge with DNFB (Nielsen et al., 2014).

Whereas DETC cells were first described in 1985, it was not until 2011 that a mouse dermal population of γδ T cells was identified and characterized. They were found to be motile and they were observed to move at the speed of 4μm/minute. Dermal γδ T cells
are radioresistant and were established to be the main source of IL-17 (an important proinflammatory cytokine that targets neutrophils and epithelial cells) in the skin and in the LN (Gray et al., 2011). In the peripheral LN γδT cells comprise 0.1% of all cells. They were demonstrated to be Vγ3− as opposed to Vγ3+ DETC. Dermal γδ T cells had similar phenotype to those found in peripheral LN: CCR6hi, IL-7Rαhi, CXCR6hi, and high for CD103. DETC on the other hand were CCR6lo and IL-7Rαlo. Dermal γδ, but not DETC, when stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycin produced high levels of IL-17A, IL-17F and IL-22. The combination of IL-1β and IL-23 also stimulated the rapid production of IL-17A by peripheral LN and dermal γδ T cells (Gray et al., 2011). Almost concurrently with Gray et al. (2011) a dermal population of γδ T cells in mouse was identified by Cai et al. (2011) and Sumaria et al. (2011). Cai et al. (2011) have described a population of dermal cells that produced IL-17 following exposure to IL-23. Analysis at the level of transcription revealed that dermal γδT cells expressed constitutively IL-23R, as well as the chemokine receptors CCR1, CCR2, CCR4, CCR5, CCR6, CXCR3, and CXCR4.

As reported by Gray et al. (2011) dermal γδ T cells are motile and migrate to the LN, however, their homing to the LN was not dependent on CCR7 expression, in contrast to conventional αβ T cells. Both δγ and αβ T cells were shown to express skin-homing receptors: CCR4 and CCR10. However, αβ cells exhibited higher levels of CCR4 transcripts, while γδ cells expressed more of CCR10 mRNA. Ligands for CCR4 and CCR10 are CCL17 and CCL27, respectively. The former chemokine is expressed by the stressed skin, while CCL27 is constitutively present in the epidermis, which gives a clue about the roles of these distinct T cell subsets, namely γδ T cells are present in the skin under physiological conditions, whereas αβ T cells are recruited to the skin during inflammation (Vrieling et al., 2012).

The role for γδ T cells in facilitating the elicitation phase of CHS has been demonstrated some years ago. It was demonstrated by Ptak et al. (1992) that γδ T cell population with phenotype CD3+, CD4−, and CD8+ was required for the transfer of sensitization to oxazolone and TNCB (Ptak and Askenase, 1992). Further studies identified that successful transfer of sensitization to PPD can only occur when both αβ and γδ T cells were transferred (Yokozeki et al., 2001), suggesting a crucial role of the γδ T cell population in sensitization to chemical allergens. It was later established that γδ T cells present in the dermis were also a source of IL-17 (Gray et al., 2011). Production of IL-
17 in the skin following exposure to DNCB has been established by Hayes (2012). He has used AhR, mice that lack γδ T cells specifically in the epidermis, while their dermal γδ T cell population is intact, and γδ T cell KO mice that do not have any γδ T cells. Hayes found that exposure to DNCB resulted in IL-17 production by the skin of AhR KO but not γδ T cell KO animals pointing to dermal γδ T cell subset as a cellular source of IL-17 following exposure to contact allergen (PhD thesis Hayes, 2012).

Contact hypersensitivity reactions have been considered for a long time to be a useful model for investigation of adaptive cutaneous immune responses. However, it has become increasingly clear that innate immune cells are necessary for the execution of both sensitization and elicitation phases. In addition to mast cells and neutrophil populations, a novel γδ T cell subset is emerging as a key participant of ACD with its ability to produce IL-17 (PhD thesis Hayes, 2012). Indeed, absence of IL-17 producing γδ T cells have been shown to render mice resistant to imiquimod-induced psoriasis-like dermatitis (Gray et al., 2013).

1.13 Characteristics of lactoferrin

Not only is contact sensitization an important occupational health issue, but it also represents a tractable system with which to investigate the mechanisms of cutaneous immune and inflammatory responses and to explore the activity of potential immunomodulatory/anti-inflammatory molecules that may lead to novel therapeutic strategies.

One such molecule in which this laboratory has had an interest is lactoferrin (LF), an 80kDa glycoprotein, and a member of the iron-binding protein family, that includes transferrin (TRF), OVA and melanotransferrin (González-Chávez et al., 2009). Lactoferrin is secreted by epithelial cells and is found in serum, milk, tears, saliva, pancreatic juice, small intestine secretions, bronchial and vaginal mucus and seminal plasma (Legrand et al., 2005). Lactoferrin is also present in secondary neutrophil granules (Yang et al., 2009). The concentration of LF depends on the physiological state of the organism. The serum concentration of LF is around 0.4-2 mg/l in healthy individuals, but it can reach as high as 200 mg/l during septicaemia. The concentration of LF in colostrum is up to 7g/l reducing to 1g/l in mature milk (Legrand et al., 2005). Indeed, the presence of LF has been recently considered to serve as a diagnostic tool for
distinguishing between inflammatory bowel disease and irritable bowel syndrome, with significantly higher LF levels found in the former condition (Sidhu et al., 2010).

Figure 1.6 Structure of human lactoferrin.

The structure of the LF molecule comprises of two domains, each consisting of an N-lobe and a C-lobe. Both domains of the protein show significant homology with one another. Each lobe is further divided into parts 1 and 2 (sections N1, N2, C1, C2). Sections N1 and C1 create domain 1 and similarly N2 and C2 form domain 2. Each domain has ability to bind one atom of iron (Fe$^{3+}$) (represented by red circles). Broken line shows location of lactoferricin (Baker et al. 2009).

The amino acid sequence of LF is highly conserved between species, with 70% homology between man, cow, pig and mouse. In addition to similarities in the primary structure, LF from the different species are alike with respect to their three dimensional organization (Baker and Baker, 2009). The LF molecule comprises of two lobes: an N-
lobe (residues 1-333) and a C-lobe (residues 345-692) with a hinge region (residues 334-344) between them, that accounts for the flexibility of LF molecule (Legrand et al., 2008). Both parts of the protein show significant homology (Figure 1.6). Metal binding by LF is dependent on simultaneous binding of two HCO$_3^-$ (or one CO$_3^{2-}$) anions. Iron sequestration by LF is a reversible process, hence the LF molecule can exist in Fe$^{3+}$ free (apo-LF; open) and bound (holo-LF; closed and resistant to proteolysis) state (Anderson et al., 1989). In the native state LF is 15-20% saturated with iron. When the level of iron binding is below 5% it is considered to be iron free or apo-LF (Steijns and van Hooijdonk, 2000). However, iron transport is not considered to be major role of LF, as there have been many reports demonstrating its importance to the immune system. Interestingly, however, LF has been shown to have 260 times higher affinity for iron than of blood serum TRF at physiological pH, and LF has ability to retain iron at much lower pH than TRF, possibly to endure acidic environment in the stomach of newborns (Steijns and van Hooijdonk, 2000).

### 1.14 Microbiocidal effects of lactoferrin

The first function ascribed to LF was its ability to inhibit bacterial growth (Oram and Reiter, 1968). At first, the bacteriostatic activity of LF was attributed to the iron-chelating properties of the protein. However, a study from Arnold et al. (1982) suggested that the action of LF is more complex than simple iron ion depletion. This study demonstrated that *Streptococcus mutans* could survive up to 72h in an iron free environment but incubation with human apo-LF resulted in a loss of viability after 1h (Arnold et al., 1982). It is recognized now that the mechanism of LF antimicrobial action lies in its capacity to bind LPS. Lipopolysaccharide is the main component of the outer membrane of Gram negative bacteria. It consists of antigen O, core polysaccharide and lipid A, with LF binding the lipid A portion (Appelmelk et al., 1994). The N-terminus of both bovine and human LF is highly cationic due to the presence of 4 arginines (Arg) (Gly$^1$-Arg$^2$-Arg$^3$-Arg$^4$-Arg$^5$) which are necessary for an effective binding of human LF to LPS, heparin and DNA (van Berkel et al., 1997).

As well as having antibacterial properties, LF has been shown to have an antiviral potential. The ability of bovine LF to reduce viral infection rates has been observed for a variety of viruses, including human cytomegalovirus, HSV 1 and 2, respiratory
syncytial virus and human immunodeficiency virus (HIV) (van der Strate et al., 2001). In addition to antibacterial and antiviral properties, LF has some antifungal and antiparasitic features. Human and bovine milk were found to inhibit in vitro growth of Entamoeba histolytica (E.histolytica), an intracellular parasite that causes amoebiasis. The antiparasitic effect was achieved due to the action of LF together with lysozyme and IgA. Since E.histolytica requires iron for its growth, perhaps it is not surprising that the greatest inhibition of parasitic growth was accomplished with the apo-LF form (León-Sicairos et al., 2006). Additionally, binding of LF to human blood monocytes increased both the number of parasites phagocytosed and the percentage of cells involved in phagocytosis, in comparison with cells cultured without LF (Lima and Kierszenbaum, 1985).

1.15 Immunomodulatory functions of lactoferrin

As described previously, LF has the ability to interact with a wide variety of microorganisms, and thereby to inhibit their invasion of the host organism by impeding their growth or blocking their entry into cells. In addition to the interactions with pathogens, as described before, LF has immunomodulatory properties. Lactoferrin has been shown to affect cell migration, cytokine and chemokine production and activation of DC (Actor et al., 2009). Lactoferrin is present in neutrophil granules, and is a member of a group of proteins called alarmins. Alarmins are part of the innate immune system and can recruit innate immune cells into a site of inflammation and subsequently activate them (Yang et al., 2009). A study by de la Rosa et al. (2008) demonstrated the selective ability of a commercially produced, recombinant human LF produced in Aspergillus niger var. awamori (trade name: talactoferrin-α; TLF) to trigger in vitro chemotaxis of human peripheral monocytes, but not DC or neutrophils. However, the same authors demonstrated that intraperitoneal administration of TLF in mice induced accumulation of neutrophils and monocytes/macrophages (de la Rosa et al., 2008). Bournazou et al. (2009) reported that in vitro human LF, independent of its iron saturation status, inhibited migration of human neutrophils, isolated from peripheral blood, towards various chemoattractants (N-Formylmethionyl-leucyl-phenylalanine, C5a, IL-8), as well as neutrophil activation, as measured by CD11b expression. Moreover, in vivo human LF decreased the number of accumulating neutrophils in a mouse thioglycollate peritonitis model (Bournazou et al., 2010; Bournazou et al., 2009).
In another study from the same group, it was found that human LF had an ability to inhibit in vitro migration of eosinophils isolated from human blood towards eotaxin (Bournazou et al., 2010). Thus, the data available on LF and cell migration of different cells types are conflicting. Possible reasons for these apparent discrepancies are the different platforms and organisms that were used, e.g. in vivo in mice versus in vitro using human cells. Additionally different LF molecules were used (recombinant versus native LF).

In line with its role as an alarmin LF has been shown to promote APC maturation. Talactoferrin-α triggered maturation of human monocyte derived DC, as assessed by up-regulation of co-stimulatory markers (CD80, CD83), MHC II expression and enhanced ability to trigger lymphocyte proliferation (de la Rosa et al., 2008). In a separate study, TLF was shown to promote DC maturation as demonstrated by increased expression of co-stimulatory molecules (CD80, CD83, CD86), HLADR, and of chemokine receptors (CXCR4, CCR5, CCR6, CCR7). Treatment with TLF also inhibited the ability of DC to process antigen and increased DC activation of T cells (Spadaro et al., 2008). Both studies demonstrated an impact of LF on APC maturation. Importantly, Spadaro et al.(2008) found that the action of TLF was not due to LPS contamination (Spadaro et al., 2008). Lactoferrin has been reported to influence chemokine and cytokine expression in both inhibitory and stimulatory ways. A study by Crouch et al. (1992) showed that human LF inhibited IL-2, IL-1 and TNF-α secretion from lymphocyte cultures (Crouch et al., 1992). Talactoferrin-α treatment of human peripheral blood derived mature DC significantly increased the secretion of IL-8, CXCL10 and decreased IL-6, IL-10 and CCL20 (Spadaro et al., 2008). Haversen et al. (2002) investigated the action of human LF on the human monocytic cell line, THP-1, and showed that LF inhibited TNF-α, IL-1β, IL-6 and IL-8 secretion from LPS stimulated cells. The inhibitory activity of human LF was observed whether LF was added to the culture 30 minutes before or after LPS introduction (Håversen et al., 2002). The experimental approach used in this latter paper (Håversen et al., 2002) supported the assumption that the effects of LF are not solely due to LPS sequestration, however, with the relatively short exposure time (30 minutes) it could still be argued that at least some of the observed inhibition was due to LPS binding. A more convincing demonstration would require removal of free LPS from the system prior to the addition of LF, or the use of an LPS-independent stimulus to promote THP-1 activation.
contrast to results from Haversen et al. (2002), were findings from Curran et al. (2006), which demonstrated in a murine macrophage cell line the ability of bovine LF to stimulate TNF-α and IL-6 production at levels comparable to LPS stimulation. Furthermore, LF was ineffective at inhibiting cytokine production in response to LPS irrespective of the time of bovine LF addition to the culture (Curran et al., 2006). Thus, data concerning LF influence on cytokine production are conflicting, and often contradictory. The discrepancies between studies by Haversen et al. (2002) and Curran et al. (2006) can be in part explained by the fact that both groups used LF preparations of different origin (derived from human or bovine sources, respectively) and the platforms used for investigation of LF action were also different (a human cell line was used by Haversen et al. (2002) and a murine cell line together with primary murine cells were utilized by Curran et al. (2006)). This suggested that despite structural and functional similarities between human LF and bovine LF molecules their precise mechanisms of action may be different and may also be cell specific.

1.15.1 The impact of lactoferrin on Langerhans’ cell migration

Langerhans’ cells are a fascinating subset of DC with multiple roles during steady state and inflammation. As mentioned before, using cytokine neutralizing antibodies, LC migration in response to allergens has been shown to be dependent on at least two cytokine signals IL-1β and TNF-α (Cumberbatch et al., 1997b). In the murine epidermis IL-1β is thought to be produced exclusively by LC (Enk et al., 1993), whereas TNF-α is likely to be primarily a keratinocyte product (Enk and Katz, 1992a). In addition to allergen-induced LC migration being mediated via these cytokines, the recombinant cytokines themselves have been shown stimulate LC mobilization. Thus, intradermal injection of TNF-α was also demonstrated to induce LC migration and DC accumulation in the draining LN (Cumberbatch et al., 1997b). The effect of injection of recombinant IL-1β was similar to that of TNF-α, however, with one important difference: the kinetics of the response was somewhat slower. Thus, TNF-α induced LC migration took place within 30 minutes, whereas the effects of IL-1β were not apparent until 2h after exposure. Higher doses of IL-1β did not alter the kinetics of LC mobilization. With respect to DC accumulation, TNF-α treatment augmented DC levels in the draining LN as soon as 2h after its delivery. In contrast, IL-1β-induced DC
accumulation was apparent 4h after injection and within 17h both cytokines stimulated similar levels DC in the LN (Cumberbatch et al., 1997a).

The authors concluded that TNF-α and IL-1β provide pivotal, but independent, signals for the reduction of LC in the epidermis and the subsequent increase in DC in the draining LN (Cumberbatch et al., 1997b). Given that oxazolone-induced migration was dependent on signals from both IL-1β and TNF-α, both anti-IL-1β and anti-TNF-α antibodies inhibit allergen induced migration. Loss of LC from the epidermis induced by exogenous IL-1β is inhibited by anti-TNF-α antibody (and vice versa), as both signals are considered to be required for LC mobilization. Administration of recombinant exogenous IL-1β alone triggers LC migration because it induces the production of TNF-α and delivery of the exogenous TNF-α alone is sufficient due to the release of endogenous IL-1β.

Furthermore, it seems that very similar mechanisms regulate LC migration in humans. Cumberbatch et al. (1999, 2003) investigated the influence of homologous recombinant TNF-α and IL-1β on LC migration in human skin and found that intradermal delivery of either cytokine caused the dose-dependent loss of LC from the epidermis (Cumberbatch et al., 2003; Cumberbatch et al., 1999d). Rambukkana et al. (1996) demonstrated that in short-term in vitro human skin explant cultures allergen treatment (DNFB, NiSO₄ and DNCB) reduced LC numbers and levels of IL-1β expression increased. Addition of anti-IL-1β antibody to the culture resulted in diminished levels of LC migration, suggesting that in humans, similar to mice, the initiation of allergen-induced LC migration depends on a signal from IL-1β (Rambukkana et al., 1996). The studies described clearly demonstrate that a regulation of LC migration is governed by the same mechanisms in human and in mice, and that they depend on a local availability of TNF-α.

Therefore, this system was chosen by Cumberbatch et al. (2000) to examine the influence of LF on allergen and cytokine induced LC migration and DC accumulation in mice. Those investigations revealed, that intradermal delivery of mouse LF was able to inhibit LC mobilization and LN DC accumulation, in response to exposure to oxazolone, and to IL-1β, but not TNF-α injection (Cumberbatch et al., 2000b). Moreover, it was established that that topically applied human native LF also was able to inhibit LC migration in mice upon oxazolone stimulation and intradermal injection of
homologous IL-1β, but not TNF-α (Almond et al., 2013). Therefore, data suggested that LF exerted its inhibitory influence on LC migration and DC accumulation in the LN by blocking *de novo* production of TNF-α (Almond et al., 2013; Cumberbatch et al., 2003; Cumberbatch et al., 2000b).

The studies in mice were followed up with experiments in healthy human volunteers. Griffiths et al. (2001) tested the impact of LF on allergen, DPC, and TNF-α induced LC mobilization. Results were similar to those obtained in mice. Topically applied homologous, recombinant LF inhibited LC migration in response to DPC, but had no effect on TNF-α induced LC mobilization. In addition to blocking the decrease in LC numbers in the epidermis, LF reduced the clinical and histological signs of inflammation in DPC treated sites (Griffiths et al., 2001). Furthermore, it was shown, that topically applied LF blocked IL-1β triggered LC migration (which, like chemical allergen-induced LC migration, is dependent on *de novo* production of TNF-α) and also inhibited IL-1β-triggered TNF-α production in the skin. These results confirmed the mechanisms of LC migration in man and mouse, and reinforced the view that LF acts via inhibition of TNF-α production (Cumberbatch et al., 2003).

### 1.16 Lactoferrin receptors

An important step in elucidating either the immunostimulatory or suppressive mechanisms of LF activity is to determine to what receptor it binds. Due to its cationic nature, LF binds to many anionic molecules, and that has impeded the identification of specific receptors. Many different receptors on various cell types have been identified to date.

The first LF receptor (LFR) to be identified was isolated from human intestine (Kawakami and Lönnerdal, 1991) and it was expressed in a variety of the adult and foetal tissues with high levels found in adult heart and foetal small intestine (Suzuki et al., 2001). This LFR was established to be species specific and had a molecular weight of approximately 114kDa (Kawakami and Lönnerdal, 1991) and subsequently has been shown to be a trimer or tetramer of 36kDa subunits which binds LF in Ca$^{2+}$ dependent, saturable fashion. Receptors for LF were also discovered in the liver. Two types of LFR also have been found on rat hepatocytes: low affinity LFR that was Ca$^{2+}$ independent,
and a high affinity Ca\(^{2+}\) dependent receptor (Bennatt and McAbee, 1997). The uptake of bovine LF by rat hepatocytes is independent of iron status and is mediated by the high affinity receptor via clathrin-dependent endocytosis. Subsequently, LFR is recycled onto the cell surface (McAbee et al., 1993). Despite similarities (presence of low and high affinity receptors; reliance on Ca\(^{2+}\)) between LFR in the intestine and liver, there is no evidence so far that these receptors are equivalent. As LF is known to have immunomodulatory effects \textit{in vivo} (Troost et al., 2003) and \textit{in vitro} (Guillén et al., 2002), considerable efforts have been made to identify LFR on the cells of the immune system. Human LF was found to bind to human peripheral blood adherent mononuclear cells, and B cell, but not T-cell, enriched lymphocytes in Ca\(^{2+}\) dependent fashion (Bennett and Davis, 1981). The THP-1 human monocytic cell line (Tsuchiya et al., 1980), that upon PMA stimulation differentiates into macrophages, is used frequently as a model for macrophage responses and has been utilized in a study in which human LF was found to bind to THP-1 macrophages via two receptors: one high affinity and one low affinity. The study showed, that iron status of human LF did not influence its binding by THP-1 macrophages and that protein, as well as oligosaccharide, structures of human LF participated in its recognition by LFR (Eda et al., 1996). After further investigation, LFR on THP-1 macrophages was identified as nucleolin (Hirano et al., 2005). Nucleolin is a 105kDa protein involved in interactions with RNA and proteins in the nucleus, nuclear transport as well as viral infection of cells, i.e. with HIV (Ginisty et al., 1999). In 2004 Legrand et al. (2004) published an extensive study that revealed nucleolin as the LFR for native human LF on the human cancerous mammary gland cell line, MDA-MB-231. They demonstrated that nucleolin binds human LF and undergoes endocytosis via a clathrin dependent mechanism. When investigating binding sites for native human LF Legrand et al. (2004) used mutant Chinese hamster ovary (CHO) cell lines that lack expression of surface proteoglycans, to establish their role in human LF binding. The wild type CHO cell line had two human LF binding sites: the higher affinity receptor (nucleolin), and lower affinity binding site, corresponding to the presence of proteoglycans. Utilizing the cell line CHO618 that lacks proteoglycan expression, it was shown that both LF lobes, namely the C- and N-lobe, participated in human LF and bovine LF binding to nucleolin, whereas, basic LF regions did not contribute to nucleolin binding. In the CHO cell line nucleolin, as well as proteoglycans, was shown to be necessary for human LF endocytosis (Legrand et al., 2004b).
Identification of LFR has been a difficult process due to the nature of LF and its non specific binding to proteoglycans. Despite these obstacles, researchers have been successful in characterizing various LFR that are cell specific. The presence of both low and high affinity binding sites for LF on different cell types and their dependency of the high affinity sites on Ca\(^{2+}\) suggest similarities between the various LFR that have been identified. However, these receptors are not identical as they have different molecular weights.

In addition to proteins described primarily as LFR, it was demonstrated that TLR4 might be required for some of LF functions (Ando et al., 2010). Indeed, it has been demonstrated recently that TLF triggers mouse and human DC maturation via interaction with TLR 2 and 4. Requirement for TLR2 and 4 in TLF-induced process of DC maturation was confirmed by utilization of small interfering (si) RNA and KO mice. First, CD14\(^+\) cells isolated from human PBMC were incubated with either siRNA against TLR2 and 4 or scrambled controls and further cultured with 1000μg/ml TLF. Cells in which TLR2 and 4 were absent (siRNA group) exhibited decreased expression of DC maturation markers (CD40, CD80 and CD83), whereas these were present in the control group (scrambled; the same nucleotide content as siRNA group but in a random order). Similarly, a crucial role for these two TLR was demonstrated in mice. When stimulated with TLF bone marrow cells from double TLR2 and 4 KO mice expressed lower levels of maturation markers compared with WT counterparts (Spadaro et al., 2014).

**1.17 Characterization of molecular mechanisms of LF activity**

Efforts have been made to characterize changes caused by LF at a molecular level. Haversen et al. (2002) showed that LF decreased TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-8 mRNA levels. Electrophoretic mobility shift assays demonstrated diminished NF-\(\kappa\)B binding to the TNF-\(\alpha\) promoter region in the presence of human LF (Håversen et al., 2002). The action of bovine LF is TLR4 independent with respect to IL-6 production, as peritoneal macrophages from wild type and TLR4 KO mice secreted IL-6 in response to bovine LF (Curran et al., 2006). A recent study by Ando et al. (2010) showed that native, LPS depleted human LF stimulated NF-\(\kappa\)B binding to DNA in THP-1 cells via a canonical pathway that involves phosphorylation of I\(\kappa\)B kinase (IKK). In addition, they found that
TLR4 is indispensable for LF-mediated NF-κB activation, a result that contradicts the previously mentioned study by Curran et al. (2006), that found macrophages deficient in TLR4 exhibited enhanced IκB degradation (IκB degradation by IKK is necessary for NF-κB activation and subsequent translocation into the nucleus). Collectively, these results again demonstrate that the immunomodulatory effects described for LF can be both stimulatory, as well as inhibitory, depending on the experimental system used. In order to confirm the dual properties of LF Ando et al. (2010) digested human LF with actinase E. That digestion resulted in a molecule with similar oligosaccharide content to the naïve molecule which retained the ability to activate NF-κB, whereas inhibition of LPS dependent NF-κB activation was compromised. Conversely, when human LF was digested with endo-β-galactosidase, the LPS inhibitory action of human LF was preserved and NF-κB activation was abolished. Digestion of LF with endo-β-galactosidase changed the sugar content of LF but conserved the polypeptide chain of the parent molecule. Interestingly, bovine LF did not activate NF-κB, although it strongly inhibited LPS triggered NF-κB activation (Ando et al., 2010) and bovine LF and human LF express different glycoprofiles (Spik et al., 1988). Ando et al. (2010) proposed, therefore, that the immunostimulatory function is attributable to carbohydrate part of the human LF molecule, whereas the immunosuppressive feature is due to the polypeptide moiety (Ando et al., 2010).

Another approach to elucidating the mechanism of action of LF involved the use of specific inhibitors to block multiple signalling pathways and observing if they change the effect of LF on cells. Spadaro et al. (2008) examined effects of multiple signalling pathways inhibitors on human monocyte derived DC activation enhanced by TLF. They found that SB203580, a selective p38 inhibitor, had strongest inhibitory action against TLF induced CD83, CXCR4 and CC7 expression. SB203580 and LY294002, a PI3K inhibitor, suppressed CXCL10 secretion from monocyte derived DC to a similar level of 80%. These results suggest that LF may signal via p38 (Spadaro et al., 2008). In further experiments pertussis toxin was used to inhibit TLF induced migration of human peripheral blood monocytes. Pertussis toxin blocks the assembly of Gia subunit with membrane bound G-coupled receptors. These results proposed that TLF potentially utilizes G-coupled receptor that still remains to be elucidated (de la Rosa et al., 2008). Finally the impact of LF on activation of the NF-κB pathway was investigated. This revealed that LF is responsible for inducting p65 subunit phosphorylation via interaction
with TNFR associated factor (TRAF) and is dependent on IKKα and IKKβ (Oh et al., 2007).

Reports addressing the immunomodulatory function of LF are often conflicting and suggest that dual role for LF exists in the immune system as both an immunostimulatory and an immunosuppressive agent. Ando et al. (2010) partially addressed this issue by demonstrating that differences in sugar content between human and bovine LF result in these two molecules having opposing properties in the process of NF-κB activation (Ando et al., 2010). However, the experiments performed by Ando et al. (2010), where they demonstrate immunosuppressive mechanism of LF, utilized LPS, which raises the question of whether LF is truly inhibiting NF-κB activation or merely reducing the availability of the stimulus (LPS). The results to date of attempts to elucidate the mechanism through which LF exerts its actions have created a complex view of LF. Indeed, as LF has many functions, it is anticipated that they can be mediated via different pathways.

In this laboratory the immunomodulatory effect of LF was thought to be mediated via inhibition of de novo TNF-α production, as concluded from the studies described previously. However, as highlighted in this section, there have been many LFR identified which can lead to multiple effects of LF depending on the target cell. Therefore, the exact mechanism of inhibition of oxazolone-induced LC migration by LF is still uncertain.

1.18 Lactoferrin derived peptides

It is of interest to identify which part of the LF molecule is responsible for its immunomodulatory effects on LC. In 1992 Bellamy et al. demonstrated the antibacterial and bactericidal properties of human and bovine lactoferricin (LFcinH and LFcinB, respectively), pepsin-derived peptides of LF (Bellamy et al., 1992). Human and bovine lactoferricins have been extensively studied in respect to their functional similarity to LF and have been found to possess many of the properties of intact LF molecule (Gifford et al., 2005). LFcinB was demonstrated to inhibit angiogenesis dependent on basic fibroblast and vascular endothelial growths factors (bFGF and VEGF, respectively) via inhibition of bFGF and VEGF binding to heparin on the surface of
endothelial cells, suggesting a role for LFcinB as an anti-cancer treatment (Mader et al., 2006).

Another LF-derived peptide is lactoferrampin (LFampin). Similarly to LFcinB it is derived from the N1 domain of bovine LF, which consists residues 268-284 and exhibits potent anti-candida and anti-bacterial properties similar to parent molecule (van der Kraan et al., 2004). Another described LF derivative is a peptide, called 'LFchimera', that constitutes of LFcinB 17-30 (N-FKCRRWQWRMKKL-G-C), a peptide derived from LFcinB, and LFampin 265-284 (N-DLIWKLLSAKEKFGKNSR-C) coupled together by a lysine amino acid. All three peptides showed the ability to inhibit the growth of strains of multidrug resistant microorganisms, obtained from the clinic: *Escherichia coli* (*E.* coli), *Staphylococcus aureus* (*S.* aureus) (Flores-Villaseñor et al., 2010) and *Pseudomonas aeruginosa* (Xu et al., 2010). All three bacterial species have a great relevance for long-term care as they target most vulnerable subjects (burn victims and bed-bound patients) and are extremely hard to eradicate.

Lactoferrin derived peptides have been extensively studied with respect to their antimicrobial roles. However, much less is known about their abilities to modulate the immune and inflammatory responses. Recent reports indicate that LFcinB acts as an anticancer agent due to its interaction with cancer cells. LactoferricinB has been shown to promote apoptosis in CCRF-CEM T-leukemia and Jurkat T-leukemia cell lines (Furlong et al., 2008). Examples of LF derived peptides described before show that they can have antimicrobial potential that is superior to that of the parent molecule. Therefore, it is of interest to identify the domain of LF that is responsible for immunomodulatory action of LF, as its effects may prove to be more pronounced than that of native LF. The peptide derived from the N lobe of human LF (GRRRRRSVQWCA), was demonstrated to specifically inhibit the enzyme myeloperoxidase in human PBMC and subsequently decrease level of ROS production following cells exposure to LPS (van der Does et al., 2012). Its ability to inhibit oxazolone induced LC mobilization was investigated in the studies described herein.
1.19 **Hypothesis, aims and experimental approach**

1.19.1 **Hypothesis**

Lactoferrin interacts with an unknown cutaneous cell or cell types and inhibits oxazolone and IL-1β induced LC migration via inhibition of cytokine signals.

1.19.2 **Aims**

This thesis sought to determine the mechanism of action of LF in modification of LC migration and inflammation.

1.19.3 **Experimental approach**

In order to examine the previously observed inhibitory effects of human LF on oxazolone and IL-1β induced migration in the mouse, it was essential to characterize cutaneous cytokine production at the level of message and protein following exposure to oxazolone in the presence and in the absence of LF treatment. Further clues to the effects of LF were gained by examining the effect of LF on oxazolone triggered changes in the LN, including proliferation and cytokine production. It has been mentioned in the previous sections that allergen triggered LC migration is dependent on signals from TNF-α and IL-1β (Cumberbatch et al., 1997b). Additionally, IL-1α, but not IL-1β, has been shown to be important in irritant-induced LC migration (Cumberbatch et al., 2002a). Therefore, these three cytokines were considered important in the process of LC migration. Consequently, cutaneous expression of selected genes was examined following intradermal delivery of these cytokines in the presence and in the absence of LF.

There are thousands of chemicals recognized for their ability to cause contact hypersensitivity (De Groot, 2008). For the purposes of investigating the mechanisms of ACD a few reference contact allergens are generally utilized in laboratory animals. The immunomodulatory effects of LF in the skin of mouse and human were examined previously using oxazolone and DPC, respectively. To broaden the knowledge about mechanism of action of LF another contact allergen, DNCB, was employed. Additional
work compared LC mobilization, cutaneous gene expression and cytokine production profiles induced by various contact allergens, respiratory allergen and an irritant. These experiments were designed to identify similarities and differences between chemicals and investigate their mechanism of action with particular emphasis on oxazolone and DNCB.

In addition to in vivo examination of effects of LF, two in vitro systems were used. The first approach aimed to investigate the mechanism of inhibition of TNF-α production by LF. The focus on TNF-α was derived from in vivo experiments in mice, which suggested that inhibitory effect of LF on oxazolone-induced LC mobilization was likely to be mediated via inhibition of de novo TNF-α production (Almond et al., 2013; Cumberbatch et al., 2003; Cumberbatch et al., 2000b). Additionally, work in humans confirmed that LF decreased TNF-α level in suction fluid following delivery of IL-1β (Cumberbatch et al., 2003). Therefore, a THP-1 cell line was utilized, which has been shown to express LFR upon treatment with PMA (Eda et al., 1996) and to produce TNF-α in a LPS-independent manner. The necessity for an LPS-independent stimulus was required due to the fact that LF was known to potently bind LPS (Appelmelk et al., 1994), therefore, it could decrease TNF-α secretion by THP-1 cells by simply sequestering the stimulus, which would impede the examination of mechanism of immunomodulation.

Another in vitro platform utilized keratinocyte cell lines (PAM 212 and HaCaT, mouse and human cell lines, respectively) and primary mouse keratinocytes. It was suspected that the production of TNF-α during LC mobilization was mediated by keratinocytes, the most abundant cell type in the epidermis. Therefore, keratinocytes were considered legitimate candidates for investigation of the immunomodulatory effects of LF. As well as examining LF, other peptides with immunomodulatory potential in vivo, namely thioredoxin and Pep-1, were considered.
Methods

2.1 Tissue culture

2.1.1 THP-1 cell line maintenance

THP-1 cells (Sigma-Aldrich, Poole, UK) were maintained in suspension culture (10⁵-10⁶ cells per ml) in T75cm² flasks in RPMI-1640 supplemented with L-glutamine (2mM) (Invitrogen, Paisley, UK) and a penicillin/streptomycin (100U/100μg/ml) cocktail (Invitrogen) (referred to as RPMI media) and 10% heat inactivated fetal calf serum (FCS) (referred to as complete RPMI media). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and passaged every 3 to 5 days.

2.1.2 Differentiation of THP-1 cells into macrophages

THP-1 cells were adjusted to a concentration of 10⁶ cells per ml following a cell count conducted using a CASY cell counter (Roche, Berlin, Germany) and 100μl or 500μl per well were seeded into flat bottomed 96 or 24 well tissue culture plates (plates), respectively (Corning, Ewloe, UK). Cells were stimulated with 100nM PMA (Sigma-Aldrich) for 3h, or for 24h, 48h, 72h, 96h and 120h. For the short incubations (3h) media was gently aspirated, replaced with RPMI media supplemented with 1% FCS and cells cultured for further 16h. The following day, the RPMI media supplemented with 1% FCS was changed. For 24-120h differentiation cells were cultured in RPMI medium with 5% FCS. Cells are referred to hereafter as 3h or 24h-120h macrophages depending on the time of incubation with PMA.

2.1.3 Stimulation of THP-1 cells

THP-1 cells were adjusted to a concentration of 10⁶ cells per ml in complete RPMI media following a cell count conducted using CASY cell counter (Roche) and 100μl per well were seeded into a flat bottomed 96 well plate (Corning). Cells were stimulated with PGN (Sigma-Aldrich) at a range of concentrations: 25μg/ml, 10μg/ml, 1μg/ml, 0.1μg/ml, 0.01μg/ml, 0.001μg/ml and 0.0001μg/ml for 6h or 24 h. Negative control cells were cultured with media alone. After 6 or 24h plates were spun down for 5
minutes at 1200 revolutions per minute (rpm) at 4°C. Culture supernatants were retained for analysis by enzyme-linked immunosorbent assay (ELISA). The cells were lysed in lysis buffer (20mM Tris-HCL (Sigma-Aldrich), 137nM NaCl (Fisher Scientific, Loughborough, UK) 20mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), 10% glycerol (Fisher Scientific), 0.5% Igepal (Sigma-Aldrich) and incubated on ice for 20 minutes. After centrifugation (5 minutes, 4°C), cell lysates were stored at -80°C until analysis by ELISA.

2.1.4 Stimulation of THP-1 macrophages

Cells in TC plates were differentiated into THP-1 macrophages as described in the section 2.1.2. Prior to use in the experiment, adherent cells in the plate were washed with PBS, and subsequently cultured with a range of doses (0.001-25μg/ml) of PGN alone. In some experiments cells were pre-treated for 1h with human native LF (Sigma-Aldrich) at a range of concentrations: 100μg/ml, 50μg/ml, 20μg/ml, 10μg/ml, 2μg/ml, 0.2μg/ml, 0.02μg/ml, and 0.01μg/ml or with medium alone (0μg/ml LF). After 1h PGN (25μg/ml) for 3h macrophages or 10μg/ml for stimulation of 24h-120h PMA activated THP-1 macrophages, or media alone was added, and the cells were incubated for a further 6h. After the incubation, cell lysates and supernatants were collected for ELISA as described in the section 2.1.3.

Additional controls for cytokine inhibition were incorporated into some experiments: dexamethasone (Sigma-Aldrich, formulated at 100nM in 0.005% dimethyl sulfoxide [DMSO]), which was added to the culture at the same time as LF. As dexamethasone was reconstituted in DMSO, the vehicle control of 0.005% DMSO was included.

2.1.5 HaCaT and PAM 212 cell line maintenance

HaCaT and PAM 212 cells were obtained from Professor Christopher E.M. Griffiths (School of Translational Medicine, University of Manchester, Manchester, UK) and Professor Alexander J. MacRobert (Institute of Biomedical Engineering, University College London, London, UK), respectively. Cells were maintained as adherent cultures in T75cm² flasks in complete RPMI media. Every 3 to 5 days cells were passaged as follows. Media was removed from the culture; cells were washed with RPMI media, subsequently washed with 0.02% EDTA solution (Sigma-Aldrich) and treated with
10mg/ml trypsin (Invitrogen) for 20 minutes at 37°C before the addition of complete RPMI media. Cells were enumerated by light microscopy following trypan blue staining and reconstituted in complete RPMI media at $10^6$ cells per ml.

### 2.1.5.1 HaCaT cell culture

Prior to use in the experiments cells were seeded at a concentration $10^5$cells per well into 12 well plates and cultured for 48h. Subsequently, cells were washed with RPMI media and incubated with a range of PGN doses (0.01-25μg/ml) for 24h or with 10μg/ml for 4h. In addition to incubation with a TLR2 ligand, HaCaT cells were cultured with cytokines. Firstly, HaCaT cells were incubated with a range of human recombinant IL-1β doses (Insight Biotechnology, Wembley, UK) (0.01-100ng/ml) for 24h or 2h or with 2ng/ml of IL-1β for 2, 4, 24 or 48h. Cells were cultured also with 10ng/ml of human recombinant TNF-α (R&D Systems Europe Ltd., Abingdon, UK) for 1, 2, 4, 6, 10 or 24h. In some experiments, prior to treatment with cytokines, cells were pre-treated for 2h with either a range (1-100μg/ml) of LF or BSA. Additional treatments included 1μM of dexamethasone in 0.05% DMSO, 200μg/ml of Pep-1 peptide (Pepceuticals, Enderby, UK; sequence: GAHWQFNALTVR) or 50μg/ml of thioredoxin peptide (Pepcueticals, sequence: CGPCKMIKP) or a scrambled peptide (Pepcueticals, sequence: IPCMPKCKG) with the same amino acid composition as thioredoxin peptide but in a random order.

### 2.1.5.2 PAM 212 cell culture

Prior to use in experiments, cells were seeded at a concentration $10^5$cells per well into 12 well plates and cultured for 48h. Subsequently, cells were washed with RPMI media and incubated with a range of TLR ligands: PGN 25μg/ml, PolyI:C 25μg/ml, LPS 10μg/ml, Flagellin 83ng/ml, R848 500ng/ml, CpG 10μg/ml as well as PMA 1μg/ml for 24, 48 or 72h. Additionally PAM cells were cultured with homologous recombinant cytokines, including a range (0.01-10ng/ml) of concentrations of IL-1β (R&D Systems) for 24h or 2h or with 2ng/ml of IL-1β for 0.5, 2, 4 or 6h and a range (0.01-100ng/ml) of concentrations of IL-1α (R&D Systems) for 2h. In some experiments, prior to treatment, cells were pre-treated for 1 or 2h with either a range (1-100μg/ml) of LF or 100μg/ml of BSA.
2.1.6 Primary keratinocyte preparation

New born (1-3 days old) BALB/c strain mice pups (bred in house from breeding pairs obtained from Harlan Olac (Bicester, UK)) were collected and terminated in accordance with Schedule 1 methods (Animal (Scientific Procedures) Act, 1986), by cervical dislocation. Following termination, pups were decapitated, limbs were removed and an incision was made down the ventral surface of the pup to allow for the skin to be peeled away. The skin was vigorously washed 3 times for the total of 10 minutes in Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich) with 5 times concentrated antibiotics-antimycotics cocktail (CELLnTEC, Bern, Switzerland). Subsequently, the skin was incubated overnight in 0.25% trypsin-EDTA solution (Sigma-Aldrich). The following morning, the skin was washed in a cold HBSS, the epidermis was peeled away, and placed (basal side down) in a 35ml Petri dish in 500μl of TrypLE Express (Gibco, Paisley, UK) for 15-20 minutes at room temperature (R/T). Afterwards, 2ml of CnT-57 media (CELLnTEC) was added to the culture dish and the epidermis was gently rubbed with blunt forceps on the base of the Petri dish. The cell suspension was centrifuged at 160g at RT for 5 minutes. The cell pellet was resuspended and seeded into 12 well plates at 4-5 x 10⁴ cells per ml. The following day, the media was changed to remove cells that have failed to adhere. The cells were monitored daily to assess their growth and the media was changed every 2 to 3 days, as required. When cells were 80% confluent media was changed to CnT-02 (CELLnTEC) and cells were used for experiments on the following day.

2.1.6.1 Primary keratinocyte culture

Cells were prepared as described in the section 2.1.6. Prior to use in the experiment, cells were washed with CnT-02 media and treated with various TLR ligands (PGN [0.1-10μg/ml], LPS [1μg/ml], CpG [1μg/ml]) for 24h. Additionally, primary keratinocytes were exposed to a range (0.001-10ng/ml) of murine recombinant IL-1β or IL-1α doses (R&D Systems) for 2h or to 2ng/ml of IL-1β or IL-1α for 2, 8 or 24h. In some experiments, prior to the addition of stimuli, cells were pretreated for 1 or 2h with either 10 or 100μg/ml of LF or 100μg/ml of BSA.
2.2 Immunohistochemistry

Cells (HaCaT, PAM212, primary keratinocytes) were grown as described previously in a 24 well plate (primary keratinocytes) or in a 24 well plate with a glass coverslip in each well (HaCaT, PAM212). After 48h, cells were washed with a phosphate buffered saline (PBS) and fixed with ice cold methanol (Fisher Scientific) for 10 minutes. Subsequently, cells were washed with PBS and incubated with 0.2μg/ml rabbit anti-keratin 14 (AF64) antibody (Covance, Leeds, UK) in 0.1% BSA in PBS at 37°C for 30 minutes. Afterwards, cells were washed again for 15 minutes with 0.05% Tween in PBS and incubated overnight at 4°C with FITC-labelled goat anti-rabbit polyclonal antibody at 10μg/ml. Then cells were washed and mounted onto a slide with VECASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK).

2.3 Mycoplasma testing

Cell lines were routinely tested for the presence of mycoplasma infection using EZ-PCR Mycoplasma Test Kit (Biological Industries, Kibbutz beit Haemek, Israel) according to the supplied protocol. Briefly, cells were spun down for 5 minutes at 1000rpm at 4°C. Afterwards, 2ml of supernatant was collected and spun for 10 minutes at 15,000rpm at 20°C, supernatant was gently aspirated and the remaining pellet was reconstituted in 20μl of buffer solution (supplied with the kit). After reconstitution, the sample was heated for 3 minutes at 95°C. The sample was amplified by PCR. The reaction mixture consisted of 15μl H2O, 3μl Reaction Mix (supplied with the kit) and 2μl sample (for positive control: 1μl of template + 1μl of H2O). PCR run parameters are presented in the Table 2.1.
Table 2.1 PCR run parameters for mycoplasma detection reaction.

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<th>Temperature</th>
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Test samples were amplified alongside positive template control. Following amplification, samples were detected by gel electrophoresis on 2% agarose gel. The presence of a band of 270kb size confirmed the presence of mycoplasma.

2.4 Enzyme-linked immunosorbent assay

2.4.1 Human TNF-α ELISA

Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated overnight at R/T with 50μl per well of mouse anti-human TNF-α (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) (both from Sigma-Aldrich) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1000pg/ml of recombinant human TNF-α (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-human TNF-α (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 250ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were
washed and blotted and 50μl of streptavidin conjugated to horseradish-peroxidase (Strep-HRP) (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of 3,3’,5,5’-tetramethylbenzidine (TMB One™; Invitrogen) was added, and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The optical density (OD) reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc, La Jola, USA). The lower limit of detection for human TNF-α ELISA was 16pg/ml.

2.4.2 Human IL-8 ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of mouse anti-human IL-8 (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 2000pg/ml of recombinant human IL-8 (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-human IL-8 (detection) antibody (R&D Systems) reconstituted in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline [TBS]) at a concentration of 20ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using
Graphpad (Graphpad Software Inc). The lower limit of detection for human IL-8 ELISA was 32pg/ml.

2.4.3 **Human IL-1β ELISA**

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of mouse anti-human IL-1β (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 250pg/ml of recombinant human IL-1β (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-human IL-1β (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 200ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for human IL-1β ELISA was 3pg/ml.

2.4.4 **Mouse TNF-α ELISA**

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of goat anti-mouse TNF-α (capture) antibody (0.8μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were
washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 2000pg/ml of recombinant mouse TNF-α (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse TNF-α (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 200ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse TNF-α ELISA was 32pg/ml.

2.4.5 Mouse IL-1β ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti-mouse IL-1β (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1000pg/ml of recombinant mouse IL-1β (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IL-1β (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 1.5μg/ml was added to each well. Following incubation on a
plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of 3TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse IL-1β ELISA was 16pg/ml.

2.4.6 Mouse IL-1α ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti-mouse IL-1α (capture) antibody (2.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1000pg/ml of recombinant mouse IL-1α (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone rather than cytokine were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IL-1α (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 100ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse IL-1α ELISA was 16pg/ml.
2.4.7 **Mouse IFN-γ ELISA**

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti-mouse IFN-γ (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 2000pg/ml of recombinant mouse IFN-γ (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IFN-γ (detection) antibody (R&D Systems) reconstituted in reagent diluent (0.1% BSA, 0.05% Tween 20 in TBS) at a concentration of 400ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450mm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse IFN-γ ELISA was 32pg/ml.

2.4.8 **Mouse IL-13 ELISA**

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti-mouse IL-13 (capture) antibody (4.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 4000pg/ml of
recombinant mouse IL-13 (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IL-13 (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 200ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of 3TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450mm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). Lower limit of detection for mouse IL-13 ELISA was 64pg/ml.

2.4.9 Mouse IL-17A ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti-mouse IL-17A (capture) antibody (2.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1000pg/ml of recombinant mouse IL-17A (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IL-17A (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 400ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes
incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse IL-17A ELISA was 16pg/ml.

2.4.10 Mouse IL-17F ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of goat anti-mouse IL-17F (capture) antibody (0.8μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1500pg/ml of recombinant mouse IL-17F (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing medium alone rather than cytokine were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse IL-17F (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 200ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse IL-17F ELISA was 23pg/ml.
2.4.11 Mouse CXCL9 ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of goat anti-mouse CXCL9 (capture) antibody (0.8μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in PBS) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 1000pg/ml of recombinant mouse CXCL9 (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse CXCL9 (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 200ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450nm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse CXCL9 ELISA was 16pg/ml.

2.4.12 Mouse CXCL10 ELISA

Plastic microtitre plates (Nunc) were coated overnight at R/T with 50μl per well of rat anti- mouse CXCL10 (capture) antibody (2.0μg/ml, R&D Systems). The plates were washed at least 3 times with wash buffer (0.05% Tween in) then plates were incubated with a 150μl of blocking solution (1% BSA in PBS) for 1h. Plates were washed as described before, and standards and samples were added. Standards were prepared by serial doubling dilutions in RPMI medium of the 4000pg/ml of recombinant mouse
CXCL10 (R&D Systems). Standards and samples were plated diluted in RPMI media in duplicate or triplicate, respectively, and were incubated on a plate shaker at R/T for 2h. Reagent blank wells containing RPMI medium alone were included on each plate and served as a blank to measure the background of the reaction. After 2h plates were aspirated, washed, blotted and 50μl of biotinylated goat anti-mouse CXCL10 (detection) antibody (R&D Systems) reconstituted in reagent diluent (1% BSA in PBS) at a concentration of 600ng/ml was added to each well. Following incubation on a plate shaker at R/T for 2h, detection antibody was aspirated, plates were washed and blotted and 50μl of Strep-HRP (R&D Systems) was added per well. After a 30 minutes incubation with the enzyme, the liquid was aspirated, plates were washed, blotted and 50μl of TMB One™ (Invitrogen) was added and the plates incubated for 20 minutes. Reaction between the enzyme and substrate (Strep-HRP and TMB) was stopped by adding 50μl of 0.5M sulphuric acid to all wells. The OD reading of each well was determined at the wavelength of 450mm using an ELISA plate reader (Titerek, Multiscan Plus). Data were analyzed using Graphpad (Graphpad Software Inc). The lower limit of detection for mouse CXCL10 ELISA was 62pg/ml.

2.5 Animal work

2.5.1 Animal source and maintenance

BALB/c and C57BL6 strain mice were obtained from Harlan Olac (Bicester, UK). Mice deficient in TNFRII (TNFRII/- strain mice; C57BL/6 strain background) (Peschon et al., 1998) were a kind gift from J.J. Peschon, Department of Molecular Immunology, Immunex Corp., Seattle, USA. Animals were 6-12 weeks old, with the exception of the experiment presented in the Figure 6.10A where animals were 20 weeks old. Mice were housed on sterilized wood bedding with materials provided for environmental enrichment. Food (Special Diet Services, Witham, UK) and water were available ad libitum. The ambient temperature was maintained at 21 +/- 2°C and relative humidity was 55 +/- 10% with a 12 h light/dark cycle. All procedures were approved by the UK Home Office and carried out in compliance with the UK Animals (Scientific Procedures) Act, 1986 under a Home Office granted Project Licence.
2.5.2 Chemicals and exposure

Mice received 25μl of 0.5% or 3% oxazolone, 0.25% or 1% DNCB, 0.25% DNFB, 25% TMA, 1% PPD, 1% DNTB all formulated in 4:1 acetone:olive oil (AOO); or 25% SLS in dimethylformamide (DMF) (all from Sigma-Aldrich) to the dorsum of both ears. The treatment time with chemicals was different depending on the endpoint that was tested. The exposure to oxazolone, DNCB, DNFB, PPD, SLS and TMA for the purpose of LC enumeration was mainly 4h, with the exception of TMA where exposure lasted 17h (unless stated differently in the figure legend). The treatment with oxazolone and DNCB for the explant experiments was either 2h or 6h (as stated in the figure legend). The exposure of mice to chemicals prior to analysis of ears by quantitative (q)PCR was for following times: oxazolone (0.5, 1, 2, 4, 6, 8, 16, 24, 72, 96 and 120h), DNCB (0.5, 1, 2, 4, 6, 8, 16 and 24h), DNTB and SLS (2, 4, 8 and 24h) and TMA (2, 4, 6, 8, 16 and 24h) ; after which time animals were sacrificed and ears were excised.

2.5.3 Exposure to lactoferrin

Native LF from human milk (Sigma-Aldrich) was reconstituted in PBS and added to aqueous cream BP (Boots, Nottingham, UK) to achieve final concentration of (0.5 or 5μg). Cream containing LF or cream alone (30μl) was applied to the dorsum of ears of mice.

2.5.4 Cytokine treatment

Mice received 30μl intradermal injections of recombinant murine TNF-α (50ng), IL-1β (50ng) or IL-1α (50ng) (R&D Systems) formulated in 0.1% BSA/PBS (or 0.1% BSA/PBS as vehicle control) to both ear pinnae using a fine gauge hypodermic needle. Four h after injection animals were sacrificed and ears were excised for LC enumeration or messenger (m) RNA extraction.
2.5.5 Antibody administration

Mice received 30μl intradermal injections of affinity purified goat anti-mouse anti-IL-1β (3μg; R&D Systems) or rabbit anti-mouse anti-TNF-α (10μg; PeproTech, London, UK) antibody to both ear pinnae using a fine gauge hypodermic needle. Normal goat serum (NGS) and normal rabbit serum (NRS; 1 in 5 dilution in PBS) were used as controls for anti-IL-1β and anti-TNF-α antibody, respectively. Two h following antibody administration mice were treated with either oxazolone or DNCB.

2.6 Preparation of epidermal sheets and LC enumeration

Ears were removed and the dorsal halves were incubated for 1.5h in 0.02 mM EDTA in PBS at 37°C. The epidermis was removed, washed in PBS and fixed in acetone at -20°C for 20 minutes. Epidermal sheets were stained for LC with 5μg/ml MHC II (FITC rat anti-mouse IA-IE 2G9 [BD Biosciences, Oxford, UK]) diluted in 0.1% BSA/PBS for 1h. Epidermal sheets were mounted onto slides using VECTASHIELD (Vector Laboratories Ltd, Peterborough, UK) and sealed with nail varnish. The frequencies of stained cells were assessed by fluorescence microscopy using an eyepiece with a calibrated grid (0.32 x 0.213) at x 40 magnification. Samples were blinded and for each sheet 10 consecutive fields were counted in the central portion of each ear. Data are expressed as the mean number of LC per mm² per group.

2.7 Homogenization of ear tissue

Ears were collected and immediately immersed in RINalater (Life Technologies) for a minimum of 12h. Subsequently, ears were cut with a scalpel into approximately 1mm² pieces and transferred into 350μl of lysis buffer with 1% of 2-mercaptoethanol (Life technologies) and homogenized with a T10 basic ULTRA-TURRAX homogenizer (IKA, Staufen, Germany) until most of the tissue was dispersed. Afterwards, samples were centrifuged at 14,000rpm for 5 minutes at 4°C, lysate was collected and mRNA was isolated as described in the section 2.9.
2.8 Preparation and analysis of lymph nodes for cytokine production

Draining LN were excised, pooled per animal and washed with PBS. Subsequently, they were placed in an individual (one per animal) well of a 24 well TC plate in complete RPMI media. Lymph nodes were disrupted and incubated with 500μg/ml collagenase (Sigma-Aldrich) and 500μg/ml DNase (Sigma-Aldrich) for 30 minutes at R/T and subsequently LN were mashed through 200μm metal gauze to obtain a single cell suspension (enzymatic method). Alternatively, LN were disrupted and mashed though metal gauze without enzyme treatment (mechanical method). Cells were counted using a CASY cell counter (Roche) and adjusted to $10^7$ cells per ml. Next, 1ml of cells was seeded into 24 well plates and incubated for 120h. Following incubation supernatants were collected, centrifuged stored at -80°C to be analyzed by ELISA.

2.8.1 Analysis of lymph node cell proliferation

Draining LN were excised and washed with PBS. Subsequently they were ruptured and mashed through metal gauze to achieve a single cell suspension (mechanical method as described in the section 2.8). Cells were enumerated using a CASY counter and 2x10^6 cells per well were seeded into a 96 well TC plate in the presence of 296Bq of [³H] thymidine (PerkinElmer, Cambridge, UK) for 24h. Following incubation, cells were harvested onto glass microfiber filter mats (Whatman, Kent, UK) using a cell harvester (Skatron, Norway). Filters were left to dry (minimum of 12h), and discs were placed in 10ml of scintillation fluid (Fisher Scientific). Radioactivity was measured by β-scintillation counting (Packard Tri Carb 2500TR Scintillation Counter, PerkinElmer, Inc., USA)

2.9 Isolation of messenger RNA

Messenger RNA was isolated using a PureLink RNA Mini Kit (Ambion, Paisley, UK) according to manufacturer’s instructions. Cells were lysed using 350μl of lysis buffer with 1% 2-mercaptoethanol. Cell or tissue lysates were collected and an equal amount of 70% ethanol was added to the sample, and was subsequently loaded onto the spin cartridge and centrifuged at 12.000g for 15 seconds (s). Next, 350μl of Wash Buffer I
was added and centrifuged at 12,000g for 15s. At this stage DNAse treatment was performed using PureLink DNase Set (Ambion). According to manufacturer’s instructions 10μl of PureLink DNase in 70μl of DNase I Reaction Buffer was added into the spin cartridge’s membrane and incubated for 15 minutes at RT. Subsequently 350μl Wash Buffer I was added and cartridges were centrifuged at 12,000g for 15s. The collection tube with the flow-through was discarded, cartridges were inserted into a new collection tube and the spin cartridge was washed twice with 500μl of Wash Buffer II. Next, the spin cartridge on its own was spun down at 12,000g for 2 minutes; 55μl of water was added into the spin cartridge and incubated for another 2 minutes. Following incubation mRNA was eluted by centrifugation for 2 minutes at 12,000g. The concentration and quality of the extracted mRNA was assessed by NanoDrop (Thermo Scientific, Wilmington, USA). Subsequently, samples were stored at -80°C until further use.

2.10 Synthesis of complementary DNA

Messenger RNA concentrations were adjusted by dilution in water and subsequently samples were used to synthesize complementary (c) DNA, using High Capacity RNA-to-cDNA Kit (Invitrogen). The kit was used according to the manufacturer’s instructions. Samples were prepared as presented in the Table 2.2. Concurrently, control samples to which no mRNA or no reverse transcriptase (RT) Enzyme Mix were added were prepared; which served as no template control and no RT control samples, respectively.

Table 2.2 Components of the cDNA synthesis reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X RT Buffer</td>
<td>10</td>
</tr>
<tr>
<td>20X RT Enzyme Mix</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease-free H₂O</td>
<td>3</td>
</tr>
<tr>
<td>mRNA Sample</td>
<td>6</td>
</tr>
<tr>
<td>Total per Reaction</td>
<td>20</td>
</tr>
</tbody>
</table>
Following sample preparation, the samples were loaded into the thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) that was set up as shown in the Table 2.3.

**Table 2.3 Cycling conditions required for the cDNA synthesis reaction.**

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td>37°C</td>
<td>95°C</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>60min</td>
<td>5min</td>
</tr>
</tbody>
</table>

After completion of cDNA synthesis, the samples were diluted by adding 180μl of water and stored for further qPCR analysis.

**2.11 Quantitative PCR**

Quantitative PCR reaction was set up as presented in the Table 2.4.

**Table 2.4 Components of the qPCR reaction.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Fast Universal PCR Master Mix (2X)</td>
<td>10</td>
</tr>
<tr>
<td>TaqMan Primer/probe</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>3</td>
</tr>
<tr>
<td>cDNA Sample</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total per Reaction</strong></td>
<td>20</td>
</tr>
</tbody>
</table>

Samples were prepared in a MicroAmp Fast 96-well reaction plate and covered by Optical Adhesive Film (both from Life Technologies). The thermo cycler used was a StepOne Plus (Applied Biosystems) with following reaction parameters (Table 2.5).
Table 2.5 Cycling conditions required for qPCR reaction.

<table>
<thead>
<tr>
<th>Holding stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>20s</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cycling stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1s</td>
</tr>
<tr>
<td>60°C</td>
<td>20s</td>
</tr>
</tbody>
</table>

40 cycles

Ct values were obtained from StepOne Plus software and fold change was calculated using the comparative Ct method or $2^{\Delta\Delta C_{T}}$ method, where $\Delta\Delta C_{T} = (C_{T, \text{sample}}) - (C_{T, \text{baseline (control)}})$. Value of $C_{T, \text{sample}}$ is the Ct value for any sample normalized to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) 1 gene and $C_{T, \text{baseline (control)}}$ is the Ct value for the calibrator (i.e. unstimulated sample, naive tissue) also normalized to the HPRT1 gene.

Table 2.6 Example of the comparative Ct method calculations.

<table>
<thead>
<tr>
<th>gene of interest (GOI)</th>
<th>average</th>
<th>first delta*</th>
<th>second delta**</th>
<th>$2^{\Delta\Delta C_{T}}$</th>
<th>HPRT1</th>
<th>average</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample 1</td>
<td>27.62739372</td>
<td>27.67078876</td>
<td>1.715829849</td>
<td>-2.804781914</td>
<td>6.987526812</td>
<td>25.991</td>
</tr>
<tr>
<td>sample 2</td>
<td>26.78629829</td>
<td>26.78186607</td>
<td>0.961769104</td>
<td>-3.558842659</td>
<td>11.7849617</td>
<td>25.845</td>
</tr>
<tr>
<td>sample 4</td>
<td>27.42993736</td>
<td>27.5025835</td>
<td>0.685917854</td>
<td>-3.834693909</td>
<td>14.26782884</td>
<td>26.856</td>
</tr>
<tr>
<td>baseline (unstimulated sample)</td>
<td>30.95965385</td>
<td>30.9257835</td>
<td>30.90592194</td>
<td>30.9257835</td>
<td>4.520611763</td>
<td>0</td>
</tr>
</tbody>
</table>

*First delta= average GOI- corresponding average HPRT1

**Second delta= first delta of sample 1- first delta of baseline
2.12 Analysis of cytokine production by explants

Ears were removed and the dorsal halves were isolated, as described previously in the section 2.6. Dorsal halves were floated on 250μl of complete RPMI medium for 16h at 37°C in a humidified atmosphere of 5% CO₂. Supernatants were harvested and analyzed for TNF-α, IL-1α and IL-1β using DuoSet ELISA.

2.13 Statistical analyses

Statistical analysis was performed using Graphpad (Graphpad Software). Non-parametric cytokine production data (ELISA) was analyzed by Mann-Whitney test or Kruskal-Wallis test with Dunn post-test when comparing two or 3 or more groups, respectively. Parametric ELISA data were analyzed by Student’s t test or one-way ANOVA with Tukey’s multiple comparisons test when comparing two or 3 or more groups, respectively. Where the values were below the limit of accurate detection, an arbitrary value of half of the value of the lower limit of detection was assigned. The LC migration data were analyzed by a Student’s t test, when comparing two groups or by one-way ANOVA with Tukey’s multiple comparisons test when comparing three or more groups. Non-parametric PCR data was analyzed by Mann-Whitney test or Kruskal-Wallis test with Dunn post-test when comparing two or 3 or more groups, respectively. Parametric PCR data were analyzed by Student’s t test or one-way ANOVA with Tukey’s multiple comparisons test when comparing two or 3 or more groups, respectively.
3 In vivo effects of lactoferrin

3.1 Introduction

Lactoferrin is an iron binding, mammalian protein present mainly in milk, but also in serum, tears, saliva, and secondary neutrophil granules. Lactoferrin is involved in both innate and acquired immunity and its roles are often contradictory. Indeed, it has been described to be both “a shield and a sword” for an organism (Legrand and Mazurier, 2010). One of the first reports regarding the role of LF demonstrated that it was a potent antimicrobial protein, and was able to exert bactericidal properties via iron sequestration in vitro (Oram and Reiter, 1968). Lactoferrin was found to have a protective role in vivo, whereby it was reported to protect mice from septic shock caused by exposure to a lethal dose of E. coli (Zagulski et al., 1989). Transgenic mice that over-expressed human LF were shown to display improved clearance of S. aureus. Spleen cells from these mice infected with S. aureus exhibited higher levels of TNF-α and IFN-γ following exposure to S. aureus (Toxic Shock Syndrome Toxin-1 [TSST-1]) antigen than did WT controls. However, in non infected animals, stimulation of spleen cells with TSST-1 resulted in higher levels of TNF-α and IFN-γ production in WT mice (Guillén et al., 2002). Also, a role for LF in humans was confirmed by Breton-Gorius et al. (1980), in a case study of a patient with LF deficiency in secondary neutrophil granules, that presented with recurrent infections starting at a very young age (Breton-Gorius et al., 1980). It was demonstrated that during infection the concentration of LF in serum increased, further confirming the role of this glycoprotein in immunity (Huang et al., 2013; Muruganand et al., 2004). In addition to increased LF levels found in systemic inflammation, it was demonstrated that levels of LF were elevated in a blister fluid following local exposure to allergen (pollen or ragweed) of patients suffering from seasonal rhinitis (Zweiman et al., 1990), suggesting that LF plays a role in the cutaneous inflammation. In fact, it was found in this laboratory, that topical application of LF inhibited LC migration triggered by exposure to contact allergen DPC or IL-1β. Moreover, LF was reported to reduce the cutaneous inflammation associated with topical exposure to DPC. There was a decrease in both erythema and cellular infiltration at the site of contact (Griffiths et al., 2001). In addition, LF was demonstrated to exert the same inhibitory effect on LC migration and DC accumulation in draining LN in
mice following treatment with another contact allergen, oxazolone (Cumberbatch et al., 2000b). However, despite the established role of LF in the immune system its mechanism of action is still not known fully. It was hypothesised that the mechanism by which LF inhibited LC migration and DC accumulation was due to decreased \textit{de novo} production of TNF-\( \alpha \) in the skin following treatment with allergen (Cumberbatch et al., 2003). That conclusion was reached following results that showed that LF inhibited migration that was dependent upon TNF-\( \alpha \), but was without effect on migration induced by delivery of homologous, exogenous TNF-\( \alpha \). The cellular and molecular processes through which LC are mobilized and migrate following exposure to contact allergens are still not understood fully. Nor is it clear exactly how LF modulates LC migration. The aim of this chapter is to describe in greater detail investigations designed to characterize the biological effects of LF. To achieve this, the impact of LF on cytokine production and expression in the skin of mice was assessed following allergen and cytokine treatment. In addition, the role of LF on cytokine production and cell proliferation in the draining LN was investigated.

\section*{3.2 Results}

Lactoferrin has been demonstrated previously to affect the cells residing in the skin: LC and DC, by impairing their migration into the draining LN (Cumberbatch et al., 2000b). In the current investigations, topical exposure to LF inhibited LC migration in mice induced by application of 0.5\% oxazolone, confirming previous observations (Chapter 6). That finding provided reassurance that the batches of LF used here possessed the \textit{in vivo} biological activity reported previously. The purpose of the experiments described herein was to examine the impact of \textit{in vivo} topical exposure to LF on the other parameters of the immune system activation caused by treatment with allergen, in order to provide further insight regarding the potential mechanism of LF. Initially, the effects of LF on allergen-induced changes in the skin, cytokines and other markers of activation such as ICAM-1 expression, were measured, at both the protein and message levels, as the skin is the site of first contact with LF. In subsequent experiments, the potential downstream effects of LF treatment on the quality of immune response provoked in the LN draining the site of topical exposure were examined, with respect to overall immunogenicity (increased cellularity and proliferation) and expression of LN cytokines. Finally, given that the stimulus of allergen is a relatively long lasting and
potent one, and that the ability of LF to inhibit oxazolone induced LC migration was transient (Chapter 6), the impact of LF on cutaneous responses induced by a somewhat weaker signal, a single bolus injection of cytokine, was examined.

3.2.1 Impact of lactoferrin on allergen-induced cutaneous cytokine expression

In initial experiments the impact of LF on oxazolone-induced cutaneous cytokine secretion was examined. The same exposure regimen was utilized as that shown to inhibit LC migration: 2h pre-treatment with LF followed by 4h exposure to oxazolone. The explant model, whereby the dorsal part of the ear is isolated after in vivo exposure to allergen, floated on and cultured in medium overnight (Cumberbatch et al., 2005), was utilized to measure cytokine (TNF-α, IL-1β, IL-1α and IL-17F) secretion in the skin.
Figure 3.1 The influence of LF on a cutaneous cytokine production following treatment with oxazolone.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO. After 4h animals were sacrificed and ears were excised. Further controls were untreated (naive). Ears were split into dorsal and ventral halves and dorsal halves were floated on 250μl of complete RPMI media, and incubated for 16h. Following incubation, supernatants were collected, and TNF-α (A), IL-1β (B), IL-1α (C) and IL-17F (D) production was determined by ELISA. Dashed line represents the lower limit of detection for ELISA (TNF-α (32pg/ml), IL-17F (23 pg/ml) and IL-1α and β (15 pg/ml). Graphs present data (mean ± SEM) from a single experiment (n=1-4 ears). The statistical significance of differences between cytokine levels in oxazolone-induced mice in the presence and in the absence of LF was compared by a Mann-Whitney test; p>0.05.
All cytokines were produced in detectable levels from naive skin, with particularly vigorous expression of IL-1α (~1000pg/ml) and relatively high, but variable, levels of IL-17. Four h exposure to oxazolone did not affect TNF-α or IL-17F production (Figure 3.1A and 3.1D). On the other hand, an increase in the production of IL-1α and β was observed. Baseline levels of IL-1α (1200pg/ml) were up-regulated to 2500pg/ml. Release of IL-1β was much lower than that reported for IL-1α (100pg/ml in the absence of allergen and 300pg/ml in the presence of allergen). Pre-treatment with LF was largely without effect on oxazolone-induced cytokine expression. Although there was a slight reduction in IL-1β, this did not reach statistical significance. The high levels of cytokine expressed in naive tissue were of some concern, and presumably reflected the trauma of tissue isolation, particularly the separation of the dorsum from the ventral half of the ear. Although only one time point was investigated, it seemed likely that this high background level could obscure both allergen-induced effects and the impact of LF. Therefore, it was decided to examine the effects of LF on cytokine mRNA levels in the skin, as this measurement could be undertaken in the whole skin, processed immediately after excision, without the need for traumatic separation of the dorsal halves from the ventral halves.

3.2.2 Lactoferrin did not markedly affect baseline dermal cytokine expression

Initially, the impact of LF on baseline dermal cytokine gene expression was studied. Mice were treated with LF and tissue was isolated 2, 4 and 6 h after the initiation of exposure. Levels of cytokine (IL-1β, Tnf-α, IL-17F) and Icam-1 transcripts were compared with naive (untreated) tissue (Figure 3.2). It was found that there was a small, but transient increase in the expression of Tnf-α, IL-1β and Icam-1 4h after treatment with LF, which reached statistical significance only for Icam-1, and returned to basal levels after 6h. Expression of IL-17F was unaffected. The impact was small at best (less than 2 fold change) and it was not clear that such changes were due to LF or a result of intra-experimental variation.
Figure 3.2 Lactoferrin induced time dependent changes in cytokine expression.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream on the dorsum of both ears. After 2, 4 or 6h mice were sacrificed and ears were excised and immersed in RNAlater. Further controls were untreated (naive; t= 0h). Subsequently, tissue was homogenised in a lysis buffer using a mechanical homogeniser. mRNA was extracted, cDNA was prepared, and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Il-17F (C) and Icam-1 (D) was measured relative to the Hprt1 gene. Results are presented as fold change in gene expression relative to untreated (naive) animals (mean ± SEM). The statistical significance of differences in gene expression following LF exposure in comparison to untreated controls was compared by a Wilcoxon test; *=p<0.05. Additionally, the statistical significance of changes in gene expression between different treatment times was assessed by Kruskal-Wallis test. Graphs present data from three independent experiments, one ear per time point (n=4-6 ears per time point).
In subsequent experiments, the effect of LF on oxazolone-induced expression of these same genes (and in addition *Il-1α* and *Cxcl11*) at various time points (0-120h) was investigated. In these experiments, changes in gene expression were measured relative to naive (untreated) control tissue, and the effect of LF on oxazolone-induced changes was compared with gene expression in tissue derived from mice that had been pre-treated with cream alone and then challenged with oxazolone (Figure 3.3). Oxazolone treatment induced time-dependent changes in the expression of all of the genes tested, although the kinetic profiles were very different, as was the maximal fold change recorded (Figure 3.3). The kinetics of changes in *Il-17F* (Figure 3.3F) and *Cxcl11* (Figure 3.3E) expression were very rapid and transient, occurring 2 and 4h following oxazolone application and reaching 2 and 150 fold increases, respectively, before returning to baseline, or in the case of *Il-17F*, a significant reduction compared to baseline levels. Conversely, expression of *Tnf-α* (Figure 3.3A) and *Icam-1* (Figure 3.3C) exhibited steady increases from 48 to 120h, reaching maximal levels at 120h post-treatment with oxazolone with ~3 and 5 fold changes, respectively. A somewhat different pattern was observed for *Il-1β* expression (Figure 3.3B), with significant increases recorded at 2 and 4 h (some 10-fold increases), with a second more vigorous increase in cytokine transcripts recorded between 96 and 120h (reaching 30 fold). *Interleukin-1α* (Figure 3.3D) showed a very different pattern of expression with down regulation of transcription observed from 4 to 24h, which then returned to baseline and again significantly decreased at 120h. However, prior treatment with LF did not alter the extent or kinetics of oxazolone-triggered gene expression for any of the genes tested, with the exception of *Il-17F* expression that increased at 4 and 120h. The increase in gene expression observed at 120h reached statistical significance (p=0.0381), whereas the change visible at 4h did not (p=0.0556) (Figure 3.3F).
Figure 3.3 The impact of LF on oxazolone-induced changes in dermal cytokine mRNA expression.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO. After 2, 4, 24, 72, 96 or 120h mice were sacrificed and ears were excised and immersed in RNAlater. Further controls were untreated (naive; t= 0h). Subsequently, tissue was homogenised in lysis buffer using a mechanical homogeniser. mRNA was extracted, and then cDNA was prepared and utilized in qPCR reaction using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Icam-1 (C), Il-1α (D), Cxcl1 (E), Il-17F (F) was measured relative to the Hprt1 gene. Results are presented as a fold change in gene expression relative to untreated (naive) animals (mean ± SEM). The statistical significance of differences in gene expression triggered by oxazolone in the presence and absence of LF was assessed by a Mann-Whitney test: #=p<0.05. Additionally, the statistical significance of differences in gene expression induced by oxazolone and untreated naive controls (t=0) was assessed by a Wilcoxon test; *=p<0.05; the results are presented in the Table 3.1. Graphs present data from a single experiment (n=6 ears per group).
Table 3.1 Results of the analysis of statistical significance\(^\#\) of differences in gene expression induced by oxazolone and untreated naive controls (t=0).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>TNF-α</th>
<th>IL-1β</th>
<th>ICAM-1</th>
<th>IL-1α</th>
<th>CXCL1</th>
<th>IL-17F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox 2h</td>
<td>1.22</td>
<td>0.15</td>
<td>8.71</td>
<td>0.03</td>
<td>0.96</td>
<td>0.68</td>
</tr>
<tr>
<td>Ox 4h</td>
<td>1.75</td>
<td>0.06</td>
<td>11.05</td>
<td>0.03</td>
<td>1.01</td>
<td>0.68</td>
</tr>
<tr>
<td>Ox 24h</td>
<td>1.08</td>
<td>0.56</td>
<td>3.71</td>
<td>0.06</td>
<td>1.05</td>
<td>0.68</td>
</tr>
<tr>
<td>Ox 72h</td>
<td>1.39</td>
<td>0.03</td>
<td>4.76</td>
<td>0.03</td>
<td>2.25</td>
<td>0.03</td>
</tr>
<tr>
<td>Ox 96h</td>
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<td>0.03</td>
<td>13.58</td>
<td>0.03</td>
<td>3.69</td>
<td>0.03</td>
</tr>
<tr>
<td>Ox 120h</td>
<td>3.34</td>
<td>0.03</td>
<td>27.36</td>
<td>0.03</td>
<td>4.94</td>
<td>0.03</td>
</tr>
<tr>
<td>LF+Ox 2h</td>
<td>1.36</td>
<td>0.03</td>
<td>7.61</td>
<td>0.03</td>
<td>1.00</td>
<td>0.84</td>
</tr>
<tr>
<td>LF+Ox 4h</td>
<td>1.25</td>
<td>0.03</td>
<td>9.45</td>
<td>0.03</td>
<td>1.14</td>
<td>0.21</td>
</tr>
<tr>
<td>LF+Ox 24h</td>
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<td>0.06</td>
<td>6.27</td>
<td>0.03</td>
<td>1.29</td>
<td>0.09</td>
</tr>
<tr>
<td>LF+Ox 72h</td>
<td>1.21</td>
<td>0.09</td>
<td>5.46</td>
<td>0.03</td>
<td>1.90</td>
<td>0.03</td>
</tr>
<tr>
<td>LF+Ox 96h</td>
<td>1.73</td>
<td>0.03</td>
<td>8.99</td>
<td>0.03</td>
<td>4.09</td>
<td>0.03</td>
</tr>
<tr>
<td>LF+Ox 120h</td>
<td>4.04</td>
<td>0.03</td>
<td>27.72</td>
<td>0.03</td>
<td>5.67</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(p^*\) assessed by Wilcoxon test

3.2.3 The effect of lactoferrin on allergen-induced lymph node activation

It was not possible to demonstrate any significant effects of LF on allergen mediated changes in the skin. In subsequent experiments the impact of LF on oxazolone-triggered events in the LN was investigated. In order to determine if LF influenced either the vigour or the quality of the induced immune response as a result of inhibited LC migration. Two general measurements of the vigour of the allergen response are: the increase in cellularity (total cell number per LN) and the proliferative activity of the lymph node cells (LNC), measured as a function of radiolabelled thymidine incorporation. Both these parameters have been used as markers of allergenicity and the extent of thymidine incorporation has been shown to correlate with the potency of a chemical allergen (Kimber and Dearman, 1991). Application of a single dose of oxazolone elicited significant increases in LN cellularity from 5x10\(^6\) cells per LN in untreated (naive) mice to 25, 28 and 32x10\(^6\) cells per LN recorded 72, 96 and 120h, respectively, following allergen treatment (Figure 3.4A).
Figure 3.4 Oxazolone-induced lymph node cell activation: the influence of LF.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO. After 72, 96 or 120h, animals alongside naive controls were sacrificed and auricular LN were excised. Further controls were untreated (naive). Lymph nodes were disaggregated and mashed through metal gauze to achieve a single cell suspension. Cells were enumerated using a CASEY counter (A) and 2x10⁶ cells per well were seeded into 96 well plate in the presence of [3H] thymidine and cultured for 24h (B). Thymidine incorporation was measured by β-scintillation counting and are displayed as dpm (disintegrations per min)(B). Graphs present data (mean ± SEM) from a single experiment (n=3 animals per group). The statistical significance of differences in LNC numbers (A) or LNC proliferation (B) between animals treated with oxazolone and those from naive animals was assessed by one-way ANOVA; **=p<0.01, ***=p<0.001. Moreover, statistical significance of differences in (A) cellularity and (B) proliferation between oxazolone exposed animals in the presence and in the absence of LF was assessed by a Mann-Whitney test (p>0.05).

In addition, the ability of LNC to proliferate after oxazolone application was examined. It was found that at all time points examined, 72, 96 and 120h, after treatment with oxazolone LNC exhibited a significant increase in proliferation (19, 9 and 6 fold, respectively), compared with untreated controls. However, a single application of LF did not significantly impact on LN cellularity or on the proliferative activity of LNC population. These data indicated that LF pre-treatment did not impact significantly on
the overall vigour of the allergic response. Therefore, the quality of the induced response was also assessed as a function of the cytokines produced by the activated LNC. Although 72h is somewhat early to observe differentiated cytokine expression and polarized Th1 and Th2 responses (Dearman et al., 1997), it has been reported previously that within this time frame allergen-activated LNC produce both Th1 and Th2 type cytokines, indicative of Th0 cell activation (Moussavi et al., 2000). Interferon-γ was chosen as a signature Th1 cytokine and IL-13 as a signature Th2 cytokine, as it has been reported that allergen-activated LNC elaborate both cytokines without the need for further in vitro re-stimulation (Dearman et al., 1996b). Thus, within the same experiment cytokine production by the LN populations was assessed by cytokine specific ELISA. In the absence of allergen-activation (naive cells) there was no detectable cytokine (below the detection limits of the ELISA) (Figure 3.5). As such, this was consistent with previous observations, in which it was found that without allergen stimulation, levels of cytokine expressed by resting (quiescent) LNC were at or around lower limits of detection (Dearman et al., 1995). Vigorous secretion of the type 1 cytokine IFN-γ was detected following oxazolone exposure, with maximal levels of 6000pg/ml recorded at 72h which declined to 3000 and 900pg/ml after 96h and 120h, respectively. There was also substantial production of the type 2 cytokine, IL-13, induced by oxazolone treatment, consistent with a Th0 undifferentiated phenotype (Dearman et al., 2003). Levels were maximal between 72 and 96h (~2700pg/ml) and had declined somewhat by 120h (1600pg/ml recorded). Although prior treatment with LF had no impact on the Th1 cytokine IFN-γ, there was an effect on expression of the Th2 cytokine IL-13, with more sustained production of this cytokine at the later (120h) time point following pre-treatment with LF (from 1600 to 3800pg/ml), albeit such did not reach statistical significance (as assessed by a Mann-Whitney test) (Figure 3.5).
**Figure 3.5 Impact of LF on allergen-activated lymph node cell cytokine production.**

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO. After 72, 96 or 120h, animals were sacrificed and auricular LN were excised. Further controls were untreated (naive). Lymph nodes were disaggregated and mashed through metal gauze to achieve a single cell suspension. Cells were enumerated using a CASY counter and \(10^7\) cells were seeded into 24 well plates and cultured for a further 120h. Subsequently, supernatants were collected and the IFN-γ and IL-13 content was determined by ELISA (the lower limit of detection was 31 and 62 pg/ml for IFN-γ and IL-13, respectively). Graphs present data (mean ± SEM) from a single experiment (n= 3 animals per group). The statistical analysis of differences between cytokine production in the presence and in the absence of LF was assessed by a Mann-Whitney test; \(p>0.05\).

Thus, there was some indication that pre-treatment with LF may impact on the quality of the response to oxazolone, with an increased bias towards the Th2 cytokine IL-13 recorded. In addition to examining the impact of LF on the Th1/Th2 skewing of the LNC cytokine response that was observed some 3 to 5 days after the initiation of exposure, the early (6 to 24h) cellular events in the LN were examined. It has been shown previously that one of the earliest changes in the LN draining the site of allergen application is the expression of the cytokine IL-17 by innate immune cells (γδ T cells) (PhD thesis Hayes, 2012). This production of IL-17 (both IL-17A and F cytokines) precedes the marked increase in cellularity stimulated by contact allergen, being detected after 6h of exposure. Mice were treated with oxazolone for either 6 or 24h, in the presence or absence of prior application of LF. After LN isolation, the standard method for preparation of a single cell suspension of LNC for subsequent
culture/analysis utilized in this laboratory has been mechanical disaggregation through wire gauze. However, in experiments designed to look at the cellular phenotype of cells, including those in which the observations of early IL-17 LN expression were recorded (PhD thesis Hayes, 2012), single cell suspensions were prepared by enzymatic digestion. Therefore, in these experiments, both methods of preparation of LNC were compared. The LNC count increased from ~6x10^6 cells per LN in naive animals to approximately 9.5x10^6 and 8x10^6 cells per LN in mice treated with oxazolone for 6h using mechanical and enzymatic methods, respectively. Exposure to oxazolone for 24h resulted in a further increase in LNC to 11x10^6 and 13.5x10^6 utilising mechanical and enzymatic methods, respectively. Allergen induced increases were similar regardless of the method of LN preparation. Application of LF was without marked effects on LN cellularity, with the exception of 24h following oxazolone application and mechanical digestion, where increased total cell numbers were recorded (Figure 3.6B). Enzymatic preparation of LNC 24h after oxazolone application resulted in the lower number of LNC in the presence of LF, however, such did not reach statistical significance. Subsequently, the production by LNC of IL-17A and IL-17F was assessed. It was found that when LNC were obtained using the mechanical method there was no detectable production of IL-17 6h after oxazolone treatment (<15 and 23pg/ml, for IL-17A and IL-17F, respectively; data not shown). When the enzymatic method was used 6h following oxazolone application, IL-17 production increased from resting levels of ~50 and 500pg/ml for A and F isoforms to 300 and 1500pg/ml, respectively (Figure 3.6A), with prior application of LF being without effect. Exposure to oxazolone for 24h resulted in more vigorous IL-17 production. When mechanical disaggregation was used, 24h exposure to oxazolone induced the release of 1200 and 3500pg/ml of IL-17 A and F, respectively, by LNC, with levels being reduced somewhat following LF pre-treatment (Figure 3.6B). In contrast, when enzymatic cell isolation was utilized for samples treated with oxazolone for 24h, there was a striking, approximately 5 fold increase, in levels of IL-17 produced (Figure 3.6C) in comparison with the level of production by cells isolated using mechanical disaggregation (Figure 3.6B). Cells that were obtained by enzymatic digestion produced 6000 and 30000pg/ml of IL-17 A and F, respectively, and prior application LF did not markedly affect IL-17 release (Figure 3.6C).
Figure 3.6 IL-17 expression by lymph node cells: the impact of disaggregation method and LF.
**Figure 3.6 IL-17 expression by lymph node cells: the impact of disaggregation method and LF.**

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO for 6 (A) or 24h (B, C). Animals alongside naive controls were sacrificed and auricular LN were excised. Lymph nodes were ruptured by gentle mechanical pressure, then incubated with 500μg/ml of collagenase and 500μg/ml of DNase for 20 minutes before being disaggregated mechanically through metal gauze (enzymatic; A, C) or simply disaggregated mechanically through metal gauze (mechanical; B) to achieve a single cell suspension. Cells were enumerated using a CASY counter and 10^7 cells per well were seeded into 24 well plates for 120h. Subsequently, supernatants were collected and the level of IL-17A and IL-17F was determined by ELISA (the lower limit of detection was 15 pg/ml and 23pg/ml for IL-17A and IL-17F, respectively). The statistical differences in LNC count and production of IL-17 A and F between oxazolone stimulated and naive animals were assessed by Kruskal-Wallis test followed by a Dunn’s post test; *=p<0.05, **=P<0.01, ***=p<0.001. Additionally, the statistical differences between oxazolone treated groups and those pre-exposed to LF were assessed by a Mann-Whitney test; #=p<0.05. Graphs present data (mean ± SEM) from two (A, C) or three (B) independent experiments (n=3-9 animals per group).

### 3.2.4 The influence of lactoferrin on cytokine-induced responses in the skin

As described before, although a dosing regimen of LF was utilized that had marked effects on allergen-induced LC migration, there were no striking or consistent effects of LF on parameters of allergen-induced activation in either the skin or the LN. Given that the stimulus of allergen is a relatively long lasting and potent one, such that even the ability of LF to inhibit oxazolone induced LC migration was transient (Chapter 6), the impact of LF on cutaneous responses induced by a more transient and “cleaner” signal, a single bolus injection of cytokine, was examined.

It has been demonstrated previously that exposure to LF inhibited IL-1β triggered LC migration without impacting on migration stimulated by the provision of exogenous TNF-α (Cumberbatch et al., 2000b). In initial experiments, the ability of LF to inhibit IL-1β induced LC migration was confirmed. Intradermal injection of 50ng of homologous IL-1β into the ears of mice resulted in a 23% decrease in LC numbers in
comparison with untreated controls. Prior treatment with LF significantly decreased the extent of LC migration to just 6% (Figure 3.7A). Exposure to IL-1β resulted in activated LC phenotype characterized by increased expression of MHC II molecules (brighter cell body appearance), more pronounced dendrites and enlarged cell body (Figure 3.7C) when compared to LC from untreated mice (Figure 3.7B). Pre-treatment with LF resulted in the change in cell morphology. Cells were larger, more dendritic and resembled those exposed to DNBC, exposure to which was demonstrated to result in morphology that was established to result mainly from high levels of IL-1β produced in the skin (Figure 3.7D).
Figure 3.7 Lactoferrin inhibited IL-1β mediated LC migration.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 6 ears per group) were treated on the dorsum of both ears with either 30μl of 0.5μg of LF in aqueous cream (D) or aqueous cream alone (C) for 2h. Subsequently, both groups received intradermal injection of 50ng of homogenous IL-1β for further 4h. Treated animals alongside non-treated controls (naive) (B) (n=3 per group; 6 ears per group) were sacrificed, ears were excised and LC enumerated by fluorescence microscopy and expressed as the mean frequency of LC per mm² per group ± SEM (A). The statistical significance of differences in LC numbers between untreated (naive) and cytokine exposed animals; ***=p<0.001 was assessed by one-way ANOVA. Representative micrographs of epidermal sheets show LC morphology (B-D). Scale bar 100μm.
To examine further the potential mechanism by which LF inhibited IL-1β induced migration, the same protocol was utilized as described in the previous experiment (Figure 3.7), but instead of assessing the extent of LC migration, the level of cutaneous expression of various genes was measured. Expression of the cytokines Tnf-α, Il-1α, Il-1β and Il-17A was measured, as well as the chemokine Cxcl1 and the adhesion molecule Icam-1, 4h after injection of cytokine, the same time point that was utilized for LC migration. It was found that exposure to IL-1β resulted in up-regulation of all of the genes tested with rank order of induction Cxcl1 < Il-1β < Il-17F < Icam-1 < Tnf-α (Figure 3.8A-3.8E), with the exception of Il-1α, expression of which was found to be decreased (Figure 3.8F) in comparison with expression in the untreated control (as represented by a dashed line). Fold changes ranged from ~4-fold increase in Tnf-α to 250-fold increase in Cxcl1. Cytokine (IL-1β)-induced changes in a cutaneous gene expression were therefore much more vigorous than those induced by allergen, particularly with respect to Il-1β (100-fold versus 12-fold), Icam-1 (40-fold versus 1-fold) and Il-17F (30-fold versus 1-fold). Application of LF prior to exposure to IL-1β resulted in the inhibition of Il-1β, Cxcl1 and Il-17F expression, however, only the decrease in Il-17F transcript levels reached statistical significance. The effects on Il-1β and Cxcl1 were to reduce levels by approximately 50% and 30%, respectively, whereas the effect on Il-17F was even more striking, reducing levels from an average of ~30-fold to less than 10-fold.
Figure 3.8 Lactoferrin significantly inhibited IL-17F expression following injection of homologous, recombinant IL-1β.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were injected intradermally with 50ng of homologous IL-1β. After 4h mice were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer using a mechanical homogeniser. mRNA was extracted, and then cDNA was prepared and utilized in qPCR reaction using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Cxcl1 (C), Il-17F (D), Icam-1 (E), Il-1α (F) was measured relative to the Hprt1 gene. Results are presented as a fold change in gene expression relative to untreated (naive) animals (mean ± SEM). The statistical significance of differences in gene expression triggered by IL-1β in the absence of LF and in the presence of LF was assessed by unpaired Student’s t test. Graphs present data from two experiments (n=12 ears per group).
It was demonstrated previously that LF had selective effects on cytokine-induced LC migration. Thus, whilst LF treatment inhibited IL-1β induced migration, it was without effect on TNF-α triggered reduction in LC frequency (Cumberbatch et al., 2000b). Therefore, it was speculated that LF should fail to affect changes in cutaneous gene expression elicited by this cytokine. Under these circumstances, TNF-α is an appropriate control for IL-1β as both cytokines have the same molecular weight (~17 kDa) and in each case 50 ng of cytokine administered intradermally was sufficient to stimulate a similar proportion of the LC to migrate from the skin, albeit with somewhat different kinetics (Cumberbatch et al., 1997b, 1999a). Intradermal delivery of homologous TNF-α, similarly to IL-1β, resulted in increased expression of all of the genes tested with rank order of induction $Cxcl1 < Il-1β < Icam-1 < Tnf-α < Il-17F$ (Figure 3.9A-3.9E). As observed for IL-1β induced gene expression, $IL-1α$ was the exception and its expression was down-regulated, albeit to a lesser extent (Figure 3.9F). Although the other 5 genes were all up-regulated by intradermal administration of both cytokines, there were differences with respect to the magnitude of the responses. Each cytokine resulted in a more vigorous induction of mRNA for the respective inducing cytokine, thus IL-1β injection stimulated a ~150-fold increase in $Il-1β$ expression, whereas TNF-α injection resulted in a ~70-fold increase. In contrast, a 4-fold and 10-fold increase, respectively, was seen for $Tnf-α$ mRNA following delivery of IL-1β and TNF-α, respectively. Induction of $Cxcl1$ was more vigorous following administration of IL-1β (~250-fold versus ~80-fold), whereas TNF-α was more effective at stimulating $Icam-1$ expression (~60-fold versus ~20-fold). The most striking difference was in the induction of $Il-17F$. Treatment with IL-1β resulted in ~40-fold increase in this cytokine, whereas injection of TNF-α resulted in much lower levels of $Il-17F$ (~2-fold increase). Interestingly, pre-treatment with LF did not modulate any TNF-α induced changes in gene expression.
Figure 3.9 Lactoferrin did not affect cytokine expression following injection of homologous, recombinant TNF-α.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were injected intradermally with 50ng of homologous TNF-α. After 4h mice were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in a lysis buffer using a mechanical homogeniser. mRNA was extracted, and then cDNA was prepared and utilized in qPCR reaction using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Cxcl1 (C), Il-17F (D), Icam-1 (E), Il-1α (F) was measured relative to the Hprt1 gene. Results are presented as a fold change in gene expression relative to untreated (naive) animals (mean ± SEM). The statistical significance of differences in gene expression triggered by TNF-α in the absence of LF and in the presence of LF was assessed by a Mann-Whitney test. Graphs present data from a single experiment (n=4-6 ears per group).
Finally, the impact of the other IL-1 family member, IL-1α, on a cutaneous gene expression, and the ability of LF to modulate IL-1α induced changes, has been explored. Interleukin-1α has a very similar profile of biological activity to IL-1β and acts through the same signalling receptor (the IL-1R1) (Jensen, 2010). However, the release of these cytokines can be induced by different stimuli. Thus, whereas IL-1β has been shown to play a role in allergen-induced LC migration (Cumberbatch et al., 1997a), IL-1α has been demonstrated to be critical for irritant (SLS)-induced LC migration (Cumberbatch et al., 2002a). Given the fact that the two molecules signal through the same receptor, it was anticipated that administration of IL-1α would induce a similar profile of gene expression changes to those observed for IL-1β, and that LF would display a similar ability to modulate these effects. The same protocol was used as that utilized in the experiments presented in the Figures 3.8 and 3.9; intradermal injection of 50ng of cytokine and isolation of tissue 4h later. Delivery of homologous IL-1α resulted in up-regulation of the majority of the genes tested with rank order of induction Cxcl1< Il-17F < Il-1β < Icam-1 < Tnf-α, similar to those changes induced by IL-1β and TNF-α. Again, the only gene that was not up-regulated was Il-1α (Figure 3.10F). Although the rank order of the increases in gene expression were very similar to those observed following administration of IL-1β, there were some differences with respect to the magnitude of the response. In particular, induction of Il-1β was considerably less vigorous (~25-fold compared with ~80-fold) and increases in Il-17F transcripts were also less marked. These differences may reflect subtle differences in kinetics or inter-animal variations. In addition, it should be noted that due to failed intradermal injections at some sites, the IL-1α control group only contains 3 replicates rather than 6 replicates. Given the rather marked inter-animal variation in Il-1β and Il-17F expression observed in the IL-1β treated animals (Figure 3.8), this reduction in group size may well explain the lower levels of these cytokines recorded. Prior treatment with LF was without effect on Il-1β and Il-17F mRNA expression, but did result in a significant decrease in expression of Cxcl1 (Figure 3.10C). Interestingly, contrary to the injection of IL-1β and TNF-α that resulted in the induction of their own transcripts, delivery of IL-1α was not able to up-regulate expression of Il-1α within the time frame investigated, suggesting a different mechanism for regulation of Il-1α transcription than that of the other proinflammatory cytokines Il-1β and Tnf-α.
Figure 3.10 Lactoferrin inhibited Cxcl1 expression following injection of homologous, recombinant IL-1α.

Young (6-8 weeks) BALB/c strain mice were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were injected intradermally with 50ng of homologous IL-1α. After 4h mice were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer using a mechanical homogeniser. mRNA was extracted, and then cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Cxcl1 (C), Il-17F (D), Icam-1 (E), Il-1α (F) was measured relative to the Hprt1 gene. Results are presented as a fold change in gene expression relative to untreated (naive) animals (mean ± SEM). The statistical significance of differences in gene expression triggered by IL-1α in the absence of LF and in the presence of LF was assessed by a Mann-Whitney test. Graphs present data from a single experiment (n=3-6 ears per group).
3.3 Discussion

In this chapter, the aim was to examine further the mechanism by which LF inhibits oxazolone-induced LC migration. The focus of the experiments described in this section was on the events in the skin, as well as in the draining LN. Various endpoints were measured to advance the understanding of the mode of action of LF.

Firstly, dermal cytokine (protein) production in ear tissue was assessed following treatment with oxazolone in the presence or the absence of LF. It was previously established that the process of LC migration is dependent on cytokine signals derived from a variety of cells present in the cutaneous environment. These in turn affect the expression of chemokines, receptors and proteases that lead to LC mobilisation (Griffiths et al., 2005). Two key cytokines in the events of LC migration are TNF-α and IL-1β (Cumberbatch et al., 1997b). However, IL-1α was also found to be important (Cumberbatch et al., 2002a). In the current investigations, the effect of LF on oxazolone-induced cytokine secretion in vivo was assessed using ear explant model. It was demonstrated that there was a relatively high production of all of the measured cytokines in naive samples. Exposure to oxazolone resulted in an up-regulation of IL-1α and IL-1β protein expression, whereas levels of TNF-α and IL-17F remained similar to those found in the naive samples. Prior treatment with LF was largely without effect on cytokine production. There was a small reduction in IL-1β levels but such did not reach statistical significance. Measurement of cytokine protein production in the skin, both in mice and in humans, is a challenging task. There is no non-invasive method to obtain human skin samples. Many studies utilize specimens from circumcisions, plastic surgeries or punch biopsies which could be utilized later in Western blot (WB) analysis of homogenised skin or assessed by IHC techniques. Alternatively, studies in humans have utilized vacuum suction to create cutaneous blisters and subsequently analyze blister fluid for the presence of various proteins; such blister fluid, which derives from the local microenvironment, is thought to resemble serum in protein content. In fact, LF was shown to inhibit TNF-α production in suction blister fluid following injection of IL-1β (Cumberbatch et al., 2003). It is considerably more difficult to measure cytokine production in mouse skin. There are reports, whereby dorsal skin biopsies were taken from mice, homogenised and analyzed by either by ELISA or WB for cytokine expression (Osborne-Hereford et al., 2008). However, the majority of murine skin is
covered in hair impeding access to the epidermis, and making sterilisation more demanding, partially explaining why mouse ears are so often used in exploration of cutaneous biology. Utilization of suction blister approach is not viable in mice, as animal would have to be anesthetised, therefore increasing the amount of experimental procedures, and further, the sample size would be minute. In our laboratory, however, an explant model was utilized, whereby following treatment with allergen ears were excised, divided into dorsal and ventral parts and dorsal part was floated onto the culture media. Next, released proteins were measured in the culture media. However, in addition to chemical and biological insults causing inflammatory cytokine production in the skin, physical insults, including heat and pressure, could have the same effect (Dearman et al., 2004; Schwacha et al., 2008). Therefore, the physical disruption of the ear was thought to be responsible for relatively high levels of all of the cytokines measured observed in naive samples. Indeed, it was reported by Cumberbatch et al. (2005) that following separation of the ear there was high constitutive production of IL-6 that was not further induced following exposure to 1% DNCB or 25% TMA (Cumberbatch et al., 2005). Interleukin 6 is a pleiotropic cytokine that has been demonstrated to be produced following induction with PAMP and DAMP by many cells types including DC, keratinocytes and endothelial cells (Tanaka and Kishimoto, 2012). It was demonstrated also that IL-6 plays a role in wound healing, as mice deficient in IL-6 displayed delayed wound healing following punch biopsy wound, as demonstrated by delayed wound closure and re-epithelialization (Lin et al., 2003).

It was reported that following treatment with an allergen there was an induction of TNF-α protein in the skin (Flint et al., 1998; Kaplan et al., 2012). However, contrary to those reports this was not observed in the current experiments. It is possible, that the trauma of ear separation obscured oxazolone-induced cytokine production. Additionally, in the current study only a single time point was utilized (4h). It is possible that TNF-α production had already peaked at an earlier time point. It was demonstrated previously that TNF-α levels in the serum of LPS injected rabbits peaked 2h after endotoxin exposure and the half life of the cytokine was established to be relatively short and was 6-7 minutes (Beutler et al., 1985). Similarly, Oliver et al. (1993) demonstrated that TNF-α production, following exposure of the whole blood to LPS, also peaked after 2h, and the TNF-α half-life was approximately 18 minutes (Oliver et al., 1993). These results suggested that in the current study oxazolone-
induced TNF-α production could have peaked before the samples were collected, explaining the lack of oxazolone-induced, TNF-α production. Additionally, explant samples were incubated ex vivo for 16h, in which time TNF-α molecule could have been degraded. Half-lives of pro-forms of IL-1α and IL-1β were established to be 15h and 3h, respectively, and their release was recorded to take place 12h and 2h after activation of human monocytes with LPS (Hazuda et al., 1988). Long half-life of IL-1α might partially explain high levels of this protein recorded in the current study. Overall, it would have been helpful to perform a time course of cytokine production by skin explants to assess the time dependent changes following exposure to allergen.

The baseline levels of measured cytokines were relatively high due to apparently traumatic nature of sample preparation, and not markedly induced by oxazolone. It was decided to investigate the baseline cytokine expression utilising a method that did not require an excessive handling of the tissue. To achieve that qPCR was used, whereby whole ear was used for mRNA isolation. It was found that treatment with LF on its own did not modulate the expression of genes tested at any of the time points with the exception of Icam-1 4h following treatment. In the skin ICAM-1 is constitutively expressed by endothelial cells; additionally its expression can be induced by IFN-γ and TNF-α in keratinocytes (Griffiths et al., 1989). Observed levels of Icam-1 expression up-regulation were very low and most probably induced in endothelial cells as there was no increase in Tnf-α expression preceding up-regulation in Icam-1 expression. Low levels of Icam-1 expression were also demonstrated in LC cultured in vitro for 12 h and interaction between ICAM-1 and LFA-1 was shown to be crucial for the ability of LC to stimulate proliferation of allogeneic T cells (Simon et al., 1991). The effect of LF on ICAM-1 expression was not without precedent. Indeed, LF was demonstrated to regulate ICAM-1 expression in human umbilical vein endothelial cells (HUVEC) following activation with TNF-α. Kim et al. (2012) reported that LF competed with NF-KB for ICAM-1 promotor, therefore inhibiting its expression (Kim et al., 2012). In contrast, this study found an increase in Icam-1 expression following exposure to LF. However, it is possible that, as Kim et al. (2012) have shown, following binding of ICAM-1 promoter and in the absence of TNF-α, LF triggered Icam-1 expression. As reported previously, LF has dual influences on immune responses and it is plausible that in the presence of stimuli (TNF-α) LF inhibits ICAM-1 expression, as reported by Kim et al. (2012), but on its own, LF was able to increase the level of Icam-1 transcription.
However, there was no lasting effect of LF treatment in the steady state. Therefore, in addition to examining expression of cytokines it would have been of interest to investigate the induction of enzymes responsible for antioxidant defence, as oxidative stress is known to be induced in the skin after exposure to allergens; that in turn contributes to the danger signalling and subsequent activation of DC (Corsini et al., 2013). Furthermore, it was observed before that LF was able to induce (by approximately 2-fold) the steady state expression of genes involved in oxidative stress management (GSH peroxidase 1 and 4, prostaglandin-endoperoxide synthase 1, peroxiredoxins and superoxide dismutase 1) in the whole blood cells, following 2h incubation with 10μg/ml of LF (Kruzel et al., 2013).

Next, the impact of LF on oxazolone-induced gene expression was investigated. As previously stated, treatment with allergen has been shown to result in the induction of epidermal TNF-α mRNA expression (Enk and Katz, 1992a). In the study by Enk and Katz (1992a) there was a rapid induction of Tnf-α, Il-1α and Il-1β expression 1h after exposure to 3% TNCB, which was still evident 24h after treatment. However, the current study did not confirm these results. There are many reasons for these discrepancies, such as different allergens and techniques used to detect expression. In the Chapter 6 it is reported that there are differences between allergens with regards to their ability to induce cytokine expression, therefore, it is possible that TNCB, especially at a somewhat high concentration of 3%, was more efficient at inducing TNF-α expression. Moreover, the technique utilized by Enk and Katz (1992) was different. Briefly, they performed PCR on cDNA and obtained cytokine specific products. Subsequently, PCR products were hybridised with cytokine-specific, radioactive probes; later electrophoresis was completed and radioactivity was assessed. That method was semi-quantitative, as the intensity of the band was assessed. It is possible that by utilising that method a weak cytokine signal could have been amplified.

In the current study the induction of Tnf-α expression was minimal until 120h after treatment with oxazolone. Similarly, expression of Il-1β was approximately 10 times higher than that in untreated animals 4h following exposure to oxazolone, until it rose to 30 fold 5 days after treatment, which is likely to represent the beginning of an elicitation response (Saint-Mezard et al., 2003). Conversely, no induction of Il-1α expression was observed at any time point. It is surprising, as an induction of IL-1α protein was observed 4h after allergen treatment. These results suggest that secretion of IL-1α in the
skin is not regulated at the level of transcription (Jensen, 2010). Expression of chemokine Cxcl2 (Mip2) was measured by Katz and Enk (1992) and it was increased 4h after application of TNCB. In the current study induction of chemokine CXCL1, which together with CXCL2 signals via the CXCR2 receptor (Zaja-Milatovic and Richmond, 2008) was measured. In accordance with the results of Enk and Katz (1992), rapid induction of Cxcl1 was observed that declined after 72h to levels 2 times higher than those observed in naive controls. It was shown previously, utilising respiratory sensitization of mice with OVA, that Cxcl1 expression was detected as soon as 1h following exposure to OVA and peaked 3h after treatment. Subsequently, neutrophils were detected in the lungs of sensitized mice after 3h and maximum neutrophil numbers were detected 6h after exposure to OVA (Knott et al., 2001). Results by Knott et al. (2001) suggested that CXCL1 is rapidly induced in allergic reactions and its primary role is to recruit neutrophils to the site of allergen exposure. It was established previously that neutrophils have a role in the challenge phase of hypersensitivity (Dilulio et al., 1999), but not in the sensitization events, therefore, it would be of particular interest to investigate the role of neutrophils in the primary phase of response to allergen. Expression of Icam-1 was still increasing 120h after allergen exposure, similar to expression of Tnf-α and Il-1β. Expression of ICAM-1 was demonstrated previously 4h following challenge with oxazolone, as well as after intradermal cytokine (TNF-α, IL-1α and IL-1β) delivery (McHale et al., 1999). Taken together results by McHale et al. (1999) and presented in the current study suggested that induction of Icam-1 expression begins following primary exposure to oxazolone and then it is further induced after allergen challenge. It was demonstrated, as previously mentioned, that the presence of ICAM-1 was shown to be important for LC-induced T cell proliferation (Simon et al., 1991). Moreover, following challenge with DNFB, presence of ICAM-1 on endothelial cells was crucial for recruitment and activation of CD8+ T cells and their subsequent production of IFN-γ and IL-17 (Kish et al., 2011). Finally, the expression of Il-17F was rapidly induced 2h after treatment with oxazolone and afterwards it decreased to a level below that present in untreated controls. Prior treatment with LF did not modulate any of the genes at the time point when an effect on LC migration was observed in vivo (4h). However, there was a statistically significant effect of LF observed at 120h, whereby LF increased Il-17F expression following oxazolone treatment accelerating the return of Il-17F expression levels to those found in untreated
controls. Although statistically significant, this result is not likely to be biologically relevant.

Exposure to oxazolone revealed a differential cytokine patterns over time that help to understand the immunological mechanisms of sensitization. Investigated kinetics spanned 5 days and it could be speculated that increase in Tnf-\(\alpha\) and \(\text{Il}-1\beta\) expression observed from 4\textsuperscript{th} and 3\textsuperscript{rd} day, respectively, could be a result of the adaptive immune response, whereby allergen was still present on the surface of the skin following initial exposure and was providing an elicitation signal (Saint-Mezard et al., 2003). It was demonstrated previously that IL-1\(\beta\) at the level of mRNA and protein was rapidly (15 and 30 minutes, respectively) detected in the skin, in both epidermis and dermis, following exposure to oxazolone (Kermani et al., 2000) and preceded TNF-\(\alpha\) production in the skin, both in the form of mRNA and protein, which was observed after 30 minutes and 1h, respectively (Flint et al., 1998). Based upon these results, it can be concluded that the increase in the expression of Tnf-\(\alpha\) and Il-1\(\beta\) observed 72h after initial exposure was due to the initiation of adaptive immune response (Asada et al., 1997). Rapid up-regulation of expression of neutrophil chemoattractant CXCL1 was observed 4h following oxazolone exposure and declined thereafter. Previous reports suggested that CXCL1 expression occurs rapidly, within 30 minutes, following challenge with 0.2% of DNFB of sensitised and non-sensitized skin of BALB/c strain mice (Dilulio et al., 1999). The presence of neutrophils was demonstrated to be necessary to achieve the optimum CHS. It was shown that neutrophils were crucial for the recruitment of CD8\textsuperscript{+} T cells, as when CXCL1 was inhibited, the CHS reaction decreased, but could be rescued by a transfer of hapten specific CD8\textsuperscript{+} T cells (Dilulio et al., 1999). Similarly, it was established that in mice deficient in CXCR2 (CXCL1 receptor) CHS reaction to DNFB and oxazolone, as measured by ear swelling, was decreased (Cattani et al., 2006).

Whilst there were no major changes in the cutaneous cytokine production and expression recorded in the presence of LF, it was decided that the downstream events of LNC proliferation and LN cytokine production would be investigated. It is known that exposure to an allergen results in LN hypertrophy caused by DC and lymphocyte migration (PhD thesis Hayes, 2012; Sikorski et al., 1996). A slight increase in LNC number was observed as soon as 6h following oxazolone exposure; by 24h the increase was statistically significant and it continued to increase until 120h. The data recorded in
this chapter were in agreement with previously published reports, whereby there was an early allergen-induced increase in LN cellularity (PhD thesis Hayes, 2012). The rapid augmentation of LN cellularity is a result of enhanced lymphocyte influx into the LN and simultaneous restriction of their exit; process termed the LN shut down. It is hypothesized that the main purpose of the LN shut down is to increase the probability of CD4+ T cells to recognize the antigen that is presented by arriving DC from the skin (McConnell et al., 1980). Furthermore, it was shown that there was a marked increase in LNC proliferation following application of oxazolone in comparison with naive animals. The proliferative response was the highest 72h after exposure to oxazolone and deceased to levels similar to those observed in naive animals after 120h. Observed changes to LNC proliferation reflect increased division of allergen specific T cells that take place following encounter with DC carrying the hapten. A subsequent decrease in the ability of cells to divide was driven by changing populations of LNC that after 120h are characterized by a presence of non-proliferating memory T cells that will become activated upon future exposure to the same allergen, as well as an increase in B cell numbers (Sikorski et al., 1996). It was found that LF did not modulate cellularity or LN proliferation at 72, 96 or 120h time points. A possible reason for that might be that contact allergens are known to persist in the skin for up to 14 days after the first exposure, and even single application of allergen (oxazolone or FITC) was able to result in CHS reaction in mice (Saint-Mezard et al., 2003). In this section it was shown that exposure to oxazolone over a somewhat longer time frame (72-120h) resulted in a steady increase of Tnf-α, Il-1β and Icam-1 expression. Therefore, it is not surprising that while LF did not affect cytokine expression after allergen treatment that it was also without effect on secondary events in the LN. In the Chapter 6 it was presented also that the effect of LF on LC migration was transient, therefore, suggesting that LF might be metabolized or not able to overcome constant stimulation from allergen present in the skin (Saint-Mezard et al., 2003).

Lymph node cells were reported to produce cytokines following exposure to allergens. The type of cytokines produced depends on the type of sensitising chemical, which in turn relies on the quality of immune response triggered by that substance (Dearman and Kimber, 1999). Chemical allergens have been demonstrated to polarize immune response, with contact and respiratory allergens resulting in Th1 and Th2-skewed responses, respectively, and subsequent production of either IFN-γ or IL-4 and IL-13.
In this section it was found that following stimulation with oxazolone for 72h to 120h LNC produced cytokines that were markers of both Th1 and Th2 immune response. The relatively short 5 day protocol that was utilized in the current study was insufficient to result in full polarisation of immune response. Typical polarisation protocols involve treatment with allergen on the flank on day 0 and day 5, followed by allergen application to the ear on days 10, 11 and 12 and termination on day 13 (Dearman and Kimber, 1999). There was no effect of LF on IFN-γ production by LNC at any of the time points. In contrast, treatment with LF resulted in increased production, albeit not statistically significant, of IL-13 at 120h. It was reported previously that the application of LF 1h before the final exposure to respiratory allergen, toluene diisocyanate, in a chronic exposure protocol, increased production of IL-4 (Zimecki et al., 2012). Accordingly, LF was demonstrated to inhibit antigen specific proliferation of Th1, but not Th2, specific cell line (Zimecki et al., 1996). Inhibitory effects on Th1 mediated response might explain an increase in Th2 involved cytokine IL-4, and could partially be responsible for the increase in IL-13 production observed in the current study, despite the lack of effect of LF on IFN-γ production by LNC. Interestingly, while the production of IFN-γ decreased with time, levels of IL-13 remained comparable at all time points tested. The observed decrease in IFN-γ production was consistent with the decrease in cell proliferation. Interferon γ was demonstrated to be derived from CD8+ cells in the LN as well as in the skin (Xu et al., 1996). It is possible that the observed decrease in LN cell proliferation and IFN-γ production was caused by CD8+ cell migration to the skin, as it was reported previously that 120h following initial exposure to oxazolone CD8+ cells and IFN-γ were detected in the skin. Moreover, ear swelling was demonstrated to increase 120h after initial exposure to allergen (Saint-Mezard et al., 2003). These reports are in accordance with data from the current study, wherein expression of Tnf-α and Il-1β rose 120h following exposure to oxazolone.

Subsequently, production of another cytokine, IL-17, was considered. Interleukin 17 (A and F) belongs to a recently identified family of cytokines. They are produced by a newly described subset of Th17 cells, but also by cells of the innate immune system: DC, macrophages, NK cells and γδ T cells (Onishi and Gaffen, 2010); and is thought to play a key role in the first line of host defence. Interleukin 17 has been identified as an early marker of ACD (PhD thesis Hayes, 2012), and the current study supported that
observation. Production of IL-17A and F was observed in the LN 6h following exposure to oxazolone. In our laboratory two LN preparation protocols were used; one that utilized enzymes (collagenase and DNase) and another that did not utilize enzymes and relied solely on mechanical disaggregation. It was found that different preparation techniques affected IL-17 production by LN following exposure to allergen. Thus, early (6h) IL-17 production was not observed when LN were prepared by mechanical disruption alone, whereas there was a detectable production of IL-17 24h after allergen treatment. However, levels of IL-17 observed 24h after oxazolone exposure from mechanically prepared LN were almost 5 times lower than those from LN cultures prepared with enzymes. It has to be noted that the early (6h) IL-17 production is attributable to innate cells of the immune system, γδ T cells, as it is too early for polarized Th or Tc responses to develop. In fact, γδ T cells have been shown to be the cellular source of IL-17 in other independent experiments in this laboratory (PhD thesis Hayes, 2012). γδ T cells comprise 3% of LN and spleen cells in rodents. The proportion of these T cells is much higher in the sites of contact with the external environment and γδ T cells can sometimes be the only T cells present in the intra-epidermal spaces. In murine skin, γδ T cells are present in the epidermis as well as in the dermis, and they participate in maintaining cutaneous homeostasis via secretion of chemokines and cytokines (Macleod and Havran, 2011). In the mouse epidermis γδ T cells are found in the proximity of LC (personal communication, Kieran Mellody). It was demonstrated in our laboratory that IL-17 is produced by LNC as soon as 6h following exposure to DNCB. It also was found that the main cell subset responsible for production of this cytokine was γδ T cells (PhD thesis Hayes, 2012). It has been established that γδ T cells produce IL-17 in response to IL-1β and IL-23 (Sutton et al., 2009). It is possible that following allergen exposure, DC arriving in the LN were a source of IL-1β, therefore stimulating IL-17 production by γδ T cells resident in the LN. It could be speculated that the lack of IL-17 signal in the absence of enzymatic digestion of LN was due to γδ T cells or DC being “trapped” within the LN capsule, and only upon treatment with collagenase and DNase these cells were retrieved and able to receive or give a cytokine (IL-1β) signal that stimulated IL-17 expression by γδ T cells. γδ T cells responsible for IL-17 production in the LN were most likely LN resident or dermal γδ T cells but not DETC. It was reported previously that dermal γδ T cells were producing IL-17 and capable of migration (Gray et al., 2011; Nielsen et al., 2014), conversely, DETC were
able to migrate to the LN following exposure to DNFB but they did not produce IL-17A or IFN-γ (Nielsen et al., 2014).

Treatment with LF prior to exposure to oxazolone had different effects depending on the single cell preparation procedure. Thus, in the samples prepared via mechanical disaggregation LF resulted in a statistically significant increase in the number of cells in the LN. However, the opposite effect was observed in the samples prepared via enzymatic method, albeit the effect was not statistically significant. Cumberbatch et al. (2000) reported that LF inhibited DC accumulation in the LN 18h following oxazolone treatment. In the aforementioned report mechanical method of LN preparation was utilized. However, the inhibition of DC accumulation observed by Cumberbatch et al. (2000) was not likely to have contributed to the change in the total LN cellularity, as reported numbers of DC comprised only 0.1% of all LN cells (Cumberbatch et al., 2000b). Opposing effects of LF on LN cellularity that depend on the method of single cell suspension preparation were perplexing. Cytokine production results suggested that there were marked differences in the effects of mechanical and enzymatic methods. It is possible that LF induced increased level of an unknown cell subset migration to the LN, and these cells were susceptible to enzyme-induced cell death and were not able to be enumerated. However, during mechanical method of LN processing that same unknown population could be enumerated and would account for the increased cellularity. Despite the differences in LN cellularity, secretion of IL-17 was measured by standardised number \(10^7\) of cells per well, allowing for comparisons between groups. To explain the differences in cytokine levels between mechanical and enzymatic LNC retrieval, one could speculate that it was possible that during a mechanical LN disaggregation some of the migratory DC from the skin were present close to the LN capsule, as they were inhibited in their migration by LF, and therefore were not completely retrieved during mechanical disaggregation. As a consequence, they could have not supplied γδ T cells with a signal to produce IL-17. In the situation where enzymes were utilized to achieve single cell suspension, the inhibitory effect of LF on IL-17 production was no longer visible. It is plausible that in the latter scenario all of the DC were “freed” and subsequently able to interact with LN γδ T cells and trigger them to release IL-17 that was no longer affected by LF.

It is not known which cell type LF is directly acting on in the skin, therefore, two hypotheses are possible. Firstly, LF could impact on γδ T cells. It was demonstrated
previously that LF is capable of interacting with T cells (Legrand et al., 1997), and expression of functional LFR was demonstrated by human γδ T cells, as measured by binding of radiolabelled LF to decidual γδ T cells (Mincheva-Nilsson et al., 1997). Interaction of γδ T cells with LF could be developmentally justified as both are present in the epithelium and mucosal barriers (Girardi, 2006; Lönnerdal and Iyer, 1995). Therefore, following application of LF to the skin it could inhibit TNF-α secretion by DETC or dermal γδ T cells; further, LF could increase DETC migration to the LN, which would explain increased cellularity in the LN and decreased IL-17 production [as DETC do not produce IL-17 in the LN (Nielsen et al., 2014)]. Secondly, LF could affect DC, LC and/or DC. It was demonstrated previously that LF had an effect on the extent of DC activation and cytokine output (de la Rosa et al., 2008), and therefore could modulate DC in a way that upon their arrival in the LN their ability to trigger IL-17 production was diminished. Alternatively, a combination of these two scenarios is possible. Indeed, it was shown that human γδ T cells from circulating blood, activated by stimulation phospho antigen, were able to induce DC maturation via TNF-α production, as measured by an increase in CD86, CD83 and MHC II expression by DC. Additionally γδ T cells increased IL-12 production by DC, which was dependent on IFN-γ secretion by γδ T cells (Ismaili et al., 2002). Further interactions between two cell types were demonstrated by Conti et al. (2005), whereby upon co-culture of DC and γδ T cells, the latter increased expression of CD25 and CD69, as well as production of TNF-α and IFN-γ (Conti et al., 2005). Results from human cells were confirmed by Dieli et al. (2004) using murine γδ T cells and DC. They demonstrated that IFN-γ production by γδ T cells was dependent upon DC-derived IL-12 (Dieli et al., 2004). Reports of interaction between DC and γδ T cells are of specific interest in the immune surveillance of the skin where these two cell types co-exist. It is possible that LF could impact on one or both cell types in order to achieve inhibition of LC migration and modulation of IL-17 production by LNC. It would be of particular importance to perform studies where mouse skin was treated with radio-labelled LF to allow for identification of the cell target of LF.

Lactoferrin was shown to decrease oxazolone triggered IL-17 production by LN cells. There were no LF-induced changes to oxazolone provoked gene expression in the skin or LNC proliferation. One of the obstacles in elucidating the mechanism of action of LF was the unknown mode of oxazolone action in the skin. Indeed, despite many advances
in our knowledge of allergens’ behaviour in the skin, it is still not known exactly how oxazolone is working. Therefore, it was decided to investigate effects of LF on cytokine induced changes to gene expression in the skin. Cytokines are one of the mediators of oxazolone effects in the skin and they were considered to be a more straightforward stimulus with which to assess effects of LF on the cytokine gene expression in the skin.

Lactoferrin was found to inhibit LC migration in response to IL-1β but not TNF-α (Almond et al., 2013; Cumberbatch et al., 2000b). The study described herein confirmed this observation. It was found that LF significantly inhibited LC migration 4h after delivery of exogenous IL-1β and subsequent changes in cell morphology were observed. Similarly to what was noted after allergen treatment, LC that did not migrate exhibited activated morphology, brighter appearance and extended dendrites. Cells that were pre-treated with LF looked even more activated and their morphology resembled that of cells treated with DNCB (Chapter 6). Langerhans’ cells were demonstrated to express IL-1R1 (Kämpfegen et al., 1994), which is the functional IL-1 receptor (Kuno and Matsushima, 1994), and is necessary for TNF-α-induced LC mobilisation (Cumberbatch et al., 1999c). Indeed, it was demonstrated previously that delivery of IL-1β into the skin resulted in decreased LC frequency and increased Ia+ (MHC II) expression (Enk et al., 1993; Lundqvist and Bäck, 1990), which is in accordance with data presented herein, as judged by the brighter LC appearance. Moreover, as demonstrated in the Chapter 5, LC are not the only cells that respond to IL-1β; keratinocytes are the target of IL-1β influence, and subsequently they express cytokines that can further stimulate LC. Additionally, LC from mice injected with IL-1β were demonstrated to stimulate T cell proliferation to a greater extent than those injected with IL-1α (Enk et al., 1993), suggesting once again that despite signalling via common receptor these two molecules have differential effects on dermal environment.

To help understand the mechanism of the inhibitory activity of LF it was decided to establish changes in a cutaneous gene expression induced by delivery of IL-1α, IL-1β and TNF-α and the impact of LF on these. In this section it was reported that there were changes in the cutaneous gene expression profile following treatment with oxazolone and that they were not significantly modulated by LF. Therefore, treatment with cytokines was considered as more targeted approach. Moreover, as presented in the Chapter 6 the effect of LF on oxazolone induced LC migration was transient, most
likely due to allergen persistence in the skin. Consequently, a more transient stimulant was required to investigate the persistence of LF effects. However, only single time point (4h) was examined following delivery of cytokine.

Delivery of IL-1β resulted in substantial up-regulation of cytokine expression. The observed cytokine profile was more vigorous (higher extent of gene induction) than that caused by oxazolone for all of the genes tested with the exception of Il-1α that was not up-regulated following exposure to any of the cytokines or allergens utilized in these investigations. In fact, expression of Il-1α was only ever modestly induced in the skin (Chapter 6), whereas its expression and secretion in primary keratinocytes and in PAM cells was readily up-regulated following exposure to both IL-1α and IL-1β. Interleukin 1α is constitutively expressed by PAM keratinocytes (Mee et al., 2005) and by distressed dermal cells, as exemplified by ear explants in this chapter, suggesting that regulation of IL-1α production is not controlled on the level of mRNA expression. Moreover, IL-1α was demonstrated previously to be produced, but not released, by murine keratinocytes, following exposure to contact allergens; levels of approximately 3000pg/ml were observed following stimulation with 10μg DNBC and 500μg oxazolone for 24h (Corsini et al., 1998). Therefore, it was somewhat surprising that in the whole ear tissue Il-1α expression was below the level present in naive animals following stimulation with IL-1β, TNF-α and IL-1α. The lack of up-regulation of Il-1α expression might suggest that mRNA for this cytokine is always present in the ear and what is modulated is the extent of its translation. However, this theory is somewhat contradicted by results from keratinocytes where cytokine stimulation (IL-1α and IL-1β) resulted in up-regulation of Il-1α expression (Chapter 5). It is also plausible that the resident immune cells in the ear provide negative feedback to keratinocytes, thereby decreasing their transcription of Il-1α. It might potentially suggest that expression of Il-1α is under stricter control than Il-1β.

These results suggest that IL-1α production is differentially regulated in the skin than Il-1β with which it shares a common receptor, IL-1R1. In fact, it was demonstrated by Enk et al. (1993) that while injection of anti-IL-1β antibody decreased TNCB induced ear swelling, anti-IL-1α antibody was without pronounced effect (Enk et al., 1993). Additionally, one might speculate that increasing intracellular levels of pro-IL-1α might negatively regulate its own expression, as it was demonstrated previously that pro-IL-1α
is capable of intracrine signalling (Werman et al., 2004). Moreover, Boraschi et al. (1990) demonstrated that IL-1α and IL-1β had the same stimulating ability on thymocytes, as well as similar pyrogenic activity in rabbits. Conversely, it was established that IL-1β, but not IL-1α, was able to enhance T cell dependent antibody production by spleen cells after injection of sheep red blood cells (SRBC). Furthermore, when IL-α and IL-1β were administered together, IL-1α could inhibit immunostimulatory action of IL-1β (Boraschi et al., 1990). Similarly, Nakae et al. (2001) showed decreased anti-SRBC antibody production in IL-1β deficient mice (Nakae et al., 2001). These results shed a light on a dichotomy between two IL-1 isoforms, partially explaining their differential regulation in the skin observed in the current investigations.

With the exception of Il-1α expression, injection of IL-1β resulted in up-regulation of all of the tested genes. The highest induction was observed in Cxcl1 expression. As discussed in the Chapter 5, treatment of primary keratinocytes and PAM cells with IL-1β resulted in a rapid and considerable induction of Cxcl1 mRNA levels. CXCL1, together with CXCL2, is responsible for neutrophil chemotaxis (Zaja-Milatovic and Richmond, 2008). Kish et al. (2012) demonstrated a crucial role for IL-1β-induced CXCL1 in recruitment of neutrophils to the allergen challenge site and subsequent infiltration of CD8+ cells into the skin (Kish et al., 2012). In addition to up-regulating other genes, IL-1β substantially induced its own expression. It was observed in the Chapter 5 that upon incubation with IL-1β primary keratinocytes and HaCaT cells moderately induced IL-1β mRNA levels, therefore suggesting that cells other than keratinocytes were responsible for an induction of IL-1β observed in the ear. Endothelial cells and/or fibroblasts potentially could be responsible for the high IL-1β mRNA levels observed in the ear tissue.

In addition to measuring expression of cytokines, the level of induction of an adhesion molecule was assessed. ICAM-1 is constitutively expressed by variety of cell types present in the ear, including: endothelial cells, epithelial cells, fibroblasts, keratinocytes. Moreover, ICAM-1 was demonstrated previously to be vigorously up-regulated following stimulation with IL-1α, IL-1β and TNF-α; with the highest degree of induction observed for combination of TNF-α and IFN-γ (Roebuck and Finnegan, 1999). Observed increase in ICAM-1 levels in the presence of inflammatory cytokines
and allergen (120h exposure) was most likely up-regulated by endothelial cells and keratinocytes and served to facilitate entry of lymphocytes expressing LFA-1 to the dermis and epidermis (Rahman and Fazal, 2009), which is a substrate for ICAM-1 (Rothlein et al., 1986). Accordingly, in the study described herein, the maximum level of ICAM-1 induction was observed after exposure to TNF-α. Expression of Tnf-α was modestly induced by the treatment with oxazolone and IL-1α, while exposure to TNF-α resulted in the highest induction of its own expression.

It has been demonstrated previously that keratinocytes treated with TNF-α up-regulated TNF-α expression, suggesting an autocrine feedback loop (Bashir et al., 2009). Banno et al. (2004) conducted a comprehensive study of effects of TNF-α on gene expression in human keratinocytes. It was found that TNF-α rapidly (within 1h) induced up-regulation of genes involved in innate immunity, in particular in leukocyte attraction (CXCL1, CXCL2, IL-8, CXCL10), as well as members of TNF-α family, IL-1β and ICAM-1. Results presented herein were in agreement with the results by Banno et al. (2004).

Interestingly, IL-17F was the cytokine that was the most differentially regulated by IL-1 and TNF-α and was the main cytokine impacted by LF pre-treatment. Induction of Il-17F mRNA was 25 and 2.5 times higher than naive levels following exposure to IL-1α/β and TNF-α, respectively. It is somewhat intriguing that such high level of IL-17F induction was observed following exposure to IL-1α/β, considering the source of IL-17 in the skin (γδ T cells) and its relative low percentage of all of cells present in the ear. It was reported that cultured mouse γδ T cells did not produce IL-17 in response to IL-1β alone, but only when combined with IL-23, IL-1β was able to induce IL-17 secretion. Similarly, CD4⁺ T cell population required a signal from both IL-1β and IL-23 in order to produce IL-17. However, when CD4⁺ T cells were cultured in the presence of anti-CD3 and anti-CD28 antibodies IL-1β on its own was able to stimulate IL-17 induction to the level similar to that previously observed for combination of IL-1β and IL-23 (Lalor et al., 2011). Therefore, it is possible to achieve IL-17 production following exposure to IL-1β. Nielsen et al. (2014) found that DNFB-induced CHS was significantly decreased in IL-17 KO and γδ T cell KO mice in comparison with WT controls, as measured by the decrease in ear thickness, suggesting that IL-17 is involved in pathogenesis of CHS. They demonstrated that DETC were capable of producing IL-17A in the skin after challenge with DNFB. Moreover, they established that DETC
migrated to the draining LN, but they did not produce IL-17. Production of IL-17A by purified and cultured DETC was up-regulated by stimulation with IL-1β and further increased when combination of IL-1β and anti-CD3 was used. The authors suggested that the IL-1β that stimulated DETC to release IL-17A was derived from keratinocytes, as demonstrated by DNBS-triggered IL-1β expression by PAM keratinocytes (Nielsen et al., 2014). Study by Nielsen et al. (2014) only considered DETC cells as the source of IL-17A.

In our laboratory production of IL-17 by skin explants following DNCB exposure was identified previously to be derived from dermal γδ T cells. It was established that γδ T cell KO animals did not produce IL-17 in response to DNCB. Conversely, AhR KO mice, which lack DETC, but not dermal γδ T cell population, had the same IL-17 production following DNCB exposure as WT counterparts (PhD thesis Hayes, 2012). It is possible that discrepancies between results of Nielsen and Hayes stemmed from the fact that Hayes (2012) measured IL-17 production following single exposure to allergen, whereas Nielsen et al. (2014) investigated IL-17 production during CHS, when in addition to dermal γδ T cells, DETC could also exhibit IL-17 production (Nielsen et al., 2014). It can be concluded that in the current study population of γδ T cells, responsible for induction of IL-17, was the one resident in the dermis.

Interleukin-17 production in the skin was found following primary exposure to allergen (DNCB) as demonstrated by Hayes (2012). It was detected during CHS reaction to DNFB treatment (Nielsen et al., 2014), as well as produced by lymphocytes in response to stimulation with IL-1β in combination with either IL-23 or anti-CD3 or anti-CD28 antibodies (Lalor et al., 2011). In the current study expression of Il-17F was detected after exposure to allergen (oxazolone, DNCB [Chapter 6]), as well as following injection of cytokine. In addition to previously mentioned γδ T cells, of both epidermal and dermal origin, IL-17 was demonstrated to derive from mast cells and neutrophils in the inflammatory models of skin inflammation, namely, application of LTB4 and tape-stripping (Keijzers et al., 2014; Lin et al., 2011). It is plausible that in the experimental system whereby IL-1α/β or TNF-α were injected, there was a recruitment of neutrophils, due to high level of mRNA induction of CXCL1, that is known to be responsible for neutrophil chemotaxis (Zaja-Milatovic and Richmond, 2008). Study by Johnson et al. (2011) demonstrated that in the rat brain peak production of CXCL1 was
12h after exposure to soman, a toxic substance with impact on nervous system, and it preceded neutrophil accumulation in the same parts of the brain where production of CXCL1 was demonstrated (Johnson et al., 2011). In a study by de Filippo et al. (2013) it was established that mast-cell derived CXCL1 was responsible for neutrophil accumulation. When mice were injected intraperitoneally with LPS (that caused CXCL1 release from mast cells), neutrophils were detected as soon as 1h after endotoxin delivery (De Filippo et al., 2013). Studies by De Filippo et al. (2013) and Johnson et al. (2011) suggest that it is possible that increased levels of CXCL1 resulted in neutrophil accumulation. Therefore, it is likely that observed increase in IL-17F expression might be partially mediated by neutrophils.

Prior application of LF significantly inhibited IL-1β induced IL-17F expression and decreased albeit not significantly Cxcl1 mRNA levels (p=0.0559). In contrast, exposure to LF before injection of IL-1α significantly decreased Cxcl1 mRNA level, but was without effect on IL-17F expression. The experiment wherein IL-1α was injected was somewhat flawed, in that the control group (without LF) had only 3 data points due to unsuccessful intradermal injections of cytokine. Nevertheless, that result was intriguing as the extent of IL-1α and IL-1β induced expression was similar and both IL-1α and IL-1β signal via the same receptor: IL-1R1 (Kuno and Matsushima, 1994). However, the disparity between the effects of LF on changes mediated by two IL-1 isoforms could be reconciled by reports that these two cytokines may have in fact differential functions in immunity, despite utilising common receptor. Results demonstrated in this chapter present an alternative way of thinking about the events surrounding LC migration and the role that LF plays in its inhibition. It would have been interesting to assess if in vivo LF could inhibit IL-1α induced LC migration. It was demonstrated previously that IL-1α induced migration was inhibited by anti-TNF-α antibody (Cumberbatch et al., 2002a), therefore suggesting that LF could potentially also be effective in inhibiting IL-1α triggered decrease in LC numbers in the epidermis. However, in the light of results presented in this chapter, it is possible that the mode of action of LF is not mediated by inhibition of TNF-α signalling. No effect of LF was observed on TNF-α-induced gene expression which was in accordance with results from LC migration studies, whereby LF did not inhibit TNF-α triggered LC evacuation (Cumberbatch et al., 2000b; Almond et al., 2013).
Lactoferrin is a pleiotropic iron-binding glycoprotein present in epithelial secretions and secondary neutrophil granules. It was shown to be involved in cell proliferation, differentiation, migration, wound healing, innate and adaptive immunity (Legrand and Mazurier, 2010). In our laboratory LF was shown to inhibit LC migration and DC accumulation following exposure to oxazolone (Almond et al., 2013; Cumberbatch et al., 2000b), as well as to decrease the level of IL-1β-induced cutaneous TNF-α in the suction blister fluid (Cumberbatch et al., 2003). The aim of experiments described in this section was to elucidate the mechanism by which LF exerted these effects. It was found that LF decreased mRNA levels of Cxcl11 and Il-17F induced by IL-1α and IL-1β, respectively. It is interesting that these cytokines were not considered previously to be involved in the process of LC migration. LF did not impact markedly on the secondary events in the LN (cell proliferation, cytokine production) with the exception of IL-13 expression, suggesting that the quality of the induced immune response may be impacted by LF. However, it is likely that the lack of an effect of LF on the vigour of the response was due to the persistence of oxazolone in the skin and continuous stimulation of dermal cytokine production. Langerhans’ cells migration is thought to depend upon signals from TNF-α and IL-1β. Following receipt of these two signals LC undergo series of changes that allow them first to leave the epidermis and subsequently to arrive in the LN (Griffiths et al., 2005). The first step in LC migration is their detachment from surrounding keratinocytes, and is achieved via a switch in chemokine receptor expression. In the epidermis LC express CCR6 receptor for keratinocyte derived CCL20 and E-cadherin that mediates their attachment to neighbouring keratinocytes (Cumberbatch et al., 2000a). Following signal from TNF-α, LC decrease expression of CCR6 and E-cadherin, facilitating their detachment from keratinocytes, and up-regulate expression of CXCR4 receptor that drives LC chemotaxis towards the dermis where production of CXCL12 occurs (Villablanca and Mora, 2008). Chemokine receptor switch is a crucial step in LC migration and it is possible that in addition to signals from TNF-α and IL-1β, cytokine IL-17 also participates in that process. Indeed, the presence of IL-17 producing γδ T cells has been demonstrated in the epidermis (Nielsen et al., 2014) as well as in the dermis (Sumaria et al., 2011). It was established by many studies that IL-17 plays an important role during skin infections (Cho et al., 2010) and diseases (Fischer-Stabauer et al., 2012), however, there are not many reports relating to the role of IL-17 in LC migration. The published literature reveals that there are several potential steps where LC mobilisation could be affected by IL-17. Effects of
IL-17A were assessed on fibroblast like cells called synoviocytes that are crucial for pathogenesis of rheumatoid arthritis (RA). It was demonstrated by Hot et al. (2012) that IL-17A up-regulated expression of CXCR4 on synoviocytes derived from patients with RA. Additionally, they reported that a combination of IL-17A and TNF-α resulted in even higher level of induction of CXCR4 (Hot et al., 2012). Data from Hot et al. (2012) implicate IL-17 in regulation of LC migration via its effects on CXCR4 expression that was demonstrated to be necessary for LC egress from the epidermis. Interleukin-17A was confirmed to affect breast cancer metastasis to bone and lungs in mice by mediating CXCL12 expression. It was established that upon systemic delivery of anti-IL-17A the percentage of metastatic tumours was diminished. It was found that anti-IL-17A antibody decreased CXCL12 levels in tissues that were targets for metastatic cells (bone and lung) (Roy et al., 2014). A study by Roy et al. (2014) revealed another potential connection between IL-17 and LC migration, whereby IL-17 is necessary for CXCL12 production that subsequently mediates LC mobilisation into the dermis. Taken together data from Roy et al. (2014) and Hot et al. (2012) suggest that IL-17 might participate in CXCR4-CXCL12 signalling axis that is responsible for LC mobilisation into the dermis. Additionally, it was established by Li et al. (2011) that IL-17A facilitates migration of hepatic carcinoma cells in vitro via up-regulation of MMP2 and MMP9 expression (Li et al., 2011). Both of these enzymes were demonstrated to be necessary for LC migration, albeit at a later stage, i.e. mobilisation from the dermis to lymphatic vessels. It is possible that this speculation is unlikely, as results implicating IL-17 in CXCR4-CXCL12 signalling came from tissues other than the skin. However, it has not been long since IL-17 has been identified as an important factor in cutaneous immunology; therefore, its exact role is still under investigation.

In conclusion, data presented in this chapter suggests an alternative mechanism by which LF inhibits oxazolone and IL-1β induced LC mobilisation. So far the focus has been on TNF-α and IL-1β. However, results presented in this chapter imply that crucial role might be played by CXCL1 and IL-17. Moreover, this data suggest that inhibitory effects of LF on LC migration could be mediated via inhibition of these two signals.
4 Investigation of the immunomodulatory potential of lactoferrin in THP-1 monocytes and macrophages

4.1 Introduction

Lactoferrin has been observed to have immunomodulatory effects in the skin. It was shown to inhibit LC migration in response to the chemical contact allergen, oxazolone, and to homologous IL-1β, in mice and in humans via TNF-α dependent mechanism (Almond et al., 2013; Cumberbatch et al., 2003; Cumberbatch et al., 2000b; Griffiths et al., 2001). Nevertheless, this mechanism is not fully understood. It was suggested that LF was exerting its effects on de novo TNF-α production. Therefore, an in vitro platform in which TNF-α was produced abundantly was required in order to investigate the mechanism further. Tumour necrosis factor-α is a cytokine mainly produced by monocytes and macrophages (Moss et al., 1997) and THP-1 cells are a widely used monocytic cell line. The THP-1 cell line was established in 1980 from a male with acute monocytic leukemia (Tsuchiya et al., 1980), and were demonstrated to express TLR (Wang et al., 2012), and to produce inflammatory cytokines, including TNF-α, following stimulation with TLR2 and TLR4 ligands (Takashiba et al., 1999). Moreover, it is widely recognized that THP-1 cells can be differentiated into macrophages by incubation with PMA (Tsuchiya et al., 1982). Thus, treatment with PMA results in inhibition of THP-1 proliferation, adherence of cells to the TC plate and increased phagocytic activity (Schwende et al., 1996). Additionally, differentiation of THP-1 cells to macrophages has been shown to result in increased levels of TNF-α production following treatment with LPS (Takashiba et al., 1999). However, LF has been demonstrated to bind LPS directly via interaction with the lipid A part of LPS (Appelmelk et al., 1994), consequently this makes LPS an unsuitable candidate for investigation of the mechanism of action of LF. Therefore, the TLR2 ligand, PGN, was chosen as the stimulus for TNF-α production. Lactoferrin was demonstrated to interact with both THP-1 monocytes (Ando et al., 2010) and macrophages (Eda et al., 1996). To control for the ability of LF to inhibit TNF-α production the steroid drug dexamethasone was chosen, as it has been shown previously to inhibit TLR mediated cytokine production by THP-1 cells (Mogensen et al., 2008).
In order to understand fully the mechanism of action of LF it was crucial to identify a receptor through which it was acting. To date many different receptors on various cell types have been identified as LFR. Surface nucleolin has been shown to be responsible for LF binding to and internalisation into THP-1 macrophages (Hirano et al., 2005). Therefore, this receptor has been investigated as the most likely candidate.

The objective of the work presented in this chapter was to utilize THP-1 monocytes and macrophages and to establish whether human native LF was able to modulate their cytokine production in response to PGN. Additionally, the aim was to elucidate further the mechanism of action of LF.

4.2 Results

4.2.1 Dose response of THP-1 monocytes to PGN

The first step in the investigations of the immunomodulatory mechanisms of LF was to establish an in vitro system in which TNF-α was produced vigorously in response to an LPS-independent stimulus. The human monocytic cell line THP-1 was selected to be stimulated with PGN, a TLR2 ligand, for either 6 or 24h. Secretion of TNF-α and IL-8 was measured by ELISA at both time points. In the absence of PGN at both 6 and 24h TNF-α production was below the limit of accurate detection of the ELISA (~20pg/ml). Treatment with PGN resulted in detectable TNF-α production only for the highest dose of the stimulant (25μg/ml) and was approximately 40pg/ml at both time points (Figure 4.1A and 4.1B). Secretion of IL-8 was considerably higher than that reported for TNF-α, such that baseline expression was ~100-150pg/ml at both time points. Incubation with PGN resulted in a dose dependent increase in IL-8 production with highest levels (1.9 and 3.8ng/ml) observed after 24h for 10 and 25μg/ml of PGN, respectively (Figure 4.1D). Despite the relatively low levels of TNF-α produced by THP-1 monocytes following PGN stimulation, the effect of pre-treatment with LF was examined. Treatment with PGN resulted in secretion of ~100pg/ml of TNF-α and addition of LF (2-100 μg/ml) was without effect on TNF-α production. However, prior incubation with the positive control dexamethasone (100nM) inhibited TNF-α secretion by 81% (Figure 4.2).
Figure 4.1 THP-1 monocytes responded to PGN with TNF-α and IL-8 production.

THP-1 cells (10⁵ cells per well) were treated with a range of PGN concentrations (0 to 25μg/ml) for 6 (A, C) or 24h (B, D). Supernatants were collected and TNF-α (A, B) and IL-8 (C, D) secretion was determined by ELISA (the lower limit of detection was 15pg/ml and 31pg/ml for TNF-α and IL-8, respectively, and is indicated by a dashed line). Statistical analyses (one-way ANOVA with Tukey’s post-test) compared TNF-α or IL-8 levels in the presence of PGN with baseline levels; *= p<0.05, **=p<0.01, ***=p<0.001. Graphs represent data from 4 independent experiments (mean ±SEM).
Figure 4.2 Lactoferrin did not modulate PGN-induced TNF-α secretion by monocytes.

THP-1 cells (10^5 cells per well) were pre-treated with a range of LF concentrations (0-100 μg/ml) or 100nM dexamethasone (Dex) formulated in DMSO, or DMSO alone, for 1h before the addition of PGN (25μg/ml) for 6h. Control cells were untreated. Supernatants were collected and TNF-α secretion was determined by ELISA (the lower limit of detection 15pg/ml, indicated by a dashed line). Graphs represent data from 3 independent experiments (mean ± SEM).

4.2.2 PGN-activated THP-1 macrophages: dose response

Given the relatively low levels of TNF-α produced by PGN stimulated THP-1 monocytes and the lack of effect of LF, an experimental system in which more vigorous TNF-α responses could be provoked was examined. Therefore, THP-1 monocytes were differentiated into macrophages by 3h treatment with PMA. Figure 3.3 illustrates the dose dependent increase in TNF-α secretion by THP-1 macrophages 6h (Figure 4.3A) and 24h (Figure 4.3B) following treatment with PGN. Differentiation into macrophages by PMA resulted in considerably higher baseline and PGN-induced cytokine production compared with THP-1 monocytes. Thus, at 6h, baseline levels of approximately
0.4ng/ml TNF-α were recorded and maximal levels of approximately 3ng/ml were reached following stimulation with 25μg/ml of PGN. After 24h culture, baseline levels of TNF-α in supernatants were markedly higher than those recorded after 6h, resulting in a less pronounced dose response to PGN. Therefore, a 6h stimulation time with a concentration of 25μg/ml of PGN was chosen for subsequent experiments with THP-1 macrophages.

Figure 4.3 THP-1 macrophages responded to PGN with TNF-α production.

THP-1 cells (10⁵ cells per well) were differentiated into macrophages by culture with 100nM PMA for 3h and left to rest overnight before being treated with a range of PGN concentrations (0 to 25μg/ml) for 6 (A) or 24h (B). Supernatants were collected and the level of TNF-α secretion was determined by ELISA (the lower limit of detection was 15pg/ml). Statistical analyzes (one-way ANOVA with a Tukey’s post-test) compared TNF-α levels in the presence of PGN with baseline levels; **=p<0.01, ***=P<0.001. Graphs represent data from 6 independent experiments (mean ± SEM).
Figure 4.4 Lactoferrin did not modulate TNF-α and IL-8 baseline secretion by THP-1 macrophages.

THP-1 cells (10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 3h and left to rest overnight before being treated with a range of LF concentrations (0-100 μg/ml) for 7h. Positive control cells were incubated with 100nM dexamethasone (Dex) formulated in DMSO, or DMSO alone in parallel. Levels of TNF-α (A) and IL-8 (B) in cell supernatants were determined by ELISA (the lower limit of detection was 15pg/ml and 31pg/ml for TNF-α and IL-8, respectively). Statistical analyzes (one-way ANOVA with a Tukey’s post-test) compared TNF-α or IL-8 levels in the presence of inhibitor with the baseline levels; ***= p<0.001. Graphs represent data from 5-6 independent experiments (mean ± SEM).

4.2.3 The influence of lactoferrin on TNF-α production by THP-1 macrophages

THP-1 macrophages were cultured with LF in the absence (Figure 4.4) or the presence (Figure 4.5) of PGN in order to examine effects of LF on both baseline and PGN-induced cytokine expression. Baseline TNF-α secretion was similar to that recorded previously (approximately 200pg/ml), and both LF and dexamethasone were without significant impact (Figure 4.4A). Relatively high baseline levels of IL-8 secretion (approximately 13ng/ml) were observed for THP-1 macrophages. Treatment with LF had little effect on IL-8 baseline expression, across a dose range used. In contrast, dexamethasone significantly inhibited IL-8 secretion. As reported previously, 6h
incubation with PGN resulted in vigorous TNF-α secretion (induction from 200pg/ml to ~1500pg/ml) (Figure 4.5). In cells that have been stimulated with PGN, all doses of LF (2 to 100μg/ml) were also without inhibitory effect (Figure 4.5A). In contrast, dexamethasone treatment inhibited significantly (by 73%) TNF-α production induced by PGN. Treatment with PGN increased IL-8 secretion approximately 3 fold, from 13ng/ml to 40ng/ml. Lactoferrin treatment was without significant or marked effects on PGN-induced IL-8 secretion. However, culture with dexamethasone inhibited significantly PGN stimulated IL-8 secretion by 60% (Figure 4.5B).

Figure 4.5 Lack of inhibition of PGN-induced THP-1 macrophage TNF-α and IL-8 secretion by lactoferrin.

THP-1 cells (10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 3h and left to rest overnight, then treated with a range of LF concentrations (0-100 μg/ml) for 1h before adding PGN for a further 6h. Positive control cells were incubated with 100nM dexamethasone (Dex) formulated in DMSO, or DMSO alone in parallel, followed by PGN. Levels of TNF-α (A) and IL-8 (B) in cell supernatants were determined by ELISA (the lower limit of detection was 15pg/ml and 31pg/ml for TNF-α and IL-8, respectively). Statistical analyzes (one-way ANOVA with Tukey’s post-test) compared PGN stimulated cytokine levels in the presence or absence of inhibitor (***= p<0.001). Graphs represent data from 5-6 independent experiments (mean ± SEM).
4.2.4 The influence of PMA on THP-1 macrophage responsiveness to LF

Thus far, LF was without inhibitory effects on THP-1 macrophages obtained following 3h stimulation with PMA with respect to TNF-α or IL-8 production (Figure 4.4 and 4.5). In order to maximise the potential impact of LF on THP-1 macrophages, alternative methods of differentiation were utilized that have been shown previously to increase membrane LFR expression, and were thus predicted to increase sensitivity to LF (Eda et al. 1996). Figure 4.6 presents morphology of THP-1 cells before (Figure 4.6A) and after differentiation by PMA for 72h (Figure 4.6B). Following treatment with PMA cells stopped proliferating, became adherent and exhibited ‘flattened’ appearance when compared with a rounded phenotype of THP-1 monocytes. Furthermore, cytoplasm has become more granular.

A PMA time course was conducted to optimise the effect of LF on TNF-α production. Figure 4.7 shows the kinetics of baseline TNF-α (Figure 4.7A) and IL-8 (Figure 4.7B) secretion by THP-1 macrophages stimulated with PMA for 24h to 120h in the presence of LF and in the absence of PGN. After 24h, 48h and 72h activation with PMA alone,
baseline TNF-α expression was below the limit of detection (<15pg/ml). After 96h and 120h cells secreted approximately 2000pg/ml TNF-α, although there was considerable inter-experimental variation. The effect of LF on THP-1 macrophages in this system was somewhat surprising. Dose dependent increases in TNF-α secretion in response to LF were recorded at all time points, contrary to expectations. Culture of THP-1 cells activated for 24h to 120h with PMA for a further 7h with LF resulted in a marked increase in TNF-α levels at all time points. In the absence of LF, PMA activation resulted in somewhat variable baseline production of IL-8 (~2ng/ml) which did not display a marked change with increasing time of culture with PMA. Treatment with LF had a similar effect to that observed for TNF-α: a dose and time dependent increase in IL-8 production. Culture of 48h PMA activated cells with the highest concentration of LF (100μg/ml) resulted in a significant increase of IL-8 secretion (Figure 4.7B). Further activation of PMA-induced macrophages with PGN resulted in a relatively vigorous TNF-α secretion at all time points (approximately 10-20ng/ml). Addition of LF was largely without effect on PGN-induced TNF-α secretion, with the exception of the 48h PMA treated cells where there was some evidence of a dose dependent increase in TNF-α production.
Figure 4.7 Lactoferrin induced TNF-α and IL-8 secretion by THP-1 macrophages.
Figure 4.7 Lactoferrin induced TNF-α and IL-8 secretion by THP-1 macrophages.

THP-1 cells (5x10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 24-120h before being cultured with a range of LF concentrations (0-100 μg/ml) for a further 7h. Levels of TNF-α (A) or IL-8 (B) in cell supernatants were determined by ELISA (the lower limit of detection was 15pg/ml and 31pg/ml for TNF-α and IL-8, respectively). Statistical analyses (one-way ANOVA with a Tukey’s post-test) compared TNF-α or IL-8 levels in baseline (0) and LF treated cells; * = p<0.05; ** = p<0.01. Graphs represent data from 3-6 independent experiments (mean ± SEM).
Figure 4.8 Lactoferrin did not inhibit PGN-induced TNF-α production by THP-1 macrophages.

THP-1 cells (5x10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 24-120h before being treated with a range of LF concentrations (0-100 μg/ml) for 1h prior to addition of 10μg/ml of PGN for a further 6h. Levels of TNF-α in cell supernatants were determined by ELISA (the lower limit of detection was 15pg/ml). Statistical analysis (one-way ANOVA with a Tukey’s post-test) compared TNF-α levels in baseline and LF treated cells; *=p<0.05. Graphs represent data from 3-6 independent experiments (mean ± SEM).
4.2.5 Stimulatory effect of lactoferrin was specific and endotoxin independent

In order to confirm that the observed stimulatory effect of LF on PMA-induced macrophages was specific, 72h macrophages were incubated with LF or various concentrations of an iron binding control protein, TRF. As demonstrated previously, treatment with 100μg/ml LF resulted in an up-regulation of TNF-α and IL-8 secretion by 100 and 8 fold, respectively, in comparison with baseline cytokine secretion. Incubation with TRF (100-1000μg/ml) did not impact on cytokine secretion at any of the concentrations tested (Figure 4.9). The assumption was that activation of THP-1 monocytes with PMA resulted in macrophages with increased ability to bind LF (Eda et al. 1996). In order to confirm this phenomenon, the impact of LF (100μg/ml) on THP-1 monocytes and 72h macrophages was compared directly. It was established that in the presence of LF monocytes secreted approximately 300pg/ml of TNF-α, whereas macrophages increased TNF-α production from 260pg/ml to 9200pg/ml in the absence and in the presence of LF, respectively (Figure 4.10).

![Graph](image)

**Figure 4.9 Transferrin does not induce TNF-α release by THP-1 macrophages.**

THP-1 cells (5x10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 72h; washed with PBS and incubated with a range of doses (100,300 and 1000μg/ml) of TRF or with 100μg/ml of LF for 7h. Levels of TNF-α (A) or IL-8 (B) in cell supernatants were determined by ELISA (the lower limit of detection was 15pg/ml and 31 pg/ml for TNF-α and IL-8, respectively). Graphs represent data from a single experiment.
Figure 4.10 Lactoferrin did not induce TNF-α release from THP-1 monocytes.

THP-1 monocytes or THP-1 macrophages (5x10^5 cells per well) differentiated into macrophages by treatment with 100nM PMA for 72h were cultured in parallel, washed with PBS and incubated in the presence or absence of 100μg/ml of LF for 7h. Levels of TNF-α in cell supernatants were determined by ELISA (the limit of detection was 15pg/ml). Graph presents data from a single experiment.

It is well documented that LF is capable of binding LPS (Appelmelk et al., 1994) and that macrophages have the ability to vigorously respond to endotoxin with TNF-α secretion (Kane et al., 2009; Takashiba et al., 1999). Therefore, it was of great importance to establish that the observed immunostimulatory effect of LF was not a result of endotoxin contamination of the LF preparation. Figures 4.11A and 4.11B, respectively, show TNF-α and IL-8 production by 72h PMA-induced macrophages following treatment with a range of doses (0.01-10μg/ml) of LPS. Incubation of 72h macrophages with 1μg/ml of LPS for 7h resulted in secretion of 3000 and 30000pg/ml of TNF-α and IL-8, respectively, equivalent to levels provoked by 100μg/ml LF. Addition of a range of doses (0.01-10μg/ml) of polymixin B (PMB; which binds LPS (Bhor et al., 2005)) prior to stimulation with LPS resulted in a dose dependent inhibition of LPS-induced cytokine production by maximum of approximately 70% and 90% at top two concentrations of PMB for TNF-α and IL-8, respectively (Figure 4.11C and 4.11D). Subsequently, 72h macrophages were cultured with PMB and either LPS or LF to ascertain whether the immunostimulatory effects of LF were LPS-independent. Treatment with both LPS and LF resulted in marked TNF-α production. Addition of
10μg/ml of PMB, as previously demonstrated, inhibited LPS-triggered cytokine production but was without effect on LF-induced TNF-α secretion (Figure 4.11E), demonstrating that the stimulatory effect of LF was independent of endotoxin. The effect of LPS on 72h THP-1 macrophages was assessed in the single experiment. Similarly, the impact of PMB on LPS-induced THP-1 macrophages was confirmed in a single experiment. It is known that LPS is capable of dose-dependently stimulating TNF-α release by macrophages (Lichtman et al., 1998; Song et al., 2000) and that PMB is able to inhibit such induction (Cardoso et al., 2007). Therefore, it was deemed suitable to perform these experiments once.
Figure 4.11 The stimulatory effect of LF was not dependent on endotoxin.

THP-1 cells (5x10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 72h; washed with PBS and treated for 7h with a range of doses (0-10µg/ml) of LPS (A and B) or pre-treated for 1h with a range of doses (0-100µg/ml) of PMB and subsequently incubated with 1µg/ml of LPS for further 7h (C and D). Alternatively, cells were incubated with medium or 10µg/ml of PMB for 1h prior to addition of either 10µg/ml of LPS or 100µg/ml of LF (E). Negative control cells were cultured with medium alone. Levels of TNF-α (A, C and E) or IL-8 (B and D) were determined by ELISA (the lower limit of detection was 15pg/ml and 32 pg/ml for TNF-α and IL-8, respectively). Graphs present data from a single experiment (Figure A-D; E for LPS stimulation) or from 5 independent experiments (Figure E [LF stimulation]) (mean ± SEM). Statistical significance of differences between TNF-α levels triggered by LF in the presence and in the absence of PMB was assessed by Student’s t test.
4.2.6 *Lactoferrin stimulated TNF-α and IL-8 production via the membrane nucleolin receptor.*

There have been various reports regarding potential LFR (Legrand et al., 2004a). Here, nucleolin was investigated as a potential receptor responsible for the stimulatory action of LF. Macrophages were treated with either anti-nucleolin antibody or isotype control for 30 minutes before the addition of LF. Consistent with the previous data, treatment with LF resulted in marked secretion of TNF-α and IL-8, and in this series of experiments production of IL-1β was also measured and found to be expressed at relatively high levels in the cell lysates (approximately 8000pg/ml). Incubation with isotype control antibody did not change the level of production of any of the measured cytokines. In contrast, anti-nucleolin antibody almost completely abolished TNF-α and IL-8 secretion (Figure 4.12A and 4.12B). Interestingly, antibody had no impact on either IL-1β secretion or intracellular production (Figure 4.12C and 4.12D).
Figure 4.12 The stimulatory effect of LF was mediated via its membrane receptor – nucleolin.

THP-1 cells (5x10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 72h; washed with PBS and incubated for 30 minutes with 2μg/ml anti-nucleolin antibody (Ab) or isotype control (iso) antibody. Subsequently, cells were treated for 7h with 100μg/ml LF. Production of TNF-α (A), IL-8 (B) and IL-1β (C) in supernatants and IL-1β (D) in cell lysates was determined by ELISA (the lower limit of detection was 15, 32 or 4pg/ml for TNF-α, IL-8 or IL-1β, respectively). Graphs present data from 1-3 independent experiments (mean ± SEM).

4.2.7 The stimulatory effect of lactoferrin was lost over time

During further investigations of the mechanism of LF activation of PMA-activated macrophages there was an unforeseen decline in cytokine levels detected following LF treatment. When data were collated over a period of some 6 months it was apparent that observed decrease was time dependent. Levels of TNF-α decreased from between
14000 pg/ml (18.07.2011) and 5000 pg/ml (27.07-07.10.2011) to below 1000 pg/ml (14.10-07.11.2011) (Figure 4.13A). This was not due to a change in the preparation of LF, which remained constant. The extent of TNF-α production by 72h macrophages triggered by LF continued to decline, although the responsiveness of the cells to LPS remained relatively stable (Figure 4.13B). Various sources of THP-1 cells and PMA have been used in order to overcome the decline in sensitivity of the cells to LF. Cells that were utilised were consistently free of mycoplasma infection. Additionally, alternative ways of differentiation of THP-1 cells to macrophages (treatment with vitamin D, IFN-γ, sodium butyrate) have been investigated (data not shown). However, none of these approaches resulted in a THP-1 macrophage phenotype that was responsive to LF. Therefore, due to the loss of LF stimulatory ability in 72h macrophages no further experiments were performed in THP-1 cells to investigate the mechanism of the stimulatory action of LF.

Figure 4.13 The immunostimulatory effect of lactoferrin was lost over time.

THP-1 cells (5×10^5 cells per well) were differentiated into macrophages by culture with 100nM PMA for 72h; washed with PBS and treated for 7h with 100μg/ml LF (A) or with 100μg/ml LF and 1μg/ml LPS for 7h (B). Production of TNF-α in supernatants was determined by ELISA (the lower limit of detection was 15pg/ml). Graphs present data from 1-2 independent experiments performed over a period of some 4 months (mean ± SEM).
4.3 Discussion

Lactoferrin is a naturally occurring iron-binding protein present in mammalian secretions which has been known for decades for its antimicrobial properties (Stephens et al., 1980). Recently, however, LF has been shown to have potential immunomodulatory properties. Results to date from our laboratory support the hypothesis that exogenous LF inhibits LC migration in vivo in response to the chemical contact allergen oxazolone via effects on the availability of TNF-α in mice (Almond et al., 2013; Cumberbatch et al., 2000b) and in humans (Griffiths et al., 2001). The aim of this chapter was to determine the impact of LF on TNF-α production by THP-1 cells, in order to elucidate further the mechanism of its action.

The first step was to establish an in vitro system in which TNF-α was produced robustly. The human monocytic cell line THP-1 was the first cell line of choice for examining the mechanism of action of LF (Tsuchiya et al., 1980). THP-1 cells have been shown previously to produce TNF-α in response to TLR agonists, for example to: LPS, lipotechoic acid, flagellin or PGN (Remer et al., 2006). As described previously, one of the properties of LF is its ability to bind LPS (Appelmelk et al., 1994). Therefore, in order to avoid inhibition via sequestration of the stimulus, LPS was not deemed suitable for assessing the action of LF. Instead the TLR2 agonist, PGN, was chosen as a trigger for TNF-α production. In the studies reported herein TNF-α production by PGN-activated THP-1 monocytes occurred at a very low levels at both 6h and 24h. Production of the chemokine IL-8 was considerably higher particularly following 24h incubation with the highest concentration of PGN. Despite the low level of TNF-α production the ability of LF to modulate PGN-induced TNF-α secretion was investigated and it was found that LF did not exert inhibitory effects in that experimental system.

The positive control for inhibition of cytokine production was dexamethasone, a corticosteroid drug well known to down-regulate inflammatory cytokine production, such as that produced by macrophages following LPS stimulation (Armstrong et al., 2009). Dexamethasone almost completely abrogated TNF-α production by THP-1 monocytes following PGN treatment. Consequently, differentiation of THP-1 cells into
macrophages was explored using methods utilized by Zhou et al. (2010)(3h PMA activation) and Eda et al. (1996)(24h-120h PMA activation). Differentiation of THP-1 cells into macrophages with PMA is a well recognized method (Tsuchiya et al., 1982). Our laboratory routinely utilized the protocol of 3h PMA differentiation with subsequent overnight incubation of the cells and that method was used first to obtain macrophages for these investigations. As predicted, 3h THP-1 macrophages produced higher levels of TNF-α than did THP-1 monocytes. Human peripheral blood monocytes have been reported previously to produce approximately 3000pg/ml of TNF-α following 2h incubation with 10μg/ml of PGN (Bonnet et al., 2008). The result reported by Bonnet et al. (2008) was similar to that observed in the current studies where THP-1 macrophages incubated for 3h with PMA followed by treatment with PGN (25μg/ml) secreted approximately 2500pg/ml of TNF-α. Dexamethasone treatment resulted in overall inhibition of both TNF-α and IL-8 production by 3h THP-1 macrophages with the exception of baseline (in the absence of PGN) TNF-α secretion. Naitoh et al. (2001) investigated the effect of PMA on TNF-α secretion by THP-1 monocytes and macrophages and found that PMA (incubation for 24h with 50ng/ml of PMA) treatment of THP-1 monocytes induced cytokine secretion (approximately 300pg/ml of TNF-α), whereas, the same treatment did not induce detectable cytokine secretion by THP-1 macrophages (differentiated with 50ng/ml PMA for 12h). From the perspective of the results reported by Naitoh et al (2001) it is difficult to determine whether the 3h PMA activation method caused TNF-α secretion in the current study, however, it is consistent with the absent or low baseline levels of TNF-α secreted by THP-1 macrophages activated with PMA for 24h-120h. PMA-induced IL-8 was reported previously to be resistant to dexamethasone mediated inhibition (Anttila et al., 1992). In the current investigations IL-8 secretion was susceptible to dexamethasone treatment. The discrepancy could be explained by the fact that levels of IL-8 demonstrated by Anttila et al. (1992) were much higher than those reported in the current study (100-300ng/ml vs.10-20ng/ml), suggesting that there might be a limit to the ability of dexamethasone with respect to inhibiting PMA-induced IL-8 production. Lactoferin had little or no inhibitory effect on TNF-α secretion by THP-1 monocytes when it was added either 1h before or 1h after (data not shown) addition of PGN to the culture.

In differentiated (3h PMA) THP-1 cells some modest effects of LF were observed with decreased baseline (approximately 25% inhibition) and PGN-induced TNF-α secretion
(25% inhibition). In addition, doses of 2-100μg/ml LF had the same slight inhibitory effect on secreted TNF-α. In parallel, experiments were conducted in which LF was added to the culture 1h after PGN (total of PGN stimulation time remained 6h) in order to examine if the time of introduction of LF to the culture influenced its effects (data not shown). In these experiments the inhibitory effects of LF were comparable to those achieved when LF was added 1h prior to PGN stimulation. In addition to inhibiting TNF-α expression, LF reduced, although to lesser extent, IL-8 secretion. Similar to the influence of LF on TNF-α, these effects were not dose dependent nor did they reach statistical significance. Although the observed inhibitory effect of LF was not statistically significant, it was consistent between experiments, different cytokines and was in keeping with the in vivo data from LC migration experiments (Cumberbatch et al., 2000b), and as such, was thought to be of physiological relevance. The data reported herein represented the first step in examining the immunosuppressive function of LF in an LPS independent system. In order to better characterize the effect of LF, experiments were performed to titrate out the inhibitory effect of LF on TNF-α production (data not shown). During these investigations it became apparent that the modest inhibition of TNF-α production was no longer observed at concentrations of LF that previously inhibited cytokine production. Thus, the effect of LF was not robust, and therefore this was not an appropriate experimental system in which to examine the mechanism of the effects of LF.

It has been shown previously that THP-1 cells differentiated into macrophages bound the highest levels of radio-labelled LF when they were activated with PMA for 120h, suggesting that there was a time dependent increase in expression of the receptor following treatment with PMA (Eda et al., 1996). Given this information on LFR expression in THP-1 macrophages, an alternative method of THP-1 differentiation was utilized. A PMA time course was performed to establish if LF treatment at time points that had been reported to show an increased expression of the receptor resulted in augmented inhibitory activity of LF. When THP-1 macrophages produced after 24h to 120h stimulation with PMA were activated with PGN no inhibitory effect of LF on TNF-α secretion was observed at any of the time points tested. Surprisingly, in 24h-120h THP-1 macrophages incubated in the absence of PGN a dose dependent, stimulatory effect of LF on TNF-α and IL-8 secretion was observed. Stimulation by LF of cytokine production by macrophages has been reported previously by other authors.
using LF from various species. For example, when the murine macrophage cell line RAW 264.7 was incubated with bovine LF (100μg/ml) approximately 6000pg/ml of TNF-α was produced (Curran et al., 2006), similar results to those observed in current investigations. Thus, the same concentration of human LF (100μg/ml) stimulated TNF-α secretion at similar levels by 72h, 96h and 120h THP-1 macrophages. Furthermore, Sorimachi et al. (1997) used bovine LF to assess its effects on macrophages derived from rat bone marrow. After 6h incubation with bovine LF release of approximately 1200 pg/ml of TNF-α and 3000 pg/ml of IL-8 was observed. Those levels were somewhat lower and ten times less, respectively, than what was found in the current investigations. However, these differences were likely to stem from the fact that the concentration that utilized by Sorimachi et al. (1997) was 10μg/ml which is 10 times less than what was used in the current study, as well as different cell type was used.

It was considered important to determine if observed activation of THP-1 macrophages was in fact due to LF, rather than to a nonspecific effect of additional protein. Therefore, TRF was used as a control. Lactoferrin and TRF both belong to the transferrin family of proteins, have the same molecular weight (80kDa), share 61.4% sequence homology and both are capable of binding two Fe³⁺ ions (Wally and Buchanan, 2007). Transferrin is the primary iron carrier in the blood and has a role in the innate immune response due to its involvement in iron metabolism (Johnson and Wessling-Resnick, 2012). Treatment with TRF did not result in either TNF-α or IL-8 secretion, whereas incubation with LF triggered release of 1.5ng/ml of TNF-α and 19ng/ml of IL-8, implying that the observed stimulatory effect was attributable to a specific property of LF. In addition, specificity of the LF effect was confirmed by demonstrating that LF did not stimulate TNF-α release by THP-1 monocytes, suggesting as Eda et al. (1996) had shown that the interaction of LF with THP-1 macrophages and subsequent TNF-α release, was a consequence of the interaction of LF with its specific receptor, which was up-regulated by PMA activation.

Moreover, it was necessary to establish that observed immunostimulatory action of LF was not due to endogenous LPS contamination of the protein preparation. Protein preparations can contain endotoxin contamination (Magalhães et al., 2007) that can lead to false positive results if the protein is not endotoxin depleted prior to use (Lin and Kuo, 2010). In our laboratory commercially available proteins have been found to have
from 0.05 endotoxin units (EU)/mg to as high as 6000EU/mg. THP-1 monocytes have been reported to respond to 200EU or less with cytokine secretion (personal communication, Dr. Rebecca Dearman). Therefore, it was critical to establish that the observed activation of TNF-α by LF was not mediated by contaminating LPS. Despite the fact that previous batches of LF were known to have relatively low endotoxin levels [10EU per mg of LF; (Almond et al., 2013)] it was decided to conduct an experiment in which THP-1 macrophages were exposed to PMB, a specific LPS binding protein, prior to addition of LF. It has been reported that THP-1 macrophages were sensitive even to low levels of endotoxin. Takashiba et al. (1999) demonstrated that THP-1 cells differentiated with 200nM PMA for 24h produced ~3ng/ml of TNF-α following 6h incubation with 0.1μg/ml of LPS (equal to 1000EU). Similarly, in the current study THP-1 macrophages vigorously responded to treatment with LPS with TNF-α and IL-8 production. Incubation of cells with PMB, prior to addition of LPS, dose dependently inhibited cytokine release, confirming PMB as LPS inhibitor. Polymixin B has been reported to efficiently inhibit stimulation of PBMC by contaminating endotoxin in the preparation of recombinant proteins produced in E. coli (Cardoso et al., 2007). In another study pre-incubation of murine macrophage cell line RAW264.7 for 30 minutes with 10μg/ml of PMB abrogated release of TNF-α following stimulation with 0.2ng/ml of LPS, as well as 1μg/ml of human recombinant Hsp70. In the absence of PMB, LPS and human recombinant Hsp70 triggered release of approximately 6ng/ml of TNF-α (Gao and Tsan, 2003). On the other hand, Tynan et al. (2012) reported that PMB inadequately inhibited LPS-induced TNF-α secretion by bone marrow derived DC. At 1μg/ml of LPS, 10μg/ml of PMB did not have inhibitory effects. With decreasing concentrations of LPS, the PMB effect was more pronounced and reached approximately 90% inhibition when 1ng/ml of LPS was used (Tynan et al., 2012). In the study reported herein, PMB inhibited LPS-induced TNF-α secretion by 80%, but was without impact on LF-triggered TNF-α production. Similarly, Na et al. (2004) reported that while PMB (at 50μg/ml) was able to abolish the ability of LPS to trigger nitric oxide (NO) production by RAW 264.7 cells, it had only minimal effect on NO production elicited by complex of bovine LF with LPS (Na et al., 2004). In the current study PMB was used to prove independence of observed immunostimulatory effect of human LF. However, the study by Na et al. (2004) suggested that use of PMB is not suitable to confirm endotoxin independence of LF. It has to be noted, that the concentration utilized in the current study was lower than that used by Na et al. (2004)
(10 vs. 50μg/ml). It was shown previously that at 50μg/ml PMB caused 21% release of chromium from chromium labelled K562 human leukemia cells, in comparison with cells treated with medium alone. Conversely, no chromium was released from cells treated with 10μg/ml of PMB, implying that PMB at 50μg/ml was toxic to cells (Duwe et al., 1986). The results of Duwe et al. (1986) also suggested that a fraction of NO released by PMB treated RAW 264.7 cells in the study by Na et al.(2004) could have come from dead or dying cells and not as a result of stimulation with un-purified bovine LF. Taken together, it was concluded that in the study reported herein the observed immunostimulatory potential of LF was not due to endotoxin contamination.

Having established the specificity of LF effects, it was decided to identify the receptor through which LF was exerting its stimulatory action. There have been many proteins that have been implicated acting as LFR in various cell types. It is logical from an evolutionary point of view that there would be a LFR present in the intestine, as LF is abundantly present in the breast milk (Suzuki et al., 2001). However, in addition to its presence in milk, LF can also be found in serum and in the secondary neutrophil granules (Wang et al., 1995), indicating its involvement in the immune response. PMA-differentiated THP-1 macrophages were observed to bind LF (Eda et al., 1996), oxidised erythrocytes (Beppu et al., 1996) and early apoptotic Jurkat cells via a protein later identified as nucleolin (Hirano et al., 2005). With nucleolin identified as an LFR present on THP-1 macrophages it was investigated whether anti-nucleolin antibody could block the observed immunostimulatory effects of LF. It was found that both TNF-α and IL-8 production was almost completely abrogated when cells were pre-incubated with anti-nucleolin antibody. Conversely, neither secretion nor intra-cellular production of IL-1β was affected. However, Ando et al. (2010) suggested that nucleolin was an unlikely candidate for transducing the signal from LF as it lacks the cytoplasmic domain, which would be responsible for such role. Nevertheless, it was possible that nucleolin has a crucial role in TLR4-mediated signal transduction (Ando et al., 2010; Curran et al., 2006), whereby its (nucleolin) blockage results in abolition of LF-mediated TNF-α and IL-8 secretion. Lactoferrin, with other proteins secreted by neutrophils, α-defensins and cathelicidins (LL-37), recently has been described as an alarmin. Alarmins are proteins that in response to the external or internal injury are able to recruit and activate cells of the immune system. Despite the lack of homology between alarmins, they are all cationic proteins (Yang et al., 2009). One of the reasons
why anti-nucleolin antibody did not inhibit IL-1β production could have been that IL-1β synthesis was achieved via an interaction of LF with P2X7 receptor and not nucleolin. It was reported that another alarmin, LL-37, was capable of activating P2X7 receptor and subsequent IL-1β production (Elssner et al., 2004). To date, there have been no reports demonstrating that blocking nucleolin resulted in an inhibition of cytokine production. However, Wang et al. (2011) have shown that the treatment of rat alveolar macrophages with anti-nucleolin siRNA inhibited TNF-α and IL-6 production following treatment with 1μg/ml of LPS. Results reported by Wang et al. (2011) may suggest that cytokine inhibition observed in the current study was due to inhibition of LPS signalling, furthermore indicating that the immunostimulatory effects of LF were in fact due to endotoxin contamination. However, as it was demonstrated by experiments utilising PMB, cytokine secretion following LF treatment in this study was endotoxin independent.

It would have been of interest to see if treatment with nucleolin agonist other than LF (eg. midkine, a low molecular weight protein involved in cell proliferation, migration and organogenesis (Muramatsu, 2002)) would have resulted in a similar increase in TNF-α production. Similarly, it would have been important to observe if a treatment with nucleolin inhibitor, pseudo-peptide HB-19, would have resulted in abolition of the stimulatory effects caused by LF (Nisole et al., 1999). However, none of these experiments could take place due to the observed loss of responsiveness of THP-1 macrophages to stimulation with LF.

While performing routine experiments that were to further characterize LF-triggered cytokine production, it was noticed that the stimulatory effect of LF on 72h THP-1 macrophages was diminishing. After collating the data, it was evident that the change in responsiveness had happened over a period of a month (from 07 October to 07 November). New THP-1 cells were acquired, however, that did not make a difference. Cells that were used were not infected with mycoplasma. Additionally, new batches of PMA and LF were sourced. Lactoferrin, both from the previously used batch and from the new stock, displayed the same level of biological activity in vivo, that is, both were capable of inhibiting oxazolone-induced LC migration. Nevertheless, after investigating every component of the system (cells, PMA and LF) the initial up-regulation of TNF-α production by LF was still not observed. It would have been useful to utilise LF from
other commercial sources than the one that was used throughout current investigations (Sigma Aldrich).

Investigation of the mechanism of action of LF started with the intention of elucidating its inhibitory effect on TNF-α production and has finished by demonstrating specific, stimulatory effects of the glycoprotein. Although at first this may seem contradictory, there are in fact many reports to support both claims of LF immunosuppressive and immunostimulatory action. Thus, injection of recombinant mouse LF into mice resulted in myelopoiesis that presented as increased levels of neutrophils, metamyelocytes and myelocytes in circulating blood. This effect of LF could be perceived as an inflammatory one, as higher levels of neutrophils appear when there is an infection, but at the same time when LF is present it serves to restore homeostasis, therefore reinforcing its role as both a stimulator and suppressant (Zimecki et al., 2013). Nishiya et al. (1982) demonstrated that human LF exerted both stimulatory and inhibitory effects on the same population of cells. The ability of LF to modulate cytotoxicity and antibody-dependent cellular toxicity of human PBMC was tested. It was found that LF increased the cytotoxicity of the adherent PBMC fraction against the K562 cell line at concentration of 1μM, while the same concentration of the control protein TRF was without effect. In contrast, when antibody-dependent cell-mediated cytotoxicity activity of PBMC was measured against antibody coated liver cells, it was established that LF dose dependently inhibited that effect, but again only in adherent fraction of PMBC (Nishiya and Horwitz, 1982). Those results suggest that the role of LF on immune system is complex and multifaceted. It is likely that its role depends on the underlying condition of the organism.

Some of the discrepancies in the reports of LF functions might stem from the fact that LF was found to exist in 3 isoforms that differ with regards to their RNase and iron binding abilities. Affinity chromatography eluted 3 LF fractions that had two distinct functions: LF α – classical iron binding form, LF β and γ – RNase, non-iron binding forms eluted from Cibacron Blue Sepharose at 0.4 and 0.2M NaCl, respectively (Furmanski et al., 1989). The concept of multiple LF isomers was further investigated and this time 5 fractions were identified, with differential functions of DNase, RNase, and ATPase as well as amylase and phosphatase (Kanyshkova et al., 2003). It was not stated what the differences were, if any, between isoforms, other than differential
strength of binding to the Cibacron Blue Sepharose. Moreover, it was suggested that the ability of LF to oligomerise might impact on its versatile functions. Indeed, it was reported that status of LF in the solution depends on the LF concentration and the presence of salts and/or LF substrates, like DNA or ATP (Nevinskii et al., 2009). Most commonly found forms of LF are monomers and tetramers (Mantel et al., 1994).

In the current investigations there was no distinction between apo- and holo-forms of LF and only native form of LF was used that was demonstrated previously to be approximately 12-25% saturated with iron (Bezwoda and Mansoor, 1989). It was demonstrated before that iron saturation status does not affect LF ability to inhibit LC migration (Cumberbatch et al., 2000b). However, there were reports that iron saturation status impacted on other aspects of LF function. Proliferation of Caco-2 cells (epithelial colorectal carcinoma cells) was more strongly stimulated by apo-LF, but not by holo-LF, despite both molecules signalling through the same receptor in a common clathrin-dependent pathway (Buccigrossi et al., 2007; Jiang et al., 2011). These differences were thought to stem from the fact that the apo-form of LF had a different conformation than the holo-form. When iron is bound by LF it causes the molecule to become more compact, therefore, potentially masking binding sites that could be recognized in the apo-form of LF (Grossmann et al., 1992).

It was reported that human LF in THP-1 and mouse embryonic fibroblast cells activated the canonical pathway of NF-κB, regardless of whether it was depleted of endotoxin using PMB. Additionally, LF activated NF-κB in the presence of inhibitors of transcription (actinomycin D) and translation (cyclohexamide) (Ando et al., 2010), suggesting that the observed effect was solely due to LF and not secondary to TNF-α, which was shown to be induced by LF and is known to potently induce NF-κB canonical pathway (Zelová and Hošek, 2013). Ando et al. (2010) established that NF-κB activation was due to the carbohydrate moiety of LF, as the effect was abolished when LF was treated with endo-β-galactosidase that destabilises glycosylation chains. However, the activation of NF-κB remained intact following incubation with a non specific proteinase, actinase E. Moreover, Ando et al. (2010) have shown that bovine LF was unable to trigger NF-κB signalling, whilst inhibiting LPS-induced NF-κB
stimulation. That result contrasted with data gathered in the current investigations (data not shown) and others (Sorimachi et al., 1997), where the treatment with bovine LF resulted in cytokine production. Ando et al. (2010) concluded that the protein part of LF molecule was responsible for the inhibitory actions of LF, whereas the carbohydrate portion of the molecule evoked stimulatory effects.

Results presented in this chapter suggest dichotomy in the function of LF that is in accordance with the literature. It is possible that the duality of LF action stems from its structure, with protein and glycan parts of the molecule responsible for opposing effects. Furthermore, various receptors utilized by LF could influence its function (Losfeld et al., 2009; Spadaro et al., 2014; Suzuki et al., 2001). In addition, the possibility of differential functions of a variety of LF isoforms makes it possible for LF molecule to exert a plethora of different functions depending on the organisms’ requirements.
5 Effects of lactoferrin on keratinocytes

5.1 Introduction

The skin is the organism’s first line of defence. It is divided into 3 layers: epidermis, dermis and hypodermis, that have distinct as well as complementary roles in maintaining the safety of the organism (Nestle et al., 2009). The epidermis is the outermost layer of the skin. It comprises of four layers (moving from the bottom outwards): basal, spinous, granular and cornified. Cells (keratinocytes) in the basal layer divide and move to the spinous layer where they change shape from columnar to polygonal, and start expressing keratins (K1 and K10) distinct from those produced by basal layer cells (K5 and K14) (Haines and Lane, 2012). Ultimately keratinocytes become corneocytes in the cornified layer, whereby they lose all of their organelles, become filled with keratin and provide the barrier function of the skin (Nestle et al., 2009).

It has been established that following allergen treatment increased epidermal levels of TNF-α and IL-1β are found (Enk and Katz, 1992a). Further, these two cytokine signals were demonstrated to be necessary for LC migration (Cumberbatch et al., 1997b). It was established that LC were the main source of IL-1β in the steady state epidermis (Enk et al., 1993; Matsue et al., 1992b). Therefore, it was hypothesised that the TNF-α necessary for LC migration was derived from keratinocytes. The fact that topical exposure to LF inhibited LC migration induced by oxazolone and exogenous IL-1β, but was without effect on LC mobilisation triggered by exogenous TNF-α (Cumberbatch et al., 2000b), suggested that LF decreases the availability of endogenous TNF-α by interaction with keratinocytes. In the series of experiments described in this chapter three keratinocyte cell types have been utilized to investigate the immunomodulatory effects of LF, primary mouse keratinocytes and the PAM 212 cell line derived from BALB/c strain mice and human immortalized keratinocytes, the HaCaT cell line. Cytokine production and expression was stimulated using the TLR2 ligand PGN, similarly to experiments described in the Chapter 4. Additionally, keratinocytes were stimulated with cytokines that have been shown to play important roles in LC migration: TNF-α, IL-1α and IL-1β.
5.2 Results

5.2.1 PAM 212 and HaCaT cell lines and primary murine keratinocytes expressed keratin-14

The first step in these investigations was to establish that the cells used were indeed keratinocytes. Immunofluorescence staining was performed for the expression of K14, present in basal keratinocytes of all stratified squamous epithelial tissues (Green et al., 2003). Both murine and human cell lines (Figure 5.1A-5.1D) were cultured on a glass coverslip, stained and mounted onto a slide. Primary murine cells failed to grow on a glass surface; therefore, staining was performed in the culture plate. Due to differences in the preparation of the samples different microscopes had to be utilized. This may partially explain differences observed in image brightness between samples. Both PAM and HaCaT cells grew to confluence on glass coverslips (as shown by DAPI staining), and clear staining of K14 fibres in the cytoplasm was observed compared with the isotype (negative) controls in both cell types. Comparatively little staining was observed for primary keratinocytes, although the pattern was also cytoplasmic (Figure 5.1E, 5.1F). This differential staining appears to be due to the different preparation method utilized for the primary keratinocytes, as when PAM cells were prepared in the same way as primary murine keratinocytes, they also displayed considerably lower levels of keratin staining (Figure 5.1G, 5.1H). Thus all three cell types expressed K14 to some extent. Keratin 14 together with K5 is expressed by cells in the basal layer of epidermis. These two keratins have been identified as markers of stratified squamous epithelial cells (epidermis) (Nelson and Sun, 1983). Therefore, detection of K14 within the tested cells confirmed their status as keratinocytes.
Figure 5.1 Primary keratinocytes and keratinocyte cell lines expressed keratin 14.
**Figure 5.1 Primary keratinocytes and keratinocyte cell lines expressed keratin 14.**

PAM cells (A, B, G, H), HaCaT cells (C, D) or primary murine keratinocytes (E, F) were cultured for 48h on glass coverslips (A-D) or in tissue culture plates (E-H), washed and fixed in ice cold methanol for 10 minutes, washed with PBS and stained with anti-K14 antibody at 37°C for 30 minutes. Subsequently, cells were washed and incubated with secondary goat anti-rabbit FITC conjugated antibody (1:50 dilution). Next, cells were mounted onto a slide in VECTASHIELD mounting medium with DAPI. Representative micrographs are shown of anti-keratin staining (A, C, E, G) as well as respective isotype staining (B, D, F, H) overlaid with DAPI image (x20 magnification). Scale bar 100μm.

**Table 5.1 PAM 212 cells did not secrete TNF-α following incubation with a variety of stimuli.**

<table>
<thead>
<tr>
<th>TLR ligand</th>
<th>TNF-α</th>
<th>IL-1α</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>CXCL9</th>
<th>CXCL10</th>
<th>IL-4</th>
<th>FNy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGN 25μg/ml</td>
<td>&lt;31pg</td>
<td>&lt;15pg</td>
<td>&lt;15pg</td>
<td>&lt;312pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
<tr>
<td>PolyIC 25μg/ml</td>
<td>&lt;31pg</td>
<td>&lt;15pg</td>
<td>&lt;15pg</td>
<td>&lt;312pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
<tr>
<td>LPS 10μg/ml</td>
<td>&lt;31pg</td>
<td>&lt;15pg</td>
<td>&lt;15pg</td>
<td>&lt;312pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
<tr>
<td>Flagellin 83ng/ml</td>
<td>&lt;31pg</td>
<td>&lt;15pg</td>
<td>&lt;15pg</td>
<td>&lt;312pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
<tr>
<td>CpG 10μg/ml</td>
<td>&lt;31pg</td>
<td>&lt;15pg</td>
<td>&lt;15pg</td>
<td>&lt;312pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
<tr>
<td>stimulus</td>
<td>PMA 1μg/ml</td>
<td>&lt;100pg</td>
<td>&lt;150pg</td>
<td>&lt;310pg</td>
<td>&lt;315pg</td>
<td>&lt;62pg</td>
<td>h.d</td>
<td>h.d</td>
</tr>
</tbody>
</table>

PAM cells were grown in complete RPMI medium. Cells were passaged every 2-3 days. For use in the experiment cells were seeded at 10⁵ cells per well in 12 well plates and cultured for 48h to become confluent. Subsequently, cells were washed and incubated with the listed stimuli for 24h. Following incubation supernatants were collected and levels of cytokines and chemokines were determined by ELISA. * n.d- not done.

**5.2.2 PAM cells did not respond to TLR ligands**

Following on from the work conducted using THP-1 macrophages (Chapter 4) it was decided to *in vitro* stimulate keratinocytes with PGN to elicit cytokine or chemokine production. Lactoferrin has been shown to bind LPS potently, thereby excluding its use as a stimulant in the current investigations, as described in the previous chapter. Primary
keratinocytes, as well as cell lines, have been widely reported to express TLR (Fukui et al., 2013; Olaru and Jensen, 2010). Therefore, it was decided to examine whether this keratinocyte cell line (PAM) also responded to TLR ligands. PAM cells were treated with PGN, as well as other TLR agonists or PMA (Table 5.1) and the production of cytokines and chemokines was determined by ELISA. PAM cells did not produce detectable levels of the main cytokines of interest, such as TNF-α, IL-1α or IL-1β, in response to TLR ligands. Expression of various chemokines (CXCL9 and CXCL10) was also below the limit of detection for all TLR ligands. The lack of vigorous cytokine protein production in response to these TLR ligands or to the mitogen PMA was the basis for exploration of other stimuli to which PAM cells could be exposed. Given their role in cutaneous inflammatory responses, it was decided to assess the response of PAM cells to IL-1α and IL-1β.

5.2.3 PAM cells produced IL-1α and IL-1β

In initial experiments, the ability of the murine keratinocyte PAM cell line to secrete cytokines in response to stimulation with IL-1α and β was investigated. Following IL-1α stimulation, IL-1β production was measured (Figure 5.2A and 5.2B) and, conversely, after IL-1β treatment levels of IL-1α were assessed (Figure 5.2C and 5.2D) by ELISA in culture supernatants and cell lysates. There was low level constitutive release of IL-1α (20pg/ml), and some evidence for a dose dependent increase in secretion of this cytokine following stimulation with IL-1β, reaching a peak of ~90pg/ml at 1ng/ml IL-1β (Figure 5.2C). Resting intracellular production of IL-1α was considerably higher (~1200pg/ml) than secreted cytokine and increased approximately 2-fold in response to treatment with 1-100ng/ml IL-1β (Figure 5.2D). In contrast, incubation of PAM cells with IL-1α failed to impact markedly on IL-1β production: constitutive levels of 75pg/ml and 20pg/ml were recorded for secretion and intracellular expression, respectively, regardless of addition of exogenous IL-1α (Figure 5.2A and 5.2B). Furthermore, there was no detectable TNF-α protein expression (<15pg/ml) recorded under any conditions (data not shown).
Figure 5.2 Effects of homologous, recombinant IL-1α and IL-1β on IL-1α and IL-1β production by PAM cells.

PAM cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and incubated with a range of concentrations (0-100ng/ml) of IL-1α (A, B) or IL-1β (C, D) for 24h. Following incubation, supernatants were collected (A, C) or cells were lysed (B, D) and the level of IL-1α and IL-1β was determined by ELISA (dashed line represents the lower limit of detection of the ELISA [15pg/ml]). Statistical significance of differences between cytokine production of cytokine treated cells versus medium treated controls was analyzed by Kruskal-Wallis test followed by Dunn’s multiple comparisons test. No statistically significant differences were found (p>0.05). Graphs present data (mean ± SEM) from 4 independent experiments.
5.2.4 PAM cells responded to stimulation with IL-1α and IL-1β

Due to the relatively low levels of IL-1α and β protein release by PAM cells, and absence of TNF-α production, the next step in the current investigations was to examine changes in mRNA levels for Tnf-α, Il-1β, Il-1α, Cxcl1 and Icam-1 expressed by PAM cells following cytokine stimulation. Quantitative PCR was utilized to determine the change in gene expression (Tnf-α, Il-1β, Il-1α, Cxcl1 and Icam-1) levels (relative to the housekeeping Hprt1 gene). In the first instance, dose dependent changes were examined following culture with homologous IL-1α and IL-1β. The 2h time point was chosen for investigation of the dose dependent effects of IL-1α and β stimulation (Figure 5.3). PAM keratinocytes responded to cytokine stimulation within this 2h time period and IL-1α was the most potent stimulus for all of the genes tested. Incubation with IL-1α at concentrations between 0.1 and 1ng/ml resulted in maximal augmentation of expression for all genes (2, 19, 64, 200 and 290 fold change for Il-1β, Il-1α, Icam-1, Cxcl1 and Tnf-α, respectively). PAM cells were somewhat less responsive to IL-1β, with peak induction recorded at 1ng/ml. The rank order of triggered responses was the same for IL-1α and IL-1β: TNF-α < CXCL1 < ICAM-1 < IL-1α < IL-1β. Interestingly, whereas TNF-α protein was not detected following cytokine treatment, Tnf-α gene expression was very markedly up-regulated by both cytokines, with a maximal induction observed following incubation with 0.1ng/ml of IL-1α (290 fold change) and with 1ng/ml of IL-1β (490 fold change).
**Figure 5.3 Homologous, recombinant IL-1α and IL-1β-induced dose dependent cytokine and Icam-1 expression by PAM cells.**

PAM cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently cells were incubated with a range (0-10ng/ml) of concentrations of IL-1α (A) or IL-1β (B) for 2h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α, Cxcl1, Il-1α, Il-1β and Icam-1 was measured relative to the Hprt1 gene and compared with the medium (0 ng/ml) treated sample. Graphs present data from a single experiment.

Based on these dose response analyses, PAM cells were incubated with 2ng/ml of either IL-1α or IL-1β for 0, 1, 2, 4, 6, 8 or 24h (Figure 5.4). The rank order of induction of the molecules investigated was the same as that recorded for the dose response studies (TNF-α < CXCL1 < ICAM-1 < IL-1α < IL-1β). Furthermore, the kinetics of cytokine-induced expression of the molecules was also similar; peaking at 1 to 2 h and returning to baseline levels within 8h. However, IL-1α was a more potent stimulator than was the equivalent amount of IL-1β. High level expression of Tnf-α was induced by stimulation with both IL-1α and β (316 and 100 increase in Tnf-α were observed following 1h treatment with IL-1α and IL-1β, respectively). Whereas Il-1β exhibited the lowest levels of up-regulation among the genes measured (maximal induction 1.9 fold increase following 4h treatment with IL-1α). On the contrary, expression of Il-1α was rapidly increased to 19 and 16 fold increase by both IL-1α and IL-1β, respectively, at concentration of 1ng/ml.
Figure 5.4 Homologous, recombinant IL-1α and IL-1β-induced time dependent increases in cytokine and Icam-1 expression by PAM cells.

PAM cells were seeded at 10^5 cells per well in 12 well plates and cultured for 48h. Subsequently, cells were incubated with 2ng/ml of IL-1α or IL-1β for 1, 2, 4, 6, 8 or 24h. After incubation cells were lysed, mRNA was extracted, cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α, Cxcl1, Il-1α, Il-1β and Icam-1 was measured relative to the Hprt1 gene and compared with the medium treated sample for each time point. Graphs present data from a single experiment.

5.2.5 Lactoferrin did not modulate cytokine stimulated gene expression by PAM cells

Having developed a protocol that resulted in vigorous induction of Tnf-α mRNA, the next step was to investigate the immunomodulatory effects of LF on cytokine stimulated PAM cells. On the basis of the experiments presented in Figures 5.3 and 5.4, the concentration of 2ng/ml cytokine and the incubation time of 2h were chosen for IL-1α and β stimulation. PAM cells were pre-treated with either 10 or 100μg/ml of LF for 1h and subsequently incubated with either IL-1α (Figure 5.5A and 5.5B) or IL-1β (Figure 5.5C and 5.5D) for a further 2h. In the absence of LF, treatment with IL-1α resulted in induction of Tnf-α and Icam-1 expression. These results were broadly consistent with data generated in the experiments presented in the Figures 5.3 and 5.4, however, there was considerable inter-experimental variation in the absolute level of IL-
1α-triggered gene expression (Figure 5.5A and 5.5B). Similarly, exposure of PAM cells to IL-1β caused up-regulation in levels of Tnf-α and Icam-1. Experiments in which 100μg/ml of LF was used resulted in higher levels of variation compared with those in which 10μg/ml of LF was used. Observed changes were in the range of 120-450 and 43-125 fold changes for Tnf-α and Icam-1, respectively (Figure 5.5C and 5.5D). Regardless of the stimuli used, LF did not impact on the expression of either Tnf-α or Icam-1 (Figure 5.5).

The concentrations used in the investigations presented in the Figure 5.5 were designed to result in maximal changes in gene expression and under these conditions LF was without effect. Therefore, in subsequent experiments, a lower, sub-maximal, concentration of cytokine (0.1ng/ml) was utilized and IL-1β only was used as a stimulant (Figure 5.6). In addition, these experiments were conducted in serum free medium, in order to maximise the potential effect of LF. PAM cells were pre-treated with various concentrations of LF (1-100μg/ml) or 100μg/ml of BSA (an irrelevant control protein) for 2h prior to treatment with 0.1ng/ml of IL-1β for a further 2h. The data are presented as the percentage of the control (gene expression following induction with 0.1ng/ml of IL-1β) in each experiment, in order to account for the variability of gene expression levels between experiments. All doses of LF resulted in some inhibition of cytokine-induced Tnf-α and Icam-1 but only 100μg/ml of LF reached statistical significance. However, these results need to be interpreted with caution, as there was no dose response observed. Additionally, similar changes in gene expression were also observed in the presence of the control protein BSA, suggesting that the effect of LF was nonspecific.
Figure 5.5 Lactoferrin did not affect IL-1β or IL-1α-induced Tnf-α or Icam-1 expression in PAM cells.

PAM cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were pre-treated with either 10μg/ml or 100μg/ml of LF for 1h. Afterwards, 2ng/ml of homologous IL-1α (A, B) or IL-1β (C, D) was added for another 2h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α (A, C) or Icam-1 (B, D) was assessed relative to the Hprt1 gene and compared to the medium treated sample. Statistical significance of changes in gene expression in IL-1β treated cells compared with LF treated cells was analyzed by Kruskal-Wallis test followed by Dunn’s multiple comparisons test. *$=p<0.05$, **$=p<0.01$. Graphs present data (mean $\pm$ SEM) from 3-6 independent experiments.
Figure 5.6 Lactoferrin significantly inhibited expression of Tnf-α and Icam-1 induced by lower concentration of IL-1β.

PAM cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media. Cells were pre-treated with 1, 10 or 100μg/ml LF or with 100μg/ml BSA for 2h. After 2h 0.1 ng/ml homologous IL-1β was added for further 2h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α (A) or Icam-1 (B) was assessed relative to the Hprt1 gene, and compared with the medium treated sample. Data are presented as a percentage of the control (0.1ng/ml of IL-1β). The statistical significance of changes in gene expression between the different LF concentrations, compared with no LF (100%), was analysed by a Kruskal-Wallis test followed by Dunn’s test; *$p<0.05$. Graphs present data (mean ± SEM) from 2-4 independent experiments.

5.2.6 PGN induced TNF-α production by primary keratinocytes

Cell lines such as PAM may not display the characteristics of freshly isolated cells, which might explain why these cells do not secrete significant amounts of TNF-α and do not respond to LF. Therefore, in parallel studies the responses of primary keratinocytes derived from new born BALB/c strain mice were investigated. Thus, TNF-α, IL-1α and β secretion was measured following TLR2 ligand (PGN) stimulation of primary keratinocyte cultures (Figure 5.7). Resting cells did not express detectable levels of secreted TNF-α, although there was detectable constitutive expression of both IL-1α and IL-1β (50 and 20pg/ml, respectively). Peptidoglycan treatment resulted in a modest increase in TNF-α secretion (50pg/ml) (Figure 5.7A) but did not impact on
either IL-1α or IL-1β release (Figure 5.7B and 5.7C). As the measurement of cytokine at the mRNA level was generally more sensitive, expression of Tnf-α and Il-1β was also examined by RT-PCR following incubation with TLR ligands. Culture with PGN resulted in up-regulated Tnf-α and Il-1β expression (by 3 and 4 fold, respectively) (Figure 5.8), whereas the TLR9 ligand, CpG, was without effect. Interestingly, treatment with LPS markedly down regulated Il-1β expression. The effect of PGN was robust and dose dependent; thus 10μg/ml of PGN was found to trigger approximately 10 and 20 fold increases in expression of Tnf-α and Il-1β, respectively (Figure 5.8C and 5.8D).

![Figure 5.7 Primary keratinocytes secreted TNF-α following PGN stimulation.](image)

Primary keratinocytes were isolated as described in the methods and cultured to confluency in 12 well plates. Subsequently, cells were washed and either media or 10μg/ml PGN was added for a further 24h. Following incubation supernatants were collected and the level of TNF-α, IL-1α and IL-1β was determined by ELISA (the lower limit of detection was 31pg/ml for TNF-α and 15pg/ml for IL-1α and IL-1β). Graph presents data from a single (A) or two (B, C) (mean ± range) independent experiments.
Primary keratinocytes were isolated as described in the methods and cultured to confluency in 12 well plates. Subsequently, cells were washed and incubated with 1μg/ml of LPS, CpG, R848, PGN (A, B) or with a range of PGN doses (C, D) for 24h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α and Il-1β was measured relative to the Hprt1 gene and compared with the medium treated sample. Graphs present data from a single experiment or 5 independent experiments (mean ± SEM).
5.2.7 *Primary murine keratinocytes responded to stimulation with IL-1α and IL-1β*

In subsequent experiments, the ability of primary keratinocytes to respond to IL-1α and IL-1β was assessed. Dose dependent changes in gene expression were examined following 2h treatment with IL-1α or IL-1β (Figure 5.9). Stimulation with IL-1α resulted in maximal responses at doses between 1 and 10ng/ml. All genes measured (*Tnf*-α, *Il-1α*, *Il-1β*, *Icam-1* and *Cxcl1*) were affected in a similar manner by IL-1α treatment resulting in a maximal fold change of between 6 and 8 compared with the control (medium treated) sample (Figure 5.9A). In contrast, treatment with 10ng/ml of IL-1β differentially affected the expression of the investigated genes. *Cxcl1* expression was increased 150 fold, *Tnf*-α and *Il-1α* levels increased by 30 fold, whereas *Il-1β* and *Icam-1* were least affected with changes of 5 fold (Figure 5.9B).

![Graph A: IL-1α dose response](image1)

![Graph B: IL-1β dose response](image2)

**Figure 5.9 IL-1α and IL-1β-induced cytokine and Icam-1 expression by murine primary keratinocytes.**

*Primary keratinocytes were isolated as described in the methods and cultured to confluency in 12 well plates. Subsequently, cells were washed and incubated with a range (0-100ng/ml) of homologous IL-1α (A) or IL-1β (B) for 2h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of *Tnf*-α, *Il-1α*, *Cxcl1*, *Il-1β* and *Icam-1* was measured relative to the *Hprt1* gene and compared with the medium treated sample. Graphs present data from a single independent experiment.*
5.2.8 *Lactoferrin did not modulate cytokine stimulated gene expression by primary keratinocytes*

Experiments depicted in the Figure 5.7 demonstrated that there were differences in the vigour of the changes caused by stimulation with IL-1α and IL-1β. The concentration of 2ng/ml was chosen for both IL-1α and IL-1β to provide, respectively, vigorous and sub-optimal levels of stimulation of primary keratinocytes. In that setting potential immunomodulatory effects of LF were examined. Lactoferrin alone was without any inhibitory effects on gene expression at any time point. There was considerable inter-experimental variation in the cytokine-induced changes in gene expression, however, some patterns emerged. Treatment with IL-1β (Figure 5.10E-5.10F) caused similar effects on *Il-1β* and *Icam-1* gene expression to those observed for IL-1α treatment (Figure 5.10B-5.10C), both with respect to the kinetics and the extent of up-regulation. The effects on *Tnf-α* expression were more variable, with most marked increases recorded at 24h following treatment with IL-1β (Figure 5.10D).
Figure 5.10 Lactoferrin did not affect Tnf-α, Il-1β or Icam-1 expression following 2, 8 or 24h cytokine stimulation in primary keratinocytes.

Primary keratinocytes were isolated as described in the methods and cultured to confluency in 12 well plates. Subsequently, cells were washed and pre-treated with 100µg/ml LF for 1h. Afterwards, cells were incubated with 2ng/ml of homologous, recombinant IL-1α (A, B, C) or IL-1β (D, E, F) for 2, 8 or 24h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of Tnf-α, Il-1β and Icam-1 was measured relative to the Hprt1 gene and compared with the medium treated sample. The statistical significance of changes between cytokine-induced gene expression in the absence versus the presence of LF for each time point was analyzed by a Wilcoxon test; p>0.05. Graphs present data (mean ± range/SEM) from 2-3 independent experiments.
5.2.9 *HaCaT cells produced TNF-α, IL-8 and IL-1β following stimulation with PGN*

Lastly, in order to facilitate comparisons between the different types of keratinocyte used in these studies, the ability of HaCaT cells (the human immortalised keratinocyte cell line) to respond to various stimuli was investigated. Initially, the response to the TLR2 agonist (PGN) was assessed at the level of both protein (Figure 5.11) and message (Figure 5.12). Modest dose-dependent production of TNF-α and IL-1β protein was observed, reaching maximal levels of 120pg/ml following treatment with 25µg/ml of PGN for 24h for both cytokines (Figure 5.11A and 5.11C). HaCaT cells produced relatively high levels of IL-8 constitutively (~2000pg/ml) and markedly up-regulated this cytokine after incubation with PGN; IL-8 levels reached 10,000pg/ml following stimulation with 10 and 25µg/ml of PGN (Figure 5.11B).

![Graph A](image1.png)  ![Graph B](image2.png)  ![Graph C](image3.png)

*Figure 5.11 Stimulation of HaCaT cells with a TLR2 ligand resulted in a production of inflammatory cytokines.*

HaCaT cells were seeded at 10^5 cells per well in 12 well plates and cultured for 48h. Subsequently, cells were incubated with a range (0-25µg/ml) of PGN for 24h. Following incubation supernatants were collected and cells were lysed and secreted (TNF-α, IL-8) and intracellular (IL-1β) levels of cytokine were determined by ELISA (the lower limit of detection was 15pg/ml, 31pg/ml and 4pg/ml for TNF-α, IL-8 and IL-1β, respectively). Graphs present data from a single experiment.
In addition to considering protein levels, gene expression was also determined 24h after PGN treatment. Interestingly, the peak fold change (at 10µg/ml of PGN) of TNF-α and IL-8 was similar (4 fold change) despite large differences recorded at the protein level. Expression of IL-1β and ICAM-1 increased 2-fold in comparison with the baseline (Figure 5.12).

![Graph of cytokine expression](image)

**Figure 5.12 Peptidoglycan-induced cytokine expression by HaCaT cells.**

HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h to become confluent. Subsequently, cells were incubated with a range (0-25µg/ml) of PGN for 24h. After incubation cells were lysed, mRNA was extracted; cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α, IL-8, IL-1β and ICAM-1 was measured relative to the HPRT1 gene and compared with the medium treated sample. Graphs present data from a single experiment.

### 5.2.10 HaCaT cells responded to stimulation with IL-1β

In initial experiments cells were treated with a range of doses of homologous IL-1β (0-10ng/ml) for 2h and activation markers (TNF-α, IL-1β, IL-8 and ICAM-1) were measured by qPCR. All four markers showed a similar dose response relationship, reaching maximal levels between 1 and 10ng/ml, with the rank order IL-1β<ICAM-1<IL-8<TNF-α with maximal responses of ~30 fold achieved for TNF-α (Figure 5.13A). Subsequently, time dependent changes triggered by IL-1β in HaCaT cells were
investigated. It was found that HaCaT cells rapidly and transiently up regulated all of the genes tested. Thus, 2h after addition of IL-1β, expression of IL-8 and TNF-α was increased by 20 and 17 fold, respectively, whereas levels of ICAM-1 and IL-1β were 7 fold higher than baseline. After 2h expression of the tested genes steadily decreased and at 48h expression was only 2 fold higher than baseline (Figure 5.13B).

![Graph A](image1)

![Graph B](image2)

**Figure 5.13 Homologous IL-1β-induced time and dose dependent changes in TNF-α, IL-1β, IL-8 and ICAM-1 expression by HaCaT cells.**

HaCaT cells were seeded at 10⁵ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were incubated for 2h with a range (0-10ng/ml) of homologous IL-1β concentrations (A) or for 2, 6, 24 or 48h with 2ng/ml of IL-1β (B). After incubation cells were lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α, IL-1β, IL-8 and ICAM-1 was measured relative to the HPRT1 gene and compared with the medium treated sample. Graphs present data (mean ± SEM) from 1-3 independent experiments.
HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were incubated with medium or pre-treated with either 10μg/ml or 100μg/ml LF for 1h. Afterwards, 2ng/ml of homologous IL-1β or medium was added for another 2h, after which cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. The expression of TNF-α (A), IL-1β (B), ICAM-1 (C) and IL-8 (D) relative to the HPRT1 gene and compared with the medium treated sample was assessed. The statistical significance of gene expression in IL-1β treated cells compared with LF treated cells was analyzed by a Kruskal-Wallis with Dunn’s post test $p > 0.05$. Graphs present data (mean ± range/SEM) from 2-3 independent experiments.
5.2.11 Lactoferrin did not impact on IL-1β-stimulated gene expression by HaCaT cells

Subsequently, the ability of LF to modulate IL-1β triggered gene expression was examined following 2h stimulation with 2ng/ml of IL-1β, a concentration and time point shown to stimulate maximal responses. Lactoferrin was applied at 10 or 100μg/ml for 1h prior to the addition of IL-1β. Consistent with the previous dose response and kinetics experiments, IL-8 and TNF-α were induced to higher levels (~15-20 fold) compared with ICAM-1 and IL-1β (~8 fold); induced cytokine expression showed relatively little inter-experimental variation whereas ICAM-1 expression was more variable. However, analysis of gene expression demonstrated that the presence of LF did not inhibit the level of gene expression at either concentration tested (Figure 5.14). In keeping with the previous work conducted with murine cells, it was investigated also if LF was able to impact on gene expression following sub-maximal levels of induction. HaCaT cells were incubated for 2h with the range of doses of LF, or with BSA, as an irrelevant control protein, prior to addition of 0.1ng/ml of IL-1β. Suboptimal levels of induction of cytokine message were recorded (~10 fold for TNF-α and IL-8 and ~2fold for IL-1β), despite the absence of FCS in the medium. Under these conditions, LF and BSA were also without effect on IL-1β induced changes in gene expression (Figure 5.15).
Figure 5.15 Lactoferrin did not inhibit TNF-α, IL-1β and IL-8 expression induced by the lower concentration of IL-1β.

HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media. Cells were pre-treated with 0, 1, 10 or 100μg/ml LF or BSA for 2h. Afterwards 0.1 ng/ml of homologous IL-1β was added to all wells for another 2h, after which cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α (A), IL-1β (B) or IL-8 (C) relative to the HPRT1 gene was assessed and compared with the medium treated sample. Graphs present data from a single independent experiment.

5.2.12 HaCaT cells responded to stimulation with TNF-α

TNF-α treatment was also utilized as a means of inducing cytokine expression in HaCaT cells. The kinetics of changes in gene expression was measured following TNF-α treatment (Figure 5.16). Expression of IL-1β, ICAM-1 and IL-8 followed a similar kinetic profile, peaking at between 1 and 4 h, although levels of maximal expression varied from approximately 140 fold (IL-8) and 7 fold (IL-1β), returning to baseline levels within 24h. As such, these profiles were similar to those observed for cytokine-induced changes in keratinocyte gene expression, in that all markers displayed transient up-regulated expression. Interestingly, expression of TNF-α mRNA showed a different profile, with sustained up-regulation in transcripts over the 24h time period, increasing
steadily from 23 fold above baseline after 1h to a 114 fold increase 24h following TNF-α stimulation.

**Figure 5.16 Effects of homologous TNF-α on time dependent changes in TNF-α, IL-1β, IL-8 and ICAM-1 mRNA expression by HaCaT cells.**

HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media with 10ng TNF-α for 0, 1, 2, 4, 6, 10 or 24h. Following incubation cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α, IL-1β, IL-8 and ICAM-1 was measured relative to the HPRT1 gene and compared with the medium treated sample. Graphs present data from a single independent experiment.

### 5.2.13 Cytokine triggered gene expression by HaCaT cells was modulated by dexamethasone and thioredoxin peptide

Given the relative insensitivity of the different keratinocyte cell types to the immunomodulatory effects of LF, additional control inhibitory molecules were employed as positive controls for the experimental system. These controls were the potent anti-inflammatory steroid drug, dexamethasone (Remick et al., 1989), and peptides: thioredoxin (Tian et al., 2013) and Pep-1 (Mummert et al., 2000). All of these controls (with the exception of Pep-1) were shown previously to exert anti-
inflammatory actions in the skin in vivo as well as in vitro in keratinocyte cell culture (Cumberbatch et al., 1999b; Tian et al., 2013). Given that gene expression following treatment with TNF-α resulted in a more delayed induction of cytokine changes than those observed for IL-1β, the 24h time point was chosen for both cytokines for further investigations of these immunomodulators. In the presence of the control irrelevant protein (BSA), there was a marked and relatively consistent TNF-α-induced up-regulation in transcripts for TNF-α (~300 fold), IL-8 (~50 fold) and IL-1β (5 fold). Similar effects were recorded for IL-1β-induced changes, although such were of a lower order of magnitude (15 fold, 10 fold and 2 fold for TNF-α, IL-8 and IL-1β respectively) (Figure 5.17), as the 24h time point was selected on the basis of optimal responses for TNF-α. Culture with Pep-1 was without significant effect on any of the parameters measured. In contrast, treatment with dexamethasone resulted in a marked down-regulation of all cytokines induced by IL-1β (at least 50% inhibition) and of TNF-α-induced TNF-α and IL-1β, although IL-8 was unaffected. It was not possible to test for the statistical significance of these findings as only 2 independent experiments were conducted with dexamethasone. Another peptide tested for its immunomodulatory potential was one derived from thioredoxin (TRX). Cells were pre-treated with the TRX peptide, or a scrambled peptide with the same amino acid content, but in a random sequence, and then stimulated with either IL-1β (Figure 5.18) or TNF-α (Figure 5.19) for a further 24h. There was some inter-experimental variation, but following pre-treatment with scrambled peptide a broadly similar profile of IL-1β-induced gene expression changes were observed to those recorded previously (Figure 5.17). In three independent experiments, TRX was without marked effect on IL-1β-induced gene expression (Figure 5.18). As reported previously, stimulation of HaCaT cells with TNF-α resulted in more vigorous gene expression, particularly for TNF-α transcripts, although there was some inter-experimental variation. TRX peptide was without effect on IL-8 levels, but the other genes (TNF-α, IL-1β and ICAM-1) all showed a trend for inhibition which reached statistical significance for TNF-α and IL-1β (Figure 5.19A and 5.19B).
Figure 5.17 Pep-1 peptide did not significantly affect TNF-α, IL-β and IL-8 expression following stimulation with homologous TNF-α and IL-1β.

HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media with 200μg of BSA, 200μg Pep-1 or 1μM of dexamethasone (Dex). After 2h 10ng/ml TNF-α (A, B, C) or 10ng/ml IL-1β (D, E, F) was added for a further 24h. Following incubation cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α (A, D), IL-8 (B, E) and IL-1β (C, F) was measured relative to the HPRT1 gene and compared with the medium treated sample. The statistical significance of changes in cytokine-induced gene expression in the presence of BSA compared with Pep-1 treated cells was assessed by a Wilcoxon test; none of the differences were significant p>0.05. Graphs present data (mean ± range/SEM) from 2-3 independent experiments.
HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media with 50μg/ml of thioredoxin peptide (TRX) or scrambled control (SCR). After 2h 10ng/ml IL-1β was added for a further 24h. Following incubation cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α (A), IL-1β (B), IL-8 (C) and ICAM-1 (D) was measured relative to the HPRT1 gene and compared with the medium treated sample. The statistical significance of differences in IL-1β-induced gene expression in the presence of scrambled peptide compared with those treated with TRX peptide was analyzed by a Wilcoxon test. No significant differences were found, p>0.05. Graphs present data from 3 independent experiments.
Figure 5.19 Thioredoxin peptide decreased significantly TNF-α-induced IL-1β expression.

HaCaT cells were seeded at $10^5$ cells per well in 12 well plates and cultured for 48h. Subsequently, cells were washed and cultured in RPMI media with 50μg/ml of TRX or SCR. After 2h 10ng/ml of TNF-α was added for further 24h. Following incubation cells were collected, lysed, mRNA was extracted, and cDNA was prepared and utilized in a qPCR reaction using TaqMan primers. Expression of TNF-α (A), IL-1β (B), IL-8 (C) and ICAM-1 (D) was measured relative to the HPRT1 gene and compared with the medium treated sample. The statistical significance of differences in TNF-α-induced gene expression in the presence of SCR compared with TRX treated cells was analyzed by a Wilcoxon test *$=p<0.05$. Graphs present data from 4-7 independent experiments.
5.3 Discussion

Keratinocytes are the main cellular component of the epidermis. They are immunologically active cells capable of producing and responding to a plethora of cytokines (Gutowska-Owsiak and Ogg, 2012). As such, they are important in the events of LC migration and maturation and have been suggested to be a potential target for the inhibitory action of LF on oxazolone and IL-1β-induced LC migration (Almond et al., 2013; Cumberbatch et al., 2000b). Therefore, in the experiments described herein keratinocytes from 3 different origins were utilized as a platform to investigate the mechanism of LF immunomodulation. In the first instance all 3 cell types were characterized in order to confirm their status as keratinocytes. The HaCaT cell line are spontaneously transformed epithelial cells from human adult skin and they do not cause malignant growths following their injection into experimental animals (Boukamp et al., 1988). The PAM (212) cell line are also spontaneously transformed cells derived from the BALB/c strain mouse (Yuspa et al., 1980). Lastly, primary murine keratinocytes from new born BALB/c strain mice were utilized and they were obtained as described in the Chapter 2. It was necessary to confirm that the process of culturing primary keratinocytes yielded pure populations of cells of interest without any contaminating cells. Similarly, in the case of cell lines, even though they previously have been confirmed to express keratinocyte markers by other authors, it is not unheard of for cell lines to become contaminated with a different cell line (e.g. HeLa) or to lose their characteristics during long-term culture (ICLAC, 2013). Therefore, cells were stained for K14. Keratin 14 is a member of the keratin protein family, filament-forming proteins found most abundantly in the skin as well as in other epithelial tissues. Keratin 14 forms a dimer with K5 and is considered to be a marker for both non- and keratinised stratified epithelia (Haines and Lane, 2012; Shetty and Gokul, 2012). The expression of both K5 and K14 is characteristic of the basal layer of the epidermis. During differentiation cells begin to express K1 and K10 which are considered to be markers of keratinised epidermis (Shetty and Gokul, 2012). It was found that all three types of cells expressed K14, thereby confirming their status as keratinocytes. It was not surprising that these cells were identified as those from basal layer, as these are the only proliferating cells within the epidermis. In addition to confirming their status a keratinocyte cells lines, PAM and HaCaT cells were routinely tested for the presence of mycoplasma infection.
All of the experiments presented in this chapter were performed with mycoplasma-free cells.

In the first instance experiments were performed using all 3 types of keratinocytes to identify a stimulus that would trigger TNF-α release. It was reported previously that keratinocytes expressed TLR (Lebre et al., 2007; Mempel et al., 2003). Furthermore, it was established that TNF-α was expressed by LC-depleted epidermal cells and that it was induced following in vivo exposure to IL-1β and allergen (TNCB) (Enk et al., 1993). Initially PAM cells were stimulated with ligands for TLR2, 3, 4, 5 and 9 as well as PMA, and the production of cytokines and chemokines was measured at the level of protein by ELISA. No detectable cytokine or chemokine production was found in response to TLR ligands. Those results were somewhat surprising as it was reported that primary human and mouse keratinocytes were the source of a plethora of cytokines (Gröne, 2002; Matsue et al., 1992a). Conversely, a study by Segawa et al. (2014) reported that PAM cells constitutively produced neither TNF-α nor IL-6 (Marthinuss et al., 1995; Segawa et al., 2014), but abundant expression of IL-1α was detected by PCR (Marthinuss et al., 1995). Of the three cell types employed in this chapter PAM cells were the least commonly utilized and thus there were few published reports whereby PAM cells were treated with TLR ligands.

Cell lines do not always fully represent characteristics of primary cells; therefore, the next step was to investigate the effects of TLR agonists on primary murine keratinocytes. Cells were obtained from new born BALB/c strain mice. Keratinocytes were used during their first passage, in order to maintain their physiological phenotype, consequently, they could not be expanded and their number depended on the litter size. As a result, some of the experiments were performed only once and not always with all the necessary controls. It was demonstrated that, unlike PAM cells, primary keratinocytes secreted TNF-α, which was further induced following treatment with PGN. Unstimulated murine epidermal cells (Matsue et al., 1992a), as well as primary cultured keratinocytes (Yarosh et al., 2000), were demonstrated previously to express TNF-α mRNA. Moreover, secretion of IL-1α and IL-1β was assessed and it was found that while primary keratinocytes produced detectable levels of both cytokines further induction was not observed following exposure to PGN. Expression of Tnf-α and Il-1β mRNA was also up-regulated after PGN treatment. Incubation with LPS did not modify Tnf-α expression and dramatically decreased mRNA levels of Il-1β. There was no gene
induction observed following CpG treatment. Lack of response to LPS and CpG was unexpected as human primary keratinocytes were demonstrated to express functional TLR4 and TLR9 receptors. Furthermore, exposure to 1μg/ml of LPS and CpG was shown previously to cause chemokine secretion by primary human keratinocytes (Lebre et al., 2007). Moreover, TLR4 was established as playing a key role in wound healing in murine models, thereby suggesting the presence of TLR4 receptor in the skin. Experiments with normal human epidermal keratinocytes confirmed that the presence of TLR4 was responsible for cell migration and IL-1β production after scratch wounding (Chen et al., 2013). In the current investigation, exposure to LPS did not result in cytokine secretion (data not shown).

Finally, HaCaT cells (a human spontaneously transformed keratinocyte cell line) were examined (Boukamp et al., 1988). HaCaT cells were found to express TLR2 at a lower level than other keratinocyte cell lines: KERTr, HEK001, but at a level comparable with that reported for primary keratinocytes (Olaru and Jensen, 2010). HaCaT cells responded to treatment with PGN in a dose dependent manner with production of TNF-α, IL-8 and IL-1β. This observation was in agreement with previously published data, whereby HaCaT cells were shown to secrete TNF-α and IL-8 following incubation with 10μg/ml of another TLR2 ligand, zymosan (Lee et al., 2009). However, there was a difference in the levels reported. In the study reported herein maximal release of ~100pg/ml of TNF-α and IL-1β and 10000pg/ml of IL-8 was observed 24h after addition of 25μg/ml of PGN to the culture. In contrast, Lee et al. (2009) demonstrated release of approximately 1800pg/ml of both TNF-α and IL-8. This big discrepancy in secreted cytokine levels is somewhat surprising, especially as Lee et al. (2009) reported similar levels of TNF-α and IL-8 while in the current investigations IL-8 has been produced at much higher levels than TNF-α. Because of the relatively low levels of TNF-α protein production it was decided that, as for PAM cells and keratinocytes, gene expression by HaCaT cells would be investigated. Exposure of HaCaT cells to PGN for 24h even at the highest concentration (25μg/ml), resulted in a modest increase of cytokines and adhesion molecule (<5 fold increase which is in agreement with study by Olaru et al. (2010) that reported 4 fold increase in IL-8 expression) (Olaru and Jensen, 2010).

Overall, results from current investigations suggest that the PAM cells are not representative of murine keratinocytes, in that they do not seem to express any TLR
receptors, as judged by the lack of their response to TLR agonists. It is possible that these cells could respond to TLR ligands with induction of gene expression but such was not measured here. On the other hand, primary murine keratinocytes and HaCaT cells appear to be a more robust tool for studying response of keratinocytes to TLR agonists, which is reflected with their widespread use in dermatology research, particularly in the case of HaCaT cells.

Despite their inability to produce cytokines in response to TLR ligands there were studies demonstrating that PAM cells were capable of cytokine expression and secretion following incubation with other stimuli. Yonei et al. (2007) demonstrated dose dependent induction of IL-10 and IL-1 following incubation with trichloroacetic acid, a widely used peeling agent, as well as constitutive production of IFN-γ by PAM cells. Additionally, PAM cells were shown to secrete IL-3 constitutively (Peterseim et al., 1993), as well as in response to UV radiation (Gallo et al., 1991). Furthermore, PAM cells were shown to express mRNA for IL-7, which was identified as a growth factor for DETC. Moreover, incubation of 7-17 cells, a DETC cell line, with PAM cell supernatants was shown to promote 7-17 DETC cell line proliferation, whereas addition of anti-IL-7 antibody partially inhibited proliferation (Matsue et al., 1993a). Furthermore, secretion of GM-CSF and M-CSF by PAM cells was reported (Chodakewitz et al., 1990; Gallo et al., 1991). Although no detectable cytokine or chemokine secretion was reported in the experiments conducted herein following stimulation with TLR ligands, IL-1 release was observed following exposure to PMA (Table 5.1). Indeed, it was established previously by McGuire et al. (1988), that the treatment with PMA increased IL-1α mRNA levels, as assessed by Northern blot. Another phorbol, 12-o-tetradecanoylphorbol-13-acetate (TPA), was used to induce cytokine production by PAM cells and there was a small but significant up-regulation of thymic stromal lymphopoietin (TSLP) production following 24h incubation with 30nM TPA (Segawa et al., 2014). Furthermore, it was reported that PMA treatment (10ng/ml) of PAM cells resulted in up-regulation of IL-10 (Enk and Katz, 1992b). Alas, secretion of neither TSLP nor IL-10 was measured in the current study, although a wide range of other cytokines and chemokines was investigated. Ansel et al. (1988) examined the ability of PAM cells to produce IL-1. It was found that IL-1α was constitutively expressed and further induced following LPS treatment and exposure to UV light. Interleukin-1β was not detected in PAM cells or in primary keratinocytes derived from
new born mice (Ansel et al., 1988). The results reported by Ansel el al. (1988) were in accordance with the results presented in the current chapter, whereby expression of IL-1β by PAM cells was least induced after stimulation with either IL-1α or IL-1β, however, IL-1β was detected in the culture supernatants albeit at a low level.

With the knowledge from the literature that PAM cells were capable of induction of cytokines it was thought that they still could prove to be a useful tool for investigating the immunomodulatory effects of LF. Therefore, it was decided to utilize cytokines IL-1α and IL-1β as stimulants. It was demonstrated previously that in vivo injection of IL-1β and, to lesser extent, IL-1α, resulted in TNF-α mRNA expression by murine epidermal cells (Enk et al., 1993). However, when PAM cells were incubated with a range of doses of IL-1α or IL-1β, secretion of TNF-α was not detected (data not shown). Despite relatively high concentration of intracellular IL-1α much lower levels were secreted in to the culture media in the investigations described herein. The difference between the level of IL-1α in cell lysates and supernatants presumably stemmed from the mechanism of IL-1 release, whereby a second signal is required for the processing and release of IL-1α and IL-1β (Lie et al., 2012). Moreover, the ELISA DuoSet that was utilized detected pro-IL-1α with the same specificity as it did the mature form of cytokine (personal communication, Joseph Ainscough), therefore explaining the high concentration of intracellular IL-1α. Conversely, overall levels of IL-1β were low and were not modulated by the exposure to IL-1α. It was difficult to compare that result with the literature, as there are few reports regarding production of IL-1β by PAM cells. Ansel et al. (1988) reported that PAM cells did not express IL-1β mRNA in the steady state or following LPS activation (Ansel et al., 1988). Presence of IL-1β was reported in the epidermal cell suspension, however, only following allergen application and in the presence of LC, indicating that in mouse epidermis the primary source of IL-1β were LC (Enk et al., 1993; Enk and Katz, 1992a). The secretion of cytokines by PAM cells under these conditions was not robust enough, particularly with respect to TNF-α, therefore, it was decided that gene expression would be measured by utilising qPCR. In the first instance, the response of PAM cells was measured following exposure to a range of doses of either IL-1α or IL-1β for 2h. Gene expression was calculated using the ddCt method, whereby a change in the gene expression was normalised against expression of the housekeeping gene Hprt1 and further compared to an untreated sample (Pfaffl, 2001). Results from experiments utilising qPCR, presented in the current
studies, could have been further validated with the use of another housekeeping gene, e.g. beta actin or 18S ribosomal RNA. Indeed, it has been shown by Beer et al. (2000) that in the wounded skin, dermal fibroblasts release keratinocyte growth factor which in turn was found to modulate expression of genes involved in DNA metabolism, i.e. HPRT, which was employed in the studies described herein. However, another study by de Kok et al. (2005) identified HPRT as one of the most stable genes from 13 most commonly utilised housekeeping genes.

Interestingly, the highest fold increase following stimulation with both IL-1α and IL-1β was observed for the TNF-α gene. That was rather surprising considering that no TNF-α protein was detected in the culture supernatants. Tumour necrosis factor α is produced as a transmembrane homotrimer protein that upon cleavage by TNF-α converting enzyme (TACE) becomes a soluble form of TNF-α (Wajant et al., 2003). Therefore, in addition to investigating TNF-α levels in culture supernatants following IL-1α or IL-1β treatment TNF-α levels were also assessed in lysed cells, to allow for the detection of TNF-α that might not have been processed, and was attached to cell membranes. Nevertheless, no TNF-α was detected in lysed PAM cells following exposure to IL-1α or IL-1β (data not shown). Quantitative PCR measures mRNA levels of genes of interest relatively to their expression in the untreated sample. It is possible, therefore, that the level of TNF-α mRNA in the control samples was very low, resulting in a relatively high apparent fold induction, which failed to result in TNF-α protein production high enough to be detected by the ELISA kit that was utilized. Another gene that was highly induced by treatment with IL-1 was CXCL1. Chemokine CXCL1 is also known as Gro-α or keratinocyte-derived chemokine. CXCL1 is expressed by neutrophils, macrophages and epithelial cells and signals via CXCR2, present on neutrophils and keratinocytes. CXCL1 is responsible for neutrophil and keratinocyte chemotaxis; and recently was demonstrated to have a role in cutaneous wound healing (Kroeze et al., 2012). A higher level of CXCL1 induction was observed following stimulation with IL-1α (approximately 200 fold increase), than after treatment with IL-1β (~100 fold increase). Despite both cytokines signalling through the same IL-1R1 receptor, there are crucial differences between them, including the ability of IL-1R1 to recognize the pro-form of IL-1α but not IL-1β (Mosley et al., 1987). Production of both forms of IL-1 is differentially regulated in human keratinocytes, with notably different half lives of 1 and 4h for IL-1α and IL-1β, respectively (Kondo et al., 1994). Moreover,
it was discovered that IL-1α has the ability to influence gene expression via an intracrine (within the cell) manner, independently of IL-1R1 (Werman et al., 2004). Results reported herein are in agreement with previously reported data. Thus, murine oral keratinocytes were reported to up-regulate expression of CXCL1, CXCL10 and ICAM-1 following treatment with IL-1α, however Wu et al. (2013) demonstrated much lower levels of CXCL1 induction; CXCL1 expression peaked 12h following exposure to 10ng of IL-1α (Wu et al., 2013). In the current investigations, treatment with 10ng of IL-1α for the period of 2h resulted in 100 fold increase in CXCL1 mRNA. In contrast, the level of expression of ICAM-1 reported by Wu et al. (2013) was comparable (~20 fold increase) to the one presented in the current investigations (~50 fold increase) after incubation with 10ng of IL-1α for 3 and 2h, respectively (Wu et al., 2013). Next, in the order of magnitude of genes induced by IL-1 was ICAM-1 followed by IL-1α. Incubation with both cytokines (IL-1α and IL-1β) resulted in comparable levels of Icam-1 and Il-1α mRNA. ICAM-1 is an adhesion molecule involved in skin inflammation and ACD; its expression was demonstrated in the skin following exposure to UV radiation (Middleton and Norris, 1995), nickel (Garioch et al., 1991) and urushiol (Griffiths and Nickoloff, 1989). Moreover, ICAM-1 was established to have a role in the CHS reaction, whereby absence of ICAM-1 on lymphatic epithelium hindered the development of CHS (Xu et al., 2001). In addition, it was demonstrated that mature DC expressed ICAM-1 and it was induced in LC upon their maturation (Cumberbatch et al., 1992b). From the panel of investigated genes the lowest expression was exhibited by IL-1β. Indeed, regardless of the stimuli concentration or incubation time Il-1β expression did not exceed a 2 fold increase.

Similarly to what was observed in PAM cells, primary keratinocytes responded to exposure to IL-1. As previously mentioned, despite signalling via a common membrane receptor, the effects of IL-1α and IL-1β on one cell type can differ in the potency of their effect. Indeed, the impact of IL-1α on gene induction was much less pronounced than that of IL-1β and had a similar effect on all of the genes tested. Treatment with IL-1β resulted in a dramatic increase in Cxcl1 mRNA level, followed by Tnf-α and Il-1α. The effect of all doses of IL-1β on expression of Icam-1 and Il-1β was comparable to that of the highest dose of IL-1α. The main difference between PAM cells and keratinocytes was the inducible expression of Il-1β. Induction of IL-1β in PAM cells was not achieved with any of the stimuli used, while Il-1β was readily induced by
primary keratinocytes. As mentioned before, IL-1β mRNA was detected in murine epidermis but the signal was lost when LC were depleted, suggesting its absence from keratinocytes (Enk et al., 1993; Enk and Katz, 1992a). It is known that both human and murine keratinocytes are a source of IL-1α, but the presence of IL-1β is much debated (Ansel et al., 1988; Kupper et al., 1986; Matsushima et al., 2010). Interleukin-1β mRNA was detected in unstimulated human keratinocytes, but no protein was detected. Conversely, IL-1α was detected in both message and protein form (Kupper et al., 1986). Therefore, it was not surprising that relatively low levels of IL-1β protein were produced by primary keratinocytes, and that IL-1β mRNA was induced by both PGN and IL-1 stimulation. Interleukin-1β was recently detected in murine skin using confocal microscopy and flow cytometry, wherein DsRed fluorescent protein was expressed under an IL-1β promoter allowing visualisation of IL-1β in the steady state and after induction with oxazolone. It was found that following exposure to oxazolone epidermal cells that expressed IL-1β were mainly CD45+ (>85%) and the remaining proportion (<15%) were keratinocytes. Further analysis of CD45+ cells showed that the majority expressed CD11b and Gr-1, additionally there were populations present expressing F4/80 and MHC II. Similarly, in the dermal cells allergen (oxazolone) induced IL-1β expression was detected in granulocytes, monocytes/macrophages and DC (Matsushima et al., 2010).

In HaCaT cells the IL-1β-induced pattern of gene expression was similar to that triggered by PGN with TNF-α and IL-8 exhibiting the highest and similar levels of induction followed by IL-1β and ICAM-1. Gene expression peaked 2h after addition of IL-1β which was in accordance with the early maximal gene induction recorded for PAM cells. Dose dependent gene expression reached its peak at 1ng/ml of IL-1β. Exposure to another cytokine involved in LC migration, TNF-α, resulted in the rapid up-regulation of ICAM-1 and IL-8 which was in agreement with the literature (Lakshminarayanan et al., 1997). The induction of IL-1β was minimal, which was in accordance with reports demonstrating that in the epidermis LC, and not keratinocytes, were the main source of IL-1β (Enk et al., 1993). TNF-α was able to induce its own expression with an interesting kinetic profile. Whereas induction of other measured cytokines had returned to baseline levels within 24h following exposure to TNF-α, the up-regulation of TNF-α was maintained up to 24h. This result was somewhat surprising. Banno et al. (2004) demonstrated that TNF-α induced its own expression most
vigorously 1h after initial exposure and decreased following 24h, albeit not to the baseline level (Banno et al., 2004), which contradicts results obtained in the study reported herein.

Following the dose response and kinetic experiments, it was decided to utilize the concentration of 2ng/ml of IL-1α and IL-1β for the induction of gene expression in PAM cells and primary keratinocytes or 2ng/ml of IL-1β for HaCaT cells. Two h incubation time, in which the immunomodulatory effects of LF were assessed, was chosen for PAM cells and HaCaT cells, while 2, 8 and 24h time points were selected for primary keratinocytes. There are reports demonstrating LF as a stimulatory and inhibitory protein, therefore it was decided to utilize two concentrations of LF (10μg/ml and 100μg/ml) in an attempt to cover the active range for PAM and HaCaT cell lines; meanwhile just one concentration of LF (100μg/ml) was utilized with primary murine keratinocytes. Exposure of PAM cells to both concentrations of LF did not result in changes in either Tnf-α or Icam-1 expression. There was a considerable variability between independent experiments potentially contributing to lack of visible effects of LF. Attempts were made to optimise the potential activity of LF by utilising a suboptimal concentration of IL-1β (0.1ng/ml) to ensure that TNF-α expression was not maximal. Additionally, experiments were conducted in the absence of any foreign protein (medium 0% FCS) to obviate any blocking effects of nonspecific protein. However, only the highest concentration of LF (100μg/ml) significantly inhibited Tnf-α and Icam-1 gene expression and this effect was similar to that observed in the presence of the irrelevant control protein, BSA. Moreover, there was no dose dependent changes present, suggesting that the observed inhibitory effect was not specific. The same was true for primary keratinocytes. Inhibition of cytokine or adhesion molecule expression was not observed at any time point for any stimulant. As reported previously in the current investigations for PAM cells, LF on its own did not modulate the expression of the investigated genes. Similarly, in the case of HaCaT cells, treatment with LF on its own neither modulated baseline gene expression nor IL-1β-induced expression, a result that was in accordance with the data gathered from PAM cells and keratinocytes. Decreasing the concentration of IL-1β to suboptimal levels did not alter the inability of LF to modulate gene expression by HaCaT cells.

Lack of inhibition of PGN and IL-1 induced keratinocyte gene expression by LF may have many potential reasons. In the investigations described in the current chapter the
main focus has been on expression of cytokines involved in LC migration, that is TNF-α, IL-1β, IL-1α as well as the adhesion molecule ICAM-1. It has been established that keratinocytes were a source of chemokines following TLR stimulation and human primary cells and cell lines were demonstrated to be the source of IL-8, CCL20, CXCL9 and CXCL10 (Olaru and Jensen, 2010). Moreover, keratinocytes were established to up-regulate expression of CCL20, CCL5, CXCL9, CXCL10 and CXCL11 following exposure to IL-1β and TNF-α (Sanmiguel et al., 2009). In the current chapter, the only chemokine investigated was IL-8 in HaCaT cells and CXCL1 in murine keratinocytes and PAM cells, as murine cells do not produce IL-8. Chemokine CXCL1 is one of the IL-8 homologues and similarly it has chemotactic effects on neutrophils (Hol et al., 2010). It is possible that the effect of LF was missed as the expression of chemokines was not investigated in greater detail, that is, the particular important chemokine was not measured. Chemokines are known to be an important part of LC migration. It was established that chemokines are immobilized by their binding to proteoglycans and heparin. It also has been demonstrated that LF is able to bind to heparin and proteoglycans and displace IL-8, therefore diminishing its effects on endothelial cells (Ellass et al., 2002). Moreover, it would seem plausible that chemokines that attract neutrophils (LF being part of the secondary granules of neutrophils), would be regulated by LF after release from secondary neutrophil granules.

In the previous chapter nucleolin was identified as a LFR on THP-1 macrophages. Due to the lack of an observed effect of LF on keratinocytes, the presence of a potential LFR on these cells was not pursued. However, in retrospect, it could have been useful to perform immunofluorescence analysis of nucleolin expression on the surface of keratinocytes in order to confirm that the lack of response was not due to lack of appropriate receptor expression. However, there are reports that demonstrated the presence of nucleolin in the murine epidermis and primary keratinocytes, by fluorescence microscopy and WB, respectively (Hwang et al., 2007). Furthermore, nucleolin was detected in HaCaT cells bound to Bcl-XI mRNA following UV radiation. Bcl-XI is an anti-apoptotic molecule which is found to be up-regulated in colorectal and breast cancer (Zhang et al., 2008). However, in both of these reports the nucleolin detected was not on the surface of the cells, but was found intracellularly, therefore it was unlikely that it served as an LFR in the same manner as it did in THP-1 macrophages. Promising data regarding LFR in the skin came from Tang el al. (2010).
who demonstrated that LF-mediated keratinocyte migration and proliferation via LRP1 protein, suggesting that it was LFR present on keratinocytes (Tang et al., 2010). Indeed, it was described previously that there exists more than one LFR on different cell types and that LRP1 was one of the potential receptors. It is important to note that despite the lack of striking inhibitory effects of LF in vitro the same batch of protein was demonstrated to be biologically active in vivo.

Although it was not possible to demonstrate that cytokine induction by IL-1 was modulated by LF it was decided to investigate the immunomodulatory potential of two different peptides: TRX and Pep-1, which are involved in the protection against ROS and inhibiting hyaluronic acid, respectively. These peptides, similarly to LF, were found to inhibit LC migration following exposure to allergen (Dearman et al., 2010; Mummert et al., 2000). Pep-1 is a 12 amino acid peptide that was developed as an inhibitor of HA (Mummert et al., 2000), as well as a probe to detect HA in tissues (Zmolik and Mummert, 2005). It was demonstrated that HA played a role in the events of LC migration and maturation, whereby inhibition of HA with Pep-1 resulted in inhibition of DNFB-stimulated LC migration and decreased expression of CD86, a marker of LC maturation (Mummert et al., 2003). Moreover, it was found that HaCaT cells express HA receptors (CD44, RHAMM, LYVE1, TLR4) and enzymes involved in the HA synthesis and degradation. Furthermore, HA was found to facilitate the migration of HaCaT cells following scratch injury (Colella et al., 2012). Additionally, human epidermal keratinocytes were demonstrated to produce and secrete HA (Akiyama et al., 1994). Therefore, it was decided to investigate, whether a specific HA inhibitor would impact on cytokine (TNF-α and IL-1β) induced gene expression by HaCaT cells. Thioredoxin and thioredoxin derived peptide, TRX, similarly to LF, were found to inhibit oxazolone-induced LC migration, but thioredoxin was thought to influence IL-1β (Dearman et al., 2010) and not TNF-α signalling (Cumberbatch et al., 2000b). Consequently, it was of particular interest to test, whether Pep-1 and TRX peptides were able to have any impact on keratinocytes in vitro, as LF was unable to have significant impact on keratinocytes.

To test the immunomodulatory ability of TRX and Pep-1, gene expression by HaCaT cells was induced by exposure to TNF-α and IL-1β. As there were differences between TNF-α and IL-1β, with respect to peak response of time induced changes, the 24h time
point was chosen, to accommodate for that. Dexamethasone was used again as a positive control for inhibition of cytokine induced gene expression. Prior exposure to Pep-1 peptide did not result in a significant inhibition of gene expression regardless of the stimuli used. Pep-1 was demonstrated previously to decrease the expression of TNF-α and TLR4 mRNA in articular tissue derived from animals with collagen-induced arthritis (Campo et al., 2013), suggesting its ability to exert inhibitory effects in vivo. It is possible that the lack of effect of Pep-1 observed in the current study was due to the fact that HA did not participate in TNF-α and IL-1β induced gene expression. Hyaluronic acid is a large molecular weight polymer comprised of D-N acetylglucosamine and D-glucuronic acid. It is expressed by all of the tissues in the body and is a major component of ECM. Under physiological conditions HA has a molecular weight of approximately 10^7 Da, however, in the event of inflammation or injury, smaller fragments of HA are found. It was demonstrated that HA of different size ranges exhibited distinct functions. High molecular weight HA displayed an anti-inflammatory properties, whereas LMW HA had a pro-inflammatory potential (Petrey and de la Motte, 2014). Therefore, it would be of interest to investigate whether gene induction stimulated via oxidative stress, (which facilitates HA degradation to LMW fragments (Esser et al., 2012)) e.g. DNCB treatment, was modulated by Pep-1 peptide. Dexamethasone inhibited induction of TNF-α and IL-1β expression and had a considerably less pronounced effect on expression of IL-8. Dexamethasone was demonstrated previously to inhibit TNF-α and IL-1β induced IL-8 protein and mRNA production in airway epithelial cells (Kwon et al., 1994). However, dexamethasone was demonstrated to have a lesser effect on LPS-induced IL-8 expression by alveolar macrophages (Armstrong et al., 2009), therefore showing that susceptibility of IL-8 production to dexamethasone could be cell specific.

Subsequently TRX, another immunomodulatory peptide, was investigated. Thioredoxin is a small, 12kDa protein crucial in the protection against oxidative stress. It works by catalyzing the reduction of disulfide bonds (Yoshihara et al., 2014). The 9mer peptide derived from thioredoxin utilized in these experiments was demonstrated previously in our laboratory to inhibit LC migration induced by oxazolone and TNF-α but not IL-1β (Cumberbatch et al., 2009). That is, analogous with LF, thioredoxin was able to inhibit LC migration, but the mechanism appears to be somewhat different, as IL-1β dependent migration was blocked (TNF-α and oxazolone induced migration), whereas exogenous
IL-1β was able to overcome the inhibition. Therefore, the effect of TRX peptide on TNF-α and IL-1β induced cytokine expression by HaCaT cells was investigated. HaCaT cells were incubated with either TRX or a peptide containing the same amino acids in a random order (scrambled) prior to addition of cytokines. Pre-incubation with TRX peptide did not impact on IL-1β induced gene expression, which was in agreement with the in vivo LC migration data (Dearman et al., 2010). However, when cytokine gene expression was induced by exposure to TNF-α, it was found that prior incubation with TRX peptide resulted in the significant inhibition of both TNF-α and IL-1β expression. The results reported in this chapter were in agreement with Tian et al. (2013), who found that treatment with recombinant human thioredoxin inhibited both protein (in vivo) production and mRNA expression (in vivo and in vitro) of TNF-α and IL-1β in murine skin and by PAM cells following exposure to croton oil (Tian et al., 2013). In another study thioredoxin was found to inhibit LPS induced neutrophil accumulation via direct interaction with neutrophils. Moreover, thioredoxin was found to suppress leukocyte migration in response to CXCL1, MCP-1 and CCL5 (Nakamura et al., 2001). The effects of thioredoxin were thought to be mediated by its ability to reduce oxidative stress but also via interaction with NF-KB and inhibitory effects on inflammatory cytokine production, i.e. IL-1β via interference with their promoter (Billiet et al., 2005). It is possible that in the study reported herein the observed inhibitory effects of TRX peptide were due to its direct interruption of cytokine induced signalling. Thioredoxin is a small protein with a molecular mass of 12kDa (Yoshihara et al., 2014). It is possible that the inhibition of cytokine expression observed in this experiment was due to the TRX peptide direct interaction with the TNF-α signalling pathway. As inhibition of TNF-α and IL-1β expression was only demonstrated after treatment with exogenous TNF-α it could be speculated that TRX peptide affected TNF-α signalling upstream from MAP3K, where IL-1β and TNF-α pathways converge (Jarvis et al., 2006). Following TNF-α binding with TNFRI, silencer of death domain (SODD) is released from the TNFRI intracellular domain (IcD) and adaptor protein TNF receptor–associated death domain (TRADD) is recruited to IcD, alongside receptor-interacting protein (RIP), TRAF2, and Fas-associated death domain (FADD) (Chen and Goeddel, 2002). TRAF2 later recruits various enzymes that ultimately can activate c-Jun NH2-terminal kinase (JNK). One of these enzymes is apoptosis-stimulated kinase 1 (ASK1) that has been indentified to be negatively modulated by thioredoxin (Saitoh et al., 1998). Therefore, it is plausible that the observed inhibitory effects of TRX peptide on
TNF-α stimulated induction of TNF-α and IL-1β expression were due to its interaction with ASK1.

The impact of thioredoxin and LF on oxazolone-induced LC migration was found to be similar: both compounds were able to prevent the decrease in LC numbers. The difference between proteins was visible when cytokines were utilized: LF inhibited IL-1β induced migration suggesting that it impacted on de novo TNF-α production (Cumberbatch et al., 2000b), whereas the opposite was true for thioredoxin, it was thought to influence IL-1β signalling (Dearman et al., 2010). It was somewhat surprising that there was an observed effect of TRX, but not LF, in HaCaT cells. It has been hypothesised that in the sequence of events of allergen (oxazolone) induced LC migration, TNF-α was synthesized by keratinocytes, TNF-α in turn stimulated LC, via TNFRII, to release IL-1β that was synthesized and present in LC in its pro-form. Subsequently, secreted IL-1β acted via IL-1R1 on LC in an autocrine manner, supplying the second signal for migration of LC. Therefore, it was rather surprising that in the current investigation it was reported that TRX was able to inhibit TNF-α-induced IL-1β expression by HaCaT keratinocytes, whereas LF failed to inhibit IL-1β-triggered TNF-α expression.

The mechanism of action of LF (either stimulatory or inhibitory) is still largely unknown, therefore it is difficult to speculate where it may exert its effects. It is possible that the end points utilized in the experiments presented in this chapter were not ideal; although such were based on known actions of LF in vivo. For example, the production of chemokines may be the key target of LF, as it has been reported that keratinocytes produce many chemokines in response to TLR stimulation (Olaru and Jensen, 2010). Overall, keratinocytes did not prove a suitable model to investigate the immunomodulatory effects of LF. It could be speculated that one cell system cannot be representative of the complex connections found in the skin. Additionally, it indicates that despite being the most abundant cell type in the epidermis, the keratinocyte may not be the target cell for LF action.
6 Differential cytokine requirements for mobilization of LC induced by oxazolone and DNBC

6.1 Introduction

The human immune system has evolved to recognize and eradicate external and internal dangers. It is very complex machinery that requires meticulous control and tuning. However, there are instances where the immune system reacts against the organism itself (autoimmunity) or it mounts a disproportionate reaction against harmless compounds (allergic reactions). Allergic contact dermatitis is a form of type IV hypersensitivity (delayed) (DTH) reaction, where the subject has become sensitized to a compound upon primary exposure, and the clinical manifestations are evident in sensitised individuals 48h or more after secondary encounter (or challenge). Causative agents are LMW molecules (usually <500Da). They are almost always unable to trigger allergic reactions on their own, as it is only after covalently binding to proteins that they become immunogenic. Over 4000 different chemical allergens have been demonstrated to cause ACD in humans. Cross-sectional studies have shown that between 20 and 30% of the population could be diagnosed with contact allergy to at least one substance (Peiser et al., 2012; Uter et al., 2005). Common contact allergens include metals: nickel, gold; cosmetics, e.g. balsam of Peru, fragrances, hair dyes (PPD); medications: antibiotics and steroids (Fonacier et al., 2010).

Despite the prevalence of ACD, and the wide range of chemicals that cause this problem, the exact mechanisms of action are incompletely understood. Considerable efforts have been made to elucidate the sequence of events following initial skin exposure to haptens. They have been shown to interact with nucleophilic amino acids, including but not restricted to, cysteine and lysine. This property stems from their electrophilic nature and was used for the development of alternative ways of identifying contact allergens (Gerberick et al., 2004). It was demonstrated previously that application of allergen results in inflammatory cytokine production in the skin (Enk and Katz, 1992a), however, the exact proteins involved are still not characterized fully. One of the allergens that has been studied most closely is the metal allergen, nickel. It was found that in human skin nickel interacts with specific histidine residues within TLR4, thus providing a direct danger signal (Schmidt et al., 2010). Moreover, other allergens
were found to trigger the generation of ROS in the skin that in turn cause the creation of LMW HA fragments that serve as a secondary inflammation signal (Esser et al., 2012).

Application of the somewhat limited range of allergens that have been examined to human and mouse skin universally results in cytokine production and LC migration. Langerhans’ cells comprise 2-5% of all epidermal cells in adult mouse skin, and are the only myeloid cells in the epidermis (Romani et al., 2010). They are positioned between keratinocytes and serve as immune sentinels, detecting danger signals and subsequently presenting antigens in the context of MHC class II (and class I) to naive T cells in the draining LN (Toebak et al., 2009). Langerhans’ cells were discovered in 1868 by Paul Langerhans and were originally thought to be a part of the nervous system (Ginhoux and Merad, 2010). Further studies showed, however, that LC derive from the bone marrow (Katz et al., 1979). The process of LC migration was considered to depend universally on cytokine signals from TNF-α and IL-1. The data supporting this conclusion came from the investigations in mice, mainly utilising oxazolone as a model contact allergen (Cumberbatch et al., 2002a; Cumberbatch et al., 1997b). In addition, LC migration has been investigated in response to UV radiation (Cooper et al., 1992), yeast (Haley et al., 2012) and virus (Byrne et al., 2001) infection. Moreover, involvement of TNF-α and IL-1 cytokines has been established both in vivo (Cumberbatch et al., 1999d) and ex vivo (Stoitzner et al., 1999). However, there is a limited amount of information regarding influence of contact allergens on LC migration in humans. In our laboratory DPC has been used as a model allergen that triggered LC mobilisation (Griffiths et al., 2001). Additionally, there are reports of DNCB use in humans (Friedmann et al., 1983) and its impact on LC migration (Oakford et al., 2011).

In the investigations described here chemical allergens have been used as a tool with which to examine the characteristics of LC biology, in particular the role of cytokines in LC function and skin inflammation. In this chapter differential cytokine regulation of LC migration between allergens is considered, with particular focus on two model compounds: oxazolone and DNCB. It was established in a mouse model that LC migration in response to the contact allergen oxazolone, two cytokine signals from TNF-α and IL-1β were required for LC mobilisation (Cumberbatch et al., 1997b). In the experiments described here the mechanisms of oxazolone- and DNCB-mediated LC migration were investigated. The belief that TNF-α is an essential signal for LC migration is challenged initially by differential inhibition of oxazolone and DNCB-
induced LC mobilisation by LF. It was described previously that LF inhibited oxazolone triggered LC migration via inhibition of de novo TNF-α production in the skin. Therefore, divergent effects of LF on oxazolone and DNBC-induced LC mobilisation were investigated further using TNFRII KO mice that lack the sole TNF-α receptor that is expressed by LC (Wang et al., 1996), and the administration of neutralising anti-cytokine antibody. Lastly, an attempt was made to elucidate the mechanisms driving the differences between allergens using of cytokine profiling. The production of cytokines, chemokine (CXCL1) and adhesion molecule ICAM-1 by allergen-treated skin tissue was examined on the level of protein secretion and gene expression.

6.2 Results

6.2.1 Contact allergens (oxazolone and DNBC) induced LC migration

Topical application of contact allergens oxazolone and DNBC has been shown previously to induce immune responses and to result in LC migration (Cumberbatch et al., 1997b; Cumberbatch et al., 1992a). In preliminary experiments the ability of oxazolone and DNBC to induce LC migration was confirmed. In the steady state there were approximately 1000 LC per mm² in BALB/c strain mouse skin. The loss of between 20 and 40% of LC is the maximum amount of cells that can migrate rapidly from the epidermis regardless of the stimulus applied to the skin (Cumberbatch and Kimber, 1990). Mice were topically exposed to a single dose of either allergen in AOO, a vehicle that was shown previously to have good topical application characteristics for organic chemicals (Heylings et al., 1996; Warbrick et al., 1999). In the current investigations controls used in in vivo experiments were either AOO treated or untreated (naive). Application of AOO for either 4 or 17h was shown previously not to impact on LC numbers in comparison with untreated animals (Cumberbatch et al., 2005). Similarly, AOO was without effect on DC accumulation in the LN when compared with untreated mice (Cumberbatch et al., 2005).

Doses of allergens that were used had been demonstrated previously to result in vigorous LNC proliferation to similar extents, and were therefore deemed to have similar levels of allergenicity (Dearman and Kimber, 1991; Van Och et al., 2002). Application of both allergens resulted in a decrease in LC numbers to approximately 800 LC per mm², representing a 21% and a 24% decrease for DNBC and oxazolone,
respectively (Figure 6.1). In addition to causing LC migration, exposure to each of the allergens resulted in a change in the morphology of LC that remained in the epidermis. Figures 6.1C to 6.1F depict LC visualised by staining with anti-MHC II antibody conjugated with FITC. In the skin of naive animals LC were small and dendrites were not very pronounced (Figure 6.1C and 6.1D). However, following allergen application the cell bodies were enlarged and the dendrites more prominent (Figure 6.1E and 6.1F), especially in DNBC-treated animals (Figure 6.1E).
Figure 6.1 Treatment with DNCB and oxazolone resulted in LC migration.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 6 ears per group) were treated on the dorsum of both ears with 25μl of 1% DNCB or 0.5% oxazolone (Ox) in AOO for 4h. Treated animals, alongside untreated controls (naive) (n=3 per group; 6 ears per group), were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM (A, B). The statistical significance of differences between LC numbers in untreated (naive) and allergen treated animals was assessed by Student’s t test; **=p<0.01. Representative micrographs of epidermal sheets show LC morphology of representative samples from naive (C, D) and DNCB (E) or oxazolone (F) treated animals. x40 magnification.
6.2.2 *Lactoferrin inhibited oxazolone-induced LC migration*

It has been demonstrated previously that in mice LC migration from the epidermis and DC accumulation in the LN induced following oxazolone treatment was inhibited by intradermal injection of mouse LF (Cumberbatch et al., 2000b) and additionally by topical application of human LF (Almond et al., 2013). The ability of human LF to inhibit oxazolone-induced LC migration in BALB/c strain mice was therefore confirmed in subsequent experiments. Firstly, it was confirmed in four independent experiments that LF was able to inhibit oxazolone-mediated LC migration (Figure 6.2). Mice were treated with LF in aqueous cream or cream alone for 2h prior to exposure to oxazolone for 4h. In untreated animals numbers of between 800 (Figure 6.2D) and 1100 (Figure 6.2A) LC per mm$^2$ were recorded. Treatment with oxazolone resulted in a significant loss of LC in each experiment, equating to a reduction in LC frequency between 17% (Figure 6.2D) and approximately 25% (Figure 6.2A-C). Prior treatment with LF resulted in almost complete abrogation of LC migration (<5%) in each of the four independent experiments. To further investigate the effects of LF, a dose response analysis was performed to assess a range of concentrations over which LF was active. In naive animals the frequency of LC was 900 LC per mm$^2$. Exposure to allergen resulted in migration of 27% of LC. Prior application of LF was found to inhibit LC migration even at the very low concentration of 0.05μg/ml and up to 50μg/ml (Figure 6.3).
Figure 6.2 Lactoferrin inhibited oxazolone-induced migration of LC.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 4-6 ears per group) were treated with 0.5μg LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO for 4h. Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated or LF treated animals was analyzed by one-way ANOVA; **=p<0.01, ***=p<0.001. Figures A-D present data from 4 independent experiments.
Figure 6.3 Lactoferrin was active over a range of concentrations.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 4-6 ears per group) were treated with a range of doses (0.05-50μg) of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% oxazolone (Ox) in AOO for 4h. Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm$^2$ per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated or LF treated animals was analyzed by one-way ANOVA; **=p<0.01, ***=p<0.001.

In these initial experiments, the 4h time point was utilized for examination of allergen-induced LC mobilisation. It was established previously (Cumberbatch et al., 2000a) and in current investigations that 4h exposure to allergen resulted in LC migration between 20 and 30% which is the maximum number of LC that are apparently capable of leaving the skin within this time frame. Indeed, it has been demonstrated before that more prolonged (96h) exposure to allergen did not dramatically change the extent of LC migration (data not shown). Therefore, the next step in these investigations of the influence of LF on LC function was to determine for how long the inhibitory effect of LF persisted. For that purpose the same protocol was performed, wherein mice were exposed to either LF in cream or cream alone for 2h and afterwards oxazolone was applied for 4h, 6h or 24h. Six and 24h time points were chosen to provide opportunity for IL-17 production by allergen-activated LNC that is described in the Chapter 3.
accordance with previously reported data it was observed that in untreated (naive) animals there were ~1100 LC per mm². Exposure to oxazolone for 4h, 6h and 24h resulted in a loss of LC of 24%, 26% and 17%, respectively. That is, the impact of oxazolone on LC frequency persisted for some 24h. It was found that the effects of LF were relatively transient; whilst migration was significantly reduced (3%) in the presence of LF at 4h post-oxazolone treatment (Figure 6.4A), LC mobilisation increased to 12% after 6h (Figure 6.4B) following oxazolone application and the inhibitory effect of LF was no longer apparent after 24h (Figure 6.4C).

**Figure 6.4 The inhibitory effect of LF on oxazolone-induced LC migration was transient.**

Young (6-8 weeks) BALB/c mice (n=3 per group, 6 ears per group) were treated with 0.5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl 0.5% of oxazolone (Ox) in AOO for 4h (A), 6h (B) or 24h (C). Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated or LF treated animals was analyzed by one-way ANOVA; *=p<0.05, **=p<0.01, ***=p<0.001.
6.2.3 The N-lobe of lactoferrin was not responsible for inhibition of LC migration

In the current investigation human LF was used in the mouse model of allergen-induced LC migration. Previous reports from our laboratory examined primarily homologous LF with regards to its inhibitory effects on LC mobilisation (Cumberbatch et al., 2000b; Griffiths et al., 2001). However, it has been established that there is a high degree of homology between LF molecules from various species, with mouse and human LF sharing 70% amino acid sequence homology (Baker and Baker, 2009). Among other roles, LF is widely recognized as an anti-microbial protein (Dalmastri et al., 1988). It has been documented that many of the anti-bacterial properties of LF can be attributed to the N-lobe of the protein, which is known to bind LPS (Drago-Serrano et al., 2012).

It was of interest, therefore, to determine whether the observed inhibitory effect of the LF on LC migration resided also in this part of the molecule. The commercially available N-lobe that was used in the current investigations was a 14 amino acid peptide which contained residues 231-245 of human LF protein. There is 67% identity between murine and human N-lobe peptide (BLAST analysis of murine and human N-lobe).

Treatment with oxazolone resulted in LC migration, albeit to a lower extent than previously reported (12%). Pre-treatment with LF was able to inhibit oxazolone-induced LC mobilisation. However, even this relatively low level of LC migration was not inhibited by prior application of N-lobe peptide, either at an equivalent concentration (with respect to total protein, μg/ml) to that of the whole molecule or in marked excess of that concentration, suggesting that this part of the molecule did not participate in the modulation of LC mobilisation (Figure 6.5).
Figure 6.5 The N-lobe of LF was not responsible for inhibition of oxazolone-induced LC migration.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 6 ears per group) were treated with 0.5μg of LF or with 0.5μg or 24.38μg of N-lobe peptide in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl 0.5% of oxazolone (Ox) in AOO for 4h. Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated or LF or N-lobe peptide treated animals was analyzed by one-way ANOVA; *=p<0.05, ns=not significant.

6.2.4 Differential effect of lactoferrin on LC migration induced by oxazolone and DNCB

In the investigations reported to date, oxazolone was used as an allergen with which the role of LF in the early sensitization events was investigated in mice. Oxazolone is a reference contact allergen used widely to examine sensitization (Antonopoulos et al., 2008; Cumberbatch et al., 1997b) and challenge events (Nakae et al., 2003). Thus, this allergen was used previously to establish that LC require 2 independent signals from TNF-α and IL-1β to migrate away from the epidermis following allergen application.
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(Cumberbatch et al., 1997b). 2,4-dinitrochlorobenzene is another chemically unrelated potent contact allergen commonly used in laboratory investigations of CHS, but in addition to its use in animal models, there is a wealth of data regarding the impact of DNCB exposure in humans (Friedmann et al., 1983), as DNCB was used to treat alopecia areata (Singh and Lavanya, 2010), warts (Kwok et al., 2012) and has been used also by at least one research group as an experimental contact allergen in humans (Friedmann et al., 1983). In subsequent experiments, DNCB was used as an additional stimulant for LC migration and comparisons were drawn with oxazolone. The influence of LF on DNCB-induced LC mobilisation was examined 4h (2 experiments), 6h (2 experiments) and 24h (1 experiment) after initiation of exposure and was compared with concurrent treatment with oxazolone. In each experiment and at each time point exposure to DNCB resulted in a significant decrease in LC numbers ranging from 14% (Figure 6.6B) to 25% (Figure 6.6C-6.6E). The extent of LC loss was similar for DNCB and oxazolone, with the exception of the experiment presented in Figure 6.6D where DNCB triggered twice as many LC to migrate as did oxazolone (25% vs. 11%). As previously demonstrated in the Figure 6.4, the inhibitory effect of LF on oxazolone-induced LC migration was transient. Thus, in both experiments at the 4h time point, a significant inhibition of LC migration was recorded following pre-treatment with LF (4% and 1% migration). In contrast, it was found that DNCB-triggered LC migration was not affected by LF at this time point (Figure 6.6A and 6.6B). As reported previously (Figure 6.3), the effect of LF on oxazolone-induced mobilisation was transient, such that at 6h and 24h after initiation of treatment, LF was unable to prevent the migration of LC (14% and 21% migration after 6h and 17% migration following 24h). Interestingly, in both experiments at the 6h time point there was some evidence for a degree of inhibition of DNCB-induced LC migration by LF. Thus, values recorded were 14% and 17% mobilisation in the presence and 25% migration in the absence of LF. However, only the experiment in the Figure 6.6C reached statistical significance. There was no impact of LF on 24h DNCB-induced LC migration (Figure 6.6E; 24% of LC were mobilized in the presence and absence of LF).
Figure 6.6 Lactoferrin was without effect on DNCB-induced LC migration.

Young (6-8 weeks) BALB/c mice (n=3 per group, 6 ears per group) were treated with 0.5μg LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of either 0.5% oxazolone (Ox) or 1% DNCB in AOO for 4h (A, B), 6h (C, D) or 24h (E). Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated or LF treated animals was analyzed by one-way ANOVA; *=p<0.05, **=p<0.01, ***=p<0.001, ns=not significant.
Given the inability of LF to affect DNCB-triggered LC migration under conditions where oxazolone-induced migration was inhibited, it was necessary to consider in more detail the concentrations of each allergen used in these comparative experiments. The initial concentrations of the allergens used in the current investigations (oxazolone 0.5% and DNCB 1%) were chosen based on the previous experience of skin sensitization in mice, whereby 0.5% oxazolone and 1% DNCB were demonstrated to trigger mobilisation of the same proportion of LC from the skin and stimulate vigorous and broadly comparable levels of cell proliferation in the draining LN (Cumberbatch et al., 2005; Cumberbatch et al., 2002b; Hilton et al., 1995).

Moreover, oxazolone and DNCB at the concentrations utilized in the current investigations to date were able to cause a similar extent of LC mobilisation in control (cream-treated) animals. However, there was a differential effect of the allergens in the skin as evidenced by changes to LC morphology, with treatment with DNCB resulting in a more activated phenotype compared with oxazolone. Therefore, it could be hypothesised that at the dose used DNCB triggered more vigorous cytokine production in the skin that LF was unable to neutralize. Subsequently, the effects of LF on LC migration induced by a lower concentration (0.25%) of DNCB were examined. Both concentrations of DNCB provoked a similar decrease in LC frequency, 28% and 22% for 1% and 0.25% of DNCB, respectively. However, prior treatment with LF did not impact significantly on LC migration at either of the DNCB concentrations used (Figure 6.7A). Thus, both concentrations of DNCB resulted in significant LC migration regardless of the presence of LF. However, the morphology of cells that were retained in the epidermis differed between 1% and 0.25% DNCB-treated skin. As previously observed, application of 1% DNCB resulted in a marked increase in the size of LC cell bodies, as well as more evident dendrites; prior application of LF did not change the appearance of the DNCB-activated LC (Figure 6.7C and 6.7D). Interestingly treatment with 0.25% DNCB did not result in similar changes to LC morphology as those observed with high dose DNCB. On the contrary, cells resembled more those that were treated with oxazolone alone, and prior administration of LF did not affect the morphology of LC treated with the low dose of DNCB (Figure 6.7E and 6.7F).
Figure 6.7 Lactoferrin did not inhibit DNCB-induced migration of LC at low or high doses of allergen.

Young (6-8 weeks) BALB/c strain mice (n=3, 5-6 ears per group) were treated with 0.5μg LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of either 1% DNCB or 0.25% of DNCB in AOO for 4h. Negative controls were untreated (naive). Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM (A). The statistical significance of differences between LC numbers in untreated (naive) or allergen treated animals (with or without LF) was analyzed by one-way ANOVA; ***=p<0.001. Representative micrographs of epidermal sheets show LC morphology from respective treatments (B-F). x40 magnification.
6.2.5 *DNCB-mediated LC migration was IL-1β dependent and TNF-α independent*

It has been demonstrated before that prior exposure to LF inhibited oxazolone-induced LC migration, as well as that triggered by delivery of homologous IL-1β, but not TNF-α (Cumberbatch et al., 2000b). It was suggested that LF inhibition of LC migration in response to oxazolone treatment was dependent on reducing the availability of TNF-α. Additionally, LF was shown to decrease the level of TNF-α in the blister fluid in suction blisters induced in human volunteers following delivery of exogenous homologous IL-1β (Cumberbatch et al., 2003). Moreover, it was established previously that LC migration was dependent on two independent cytokine signals from TNF-α and IL-1β (Cumberbatch et al., 1997b). Taken together, these data suggested that LF was capable of inhibiting TNF-α dependent migration (oxazolone and IL-1β-induced migration), possibly through inhibition of TNF-α production. Lactoferrin is unlikely to be blocking TNF-α signalling (at the TNF-α receptor level), as LF is unable to inhibit migration when exogenous TNF-α was provided (Almond et al., 2013; Cumberbatch et al., 2000b). It was intriguing, therefore, that as demonstrated in the Figure 6.6 LF was unable to inhibit DNCB-mediated LC migration, suggesting that such migration might be TNF-α independent. This hypothesis was particularly interesting as LC migration provoked by a variety of other stimuli, including: UV radiation (Moodycliffe et al., 1994), physical trauma (Ghaznawie et al., 1999), irritants (Cumberbatch et al., 2002a) and certain pathogens (Shankar et al., 1996), have been reported to be dependent upon this cytokine. It was demonstrated previously that delivery of TNF-α resulted in LC migration in mice (Cumberbatch et al., 1999a) and in humans (Cumberbatch et al., 1999d). In addition, TNF-α was reported be necessary for DC accumulation in the LN following treatment with the fluorescent allergen, FITC (Cumberbatch and Kimber, 1995).

The role of TNF-α and IL-1β cytokines in DNCB-triggered LC mobilisation was investigated further. The initial approach chosen was to utilize neutralising antibodies directed against either TNF-α or IL-1β and to compare their impact on LC migration stimulated following application of oxazolone or DNCB. In the first instance a polyclonal anti-TNF-α antibody was employed. Intradermal delivery of normal rabbit serum did not impact on LC migration following allergen application, thus, levels of migration of 18% and 22% were induced by oxazolone and DNCB, respectively, similar
to the levels seen in the absence of control intradermal injection. Consistent with previous publications (Cumberbatch et al., 1997b; Cumberbatch and Kimber, 1995), it was found that anti-TNF-α antibody pre-treatment completely inhibited oxazolone-mediated migration. However, it was without effect on DNCB-triggered migration (Figure 6.8A). Secondly, the role of IL-1β signalling in LC migration provoked by DNCB application was established with the use of anti-IL-1β antibody. Interleukin 1β signalling was reported previously to be necessary for oxazolone and TNF-α induced DC accumulation in the LN, as well as TNF-α triggered LC migration (Cumberbatch et al., 1999c). In the current investigations it was discovered that neutralisation of IL-1β inhibited effectively oxazolone-induced migration; 24% and 2% decreases in LC numbers was observed in the absence and in the presence of anti-IL-1β antibody, respectively. The influence of anti-IL-1β antibody on DNCB-mediated LC migration was somewhat less pronounced and resulted in 12% decline in LC numbers compared with 23% migration observed in the absence of the antibody (Figure 6.8B), however the degree of inhibition was still statistically significant (p<0.05).
Figure 6.8 DNBC-induced LC migration was TNF-α independent but IL-1β dependent.

Young (6-8 weeks) BALB/c strain mice (n=3, 6 ears per group) received 30μl intradermal injections of affinity purified rabbit anti-mouse anti-TNF-α (A) or goat anti-mouse anti-IL-1β (B) antibody. Normal goat serum (NGS) and normal rabbit serum (NRS; 1 in 5 dilution in PBS) were used as controls for anti-IL-1β and anti-TNF-α antibody, respectively. Two h following antibody administration mice were treated with 25μl of either 0.5% oxazolone (Ox) or 1% DNBC. Negative controls were untreated (naive). After 4h mice were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) or allergen treated animals (#) as well as allergen treated in the presence or absence of an antibody (*) was analyzed by one-way ANOVA; *p<0.05; ###, ***p<0.001; ns=not significant.

In order to provide further evidence regarding whether DNBC-induced LC migration is dependent on TNF-α, mice were utilized that lack TNF-α receptor II (TNFRII), the only TNF-α receptor present on LC (Tartaglia and Goeddel, 1992), rendering LC from these animals unresponsive to TNF-α signalling (Wang et al., 1996). In untreated, WT (TNFRII+/+) and KO (TNFRII/-) mice, resting levels of LC were ~600 cells per mm² (Figure 6.9). The animals used in this experiment were on a C57BL/6 strain background and had a considerably lower baseline LC frequency than that observed for BALB/c.
strain mice (between 1000-1200 LC per mm$^2$) used in previous experiments, which was consistent with previous reports from our laboratory (PhD thesis Eaton, 2012). Treatment with a range of DNCB concentrations (0.25% to 1%) resulted in significant LC migration in WT as well as KO animals when compared to untreated controls (Figure 6.9A). In WT animals the extent of migration was between 14% and 16%, whereas in the KO mice between 31% and 36% of cells migrated from the epidermis. Despite different resting levels of LC being recorded in C57BL/6 and BALB/c strain mice, application of allergen resulted in a similar extent of LC migration with respect to the proportion of cells ready to migrate rapidly. Interestingly, although all of the concentrations of DNCB triggered the same extent of LC migration, cells that did not migrate, both in WT and KO animals had differential cell morphology. Langerhans’ cells, in both WT and KO animals, exposed to the highest concentration of DNCB exhibited morphological changes that were described previously (Figure 6.1), consisting of enlarged cell bodies and more pronounced dendrites (Figure 6.9D and 6.9E). In the intermediate dose, the majority of cells showed this activated phenotype, whereas very few cells looked activated at the 0.25% dose. The observed morphological changes, LC size (area) and length of dendrites were quantified. It was found that following treatment with 1% and 0.5% DNCB in both WT and KO mice the size of LC was significantly increased when compared with cells from concurrent untreated controls (Figure 6.9B). The length of LC (the ‘reach’ of dendrites) was also significantly higher than untreated controls for 0.5% and 1% of DNCB in WT animals and 1% of DNCB in KO mice (Figure 6.9C).
Figure 6.9 DNCB induced the same level of LC migration at different concentrations.
Figure 6.9 DNCB induced the same level of LC migration at different concentrations.

Young C57BL6 strain mice (8-12 weeks) (TNFRII+/+ or TNFRII−/−) (n=3, 6 ears per group) received topical applications of 0.25%, 0.5% or 1% DNCB in AOO or AOO alone to the dorsum of both ears for 4h. Negative controls were untreated (naive). After 4h mice were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM (A). Surface area (B) and length (C) of LC were measured using Fuji Image J software and expressed as the mean area (μm²) (B) and the mean length (μm) (C) of LC in TNFRII+/+ and TNFRII−/− strain mice. The statistical significance of differences between LC numbers, mean LC area and length in untreated (naive) or allergen treated animals was analyzed by one-way ANOVA: *=p<0.05, **=p<0.01, ***=p<0.001. Representative micrographs display LC morphology from respective treatments in TNFRII−/− and TNFRII+/+ mice (D). x40 magnification.
6.2.6 Members of dinitrohalobenzene family induced LC migration in the absence of TNF-α signalling

After establishing, using a variety of approaches, that DNCB can indeed cause LC migration in the absence of TNF-α signalling, other stimuli that previously have been shown to trigger LC migration were investigated with respect to their ability to cause LC migration in the absence of TNFRII. Knockout mice and their WT counterparts were treated with a number of different contact allergens (DNCB, DNFB, PPD), the respiratory allergen (TMA) or the non-sensitizing skin irritant (SLS). With the exception of PPD, all these stimuli have been shown previously to induce LC migration in BALB/c strain mice (Antonopoulos et al., 2008; Cumberbatch et al., 2005; Cumberbatch et al., 2002a). 2,4-dinitrofluorobenzene is a member of the same family of chemicals (dinitrohalobenzenes) as DNCB, and similarly to DNCB, it is a contact sensitizer (Watanabe et al., 2008). Paraphenylenediamine is a contact allergen found in hair dyes, and as a known skin sensitizer, it was expected to cause LC migration (Pot et al., 2013). Trimellitic anhydride is a recognized respiratory allergen (Dearman et al., 1991; Grammer et al., 1997) and has been shown previously in mice to result in LC migration following topical exposure, albeit with somewhat delayed kinetics (Cumberbatch et al., 2005). Lastly, SLS is not an allergen, but a skin irritant, nevertheless, when applied topically it was reported to trigger LC migration (Cumberbatch et al., 2002a). The hypothesis was that when applied topically these chemicals would not cause LC migration in TNFRII/-/- mice. In the untreated (naive) or vehicle (AOO) treated WT and KO mice resting LC levels were ~800 and ~600 cells per mm², respectively. In WT mice treatment with all of the stimuli resulted in LC migration in the expected range of between 20 and 30% (Figure 6.10A). However, in the KO animals only members of the dinitrohalobenzene family (DNCB and DNFB) were capable of triggering significant LC migration resulting in LC loss comparable to that reported for WT mice (between 20% and 37%) (Figure 6.10B). For the other stimuli the loss of LC from the skin ranged between 2% and 4%; none of which reached statistical significance. In the series of experiments reported in the Figure 6.10 oxazolone was not used as one of the stimuli, despite this chemical being used routinely as a reference contact allergen in previous studies of the cytokine regulation of LC migration (Cumberbatch et al., 2001). This was due to the fact that oxazolone failed to
cause LC migration in C57BL/6 strain mice at the concentration that caused vigorous migration in BALB/c strain mice (0.5%). Only when applied at the concentration of 3% was oxazolone capable of influencing LC frequency, and even then responses were not robust (PhD thesis Eaton, 2012).

Figure 6.10 Dinitrohalobenzenes (DNCB and DNFB) did not require TNF-α signalling to trigger LC migration.

C57BL6 strain mice (TNFRII+/

[A: 8-12 weeks, B: 20 weeks] or TNFRII−/− [8-12 weeks]) (n=3-4, 6-8 ears per group) were treated with 25μl of 1% DNCB, 25% TMA, 0.25% DNFB or 1% PPD in AOO or with 10% SLS in DMF on the dorsum of both ears for 4h or 17h (TMA). Treated animals together with untreated or AOO controls were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in naive or AOO group and allergen treated animals was analyzed by one-way ANOVA; ***=p<0.001.
6.2.7 Oxazolone and DNCB induced divergent cutaneous cytokine expression profiles

In the current investigations it was found that there were differences between these two allergens with regards to their ability to induce LC migration, particularly with respect to cytokine requirements for LC migration. Therefore, it was of particular interest to investigate the kinetics, vigour and quality of cutaneous cytokine mRNA profiles induced by oxazolone and DNCB. It was decided that measurement of gene expression in excised full thickness skin, sampled from the ear, would be most appropriate, as this tissue could be quickly excised without further manipulation and fixed in RNAlater immediately after excision, therefore, reducing the impact of trauma on gene expression (as observed in the explant experiments; Figure 3.1).

The figure 6.11 presents a comparison between two allergens: with respect to the expression of various IL-1 family members (IL-1α and IL-1β; IL-18 and IL-33), the proinflammatory cytokines TNF-α and IL-17F, the chemokine CXCL1 and the membrane marker ICAM-1, as a downstream indicator of inflammation. Expression of these molecules was assessed relative to baseline expression in control naive animals and a single animal was used at each time point (0.5h to 24h). Exposure to both allergens resulted in remarkably similar kinetic profiles for each molecule, although in many cases, the extent of activation was markedly higher for the DNCB-treated skin. A number of genes (TNF-α, IL-1β, CXCL1, ICAM-1 and IL-17F) were up-regulated upon allergen exposure; all followed a similar, transient, profile, returning to baseline levels within 24 h, with the exception of TNF-α, which peaked at approximately between 1 and 2h. Peak expression of markers was generally at 6h after initiation of exposure. In contrast, other IL-1 family members (IL-1α, IL-18 and IL-33) all exhibited down-regulation, also reaching maximal levels at around 6h and returning back to baseline levels by 24h. As indicated previously, although the patterns of expression were very similar for both allergens for all markers, there were some differences with respect to the vigour of the reaction between DNCB and oxazolone. The most striking differences were observed with respect to IL-1β and Cxcl1 expression. At peak expression (6h) the fold increases recorded were 8 and 18 for oxazolone and 55 and 130-fold for DNCB, for IL-1β and Cxcl1, respectively. DNCB-triggered IL-1β expression was higher than that observed following oxazolone treatments at all times, whereas Cxcl1 expression was
higher than oxazolone at the 6h time point, but then declined more rapidly than oxazolone-induced expression (Figure 6.11).

![Graphs showing cytokine expression over time for various cytokines: TNF-α, IL-1β, CXCL-1, IL-1α, ICAM, IL-18, IL-17F, IL-33.](image)

*Figure 6.11 Oxazolone and DNCB induced cutaneous cytokine expression in BALB/c strain mice.*
Young (6-8 weeks) BALB/c strain mice were treated with 25μl of either 0.5% oxazolone (Ox) or 1% DNCB on the dorsum of both ears. After 0.5, 1, 2, 4, 6, 8, 16 or 24h animals (n=1 per group) were sacrificed and ears were excised and immersed in RNAlater. Next, ears were homogenised in lysis buffer using homogeniser. Subsequently, tissue was homogenised in lysis buffer, mRNA was extracted and cDNA was prepared and utilized in qPCR reactions using TaqMan primers. Expression of Tnf-α, Il-1β, Cxcl1, Il-1α, Icam-1, Il-18, Il-17F and Il-33 was measured relative to the Hprt1 gene. Results are presented as a fold change (mean ± range) in the gene expression relative to untreated (naive) animals presents data from a single experimental animal (2 ears per group).

It must be noted that these kinetic experiments were conducted using a single animal at each time point, thus it was not possible to assess the statistical significance of any differences between oxazolone and DNCB. In order to confirm any differences between oxazolone and DNCB-induced cytokine and ICAM-1 transcript expression, the 4h time point was chosen (time of LC migration measurement) and additional animals were treated with oxazolone (0.5%) and DNCB (1%) (Figure 6.12). The results largely confirmed the previous kinetics experiment (Figure 6.11), with relatively vigorous up-regulation of Il-1β and Cxcl11 expression. There was a little effect (or a slight down-regulation) of Il-1α, Il-18 or Il-33, consistent with the previous experiment in which these cytokines were down-regulated transiently and then returned to baseline. Effects on Tnf-α, Il-17F and Icam-1 were relatively modest (2 or 3-fold increases only) and in general responses to DNCB were more vigorous than those recorded for oxazolone treatment. Similarly, higher levels of Il-1β and Cxcl11 were induced by DNCB compared with oxazolone.
Figure 6.12 Differential induction of cutaneous IL-1β and Cxcl1 expression by DNCB compared with oxazolone.

Young (6-8 weeks) BALB/c strain mice were treated with 25μl of either 0.5% oxazolone (Ox) or 1% DNCB on the dorsum of both ears. After 4h animals (n=2-4 per group) were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer, mRNA was extracted and cDNA was prepared and utilized in qPCR reactions using TaqMan primers. Expression of Tnf-α (A), Il-1β (B), Il-1a (C), Cxcl1 (D), Icam-1 (E), Il-17F (F), Il-18 (G) and Il-33(H) was measured relative to the Hprt1 gene. Results are presented as a fold change in the gene expression relative to untreated (naive) animals (mean ± SEM) and data from a single experiment (4-8 ears per group). The statistical significance of differences between gene expression between different treatment conditions was analyzed by a Kruskal-Wallis with Dunn’s post-test; *=P<0.05, **=p<0.01.

It was of considerable interest that more vigorous up-regulation of IL-1β mRNA was observed for DNCB compared with oxazolone treated tissue, given that this might represent a mechanism by which DNCB induced migration independently of TNF-α (by more vigorous expression of this cytokine). However, IL-1β production is controlled at many levels, not just at the level of message. Interleukin-1β protein is translated and produced as an inactive precursor protein, which must be cleaved to its active form by
the enzyme caspase 1, which also exists as an inactive precursor. In addition, it also is regulated at the level of secretion, with a second signal required for the release of this cytokine (Netea et al., 2010). Therefore, in addition to measuring this cytokine at the level of transcription, it was important to measure the secretion of the IL-1β. Thus, animals were treated with 0.5% oxazolone or 1% DNCB for either 2h or 6h (Figure 6.13). Ears were excised, explants prepared and floated on medium overnight, then cytokines (TNF-α, IL-1α and β) that had been secreted into the supernatant were analyzed by ELISA. Production of TNF-α in the absence of allergen treatment was ~100pg/ml. Application of allergen was largely without effect, apart from 2h exposure to oxazolone where TNF-α levels of 200pg/ml, were recorded. High constitutive production of IL-1α (~2000pg/ml) was observed and this was not modulated by exposure to allergens. Baseline release of IL-1β was ~100pg/ml, and was up-regulated slightly by treatment with oxazolone at both time points to approximately ~200pg/ml. However, the most vigorous production of this cytokine was reported 6h after DNCB application (approximately 5-fold increase) (Figure 6.13C).

**Figure 6.13 Allergen treatment provoked release of cytokines from the skin.**

Young (6-8 weeks) BALB/c strain mice were treated with 25μl of either 0.5% oxazolone (Ox) or 1% DNCB on the dorsum of both ears. After 2 or 6h (A, B, C; n=1 per group; 2 ears) treated animals and untreated controls (naive) were sacrificed and ears were excised. Ears were split into dorsal and ventral halves and the dorsal part was floated on 250μl of complete RPMI media and incubated for 16h. Following incubation supernatants were collected and TNF-α (A), IL-1α (B) or IL-1β (C) production was determined by ELISA. The dashed line represents the lower limit of detection for each ELISA (TNF-α [32pg/ml], IL-1α and β [15pg/ml]). Graphs present mean ± range (for n=1; 2 ears per group).
This increased expression of IL-1β, therefore, represented a possible mechanism whereby DNCB could cause TNF-α-independent mobilisation of LC. In subsequent experiments, the dose dependence of the effects of allergen on cytokine was explored. In the majority of experiments reported herein, doses of 0.5% oxazolone and 1% DNCB were used routinely, as these each provoked significant mobilisation of LC following a single topical application and caused downstream events such as vigorous proliferation of LNC (Cumberbatch et al., 2005; Cumberbatch et al., 2002b; Hilton J et al., 1995). At these specific concentrations of allergen, it has been shown herein that oxazolone and DNCB exhibit a differential dependence on TNF-α for LC mobilisation. Furthermore, in the case of DNCB it was shown that at much lower concentrations (0.25%) the same pattern of TNF-α-independent LC migration was observed. In these experiments the primary purpose was to determine whether at very high oxazolone concentrations (3%) this chemical could overcome requirement for TNF-α. Thus, animals were dosed with 0.5% and 3% of oxazolone, and 0.25% and 1% of DNCB and allergen-induced effects on cutaneous cytokine production (secretion) examined. As demonstrated previously, there was a constitutive expression of both TNF-α and IL-1α, which was unaffected by treatment with either allergen at any dose (Figure 6.14A and 6.14B). On the other hand, there was a significant allergen-induced production of IL-1β. In the naive animals IL-1β production was ~75pg/ml. Following application of 0.5% oxazolone, relatively low level of induction of IL-1β was recorded (~300pg/ml); whereas application of 3% of oxazolone or 0.25% and 1% of DNCB resulted in more vigorous IL-1β release (800, 600 and 700pg/ml, respectively). The release of IL-1β was induced by the higher oxazolone and both concentrations of DNCB reached statistical significance compared with baseline expression. Additionally, there was a statistically significant difference between the levels of IL-1β production triggered by 0.5% and 3% oxazolone (Figure 6.14C).
Figure 6.14 Cutaneous cytokine release induced by contact allergens was dose dependent.

Young (6-8 weeks) BALB/c strain mice were treated with 25μl of either 0.5% or 3% oxazolone (Ox) or 0.25% or 1% DNCB on the dorsum of both ears. After 6h mice were sacrificed and ears were excised. Ears were split into dorsal and ventral halves and the dorsal part was floated on 250μl of complete RPMI media and incubated for 16h. Following incubation, supernatants were collected and TNF-α (A), IL-1α (B) or IL-1β (C) production was determined by ELISA. The dashed line represents the lower limit of detection for each ELISA (TNF-α [32pg/ml], IL-1α and β [15pg/ml]). Graphs present data ± SEM (for n=2; 4 ears per group). The statistical significance of differences between naive samples and allergen induced cytokine production was analyzed by a Kruskall Wallis test; **p<0.01.

6.2.8 LC migration induced by high dose of oxazolone is TNF-α independent

It was reported that high IL-1β levels were detected not only following 1% DNCB treatment but also after exposure to 3% oxazolone (Figure 6.14C). It had been established that DNCB was able to induce LC migration in the absence of TNF-α (Figure 6.8A), that was hypothesised to be due, in part, to vigorous IL-1β expression. Therefore, it was important to ascertain if this higher (3%) dose of oxazolone was also capable of causing TNF-α-independent LC migration. Exposure to both doses of oxazolone resulted in the usual loss of the proportion of LC that are able to rapidly mobilize; 20% LC migration. Further, it was demonstrated that prior treatment with LF, a protein capable of inhibiting LC migration in a TNF-α-dependent manner, inhibited significantly LC migration triggered by 0.5% oxazolone, but was without effect on the decrease in LC numbers caused by exposure to 3% oxazolone (Figure 6.15). Thus, treatment with 3% oxazolone resulted in 18% and 15% loss of LC in the absence and in
the presence of LF, respectively. Application of 0.5% oxazolone resulted in the change in morphology as previously described (Figure 6.1C). However, treatment with 3% oxazolone caused changes in LC phenotype that resembled those observed following exposure to 1% DNCB, regardless of the presence of LF.

![Graph showing LC frequency](image)

**Figure 6.15** Lactoferrin did not inhibit LC migration caused by high concentration of oxazolone.

Young (6-8 weeks) BALB/c strain mice (n=3 per group, 4-6 ears per group) were treated with 5μg of LF in 30μl of aqueous cream or cream alone on the dorsum of both ears. Two h later animals were challenged on the same site with 25μl of 0.5% or 3% oxazolone (Ox) in AOO for 4h. Animals were sacrificed, ears were excised and LC frequency was enumerated by fluorescence microscopy and is expressed as the mean frequency of LC per mm² per group ± SEM. The statistical significance of differences between LC numbers in untreated (naive) and allergen treated and LF treated animals was analyzed by one-way ANOVA; *=p<0.05, **=p<0.01. Representative micrographs display LC morphology from respective treatments.
6.2.9 Chemicals provoked distinct cutaneous cytokine expression profiles

Finally, a series of experiments was conducted to explore the nature of cutaneous cytokine expression following topical exposure to other types of chemical insult. Animals were exposed to a respiratory allergen (TMA) (Grammer et al., 1997), DNTB a chemical that has been reported previously to be a tolerogen and to failed to provoke LC migration (Udey, 2012) or to a skin irritant, SLS (Agner et al., 1989), and time dependent changes in cutaneous gene expression were examined.

Quantitative PCR was used to assess time dependent changes in cutaneous cytokine (TNF-α, IL-1β, IL-1α, IL-33, IL-18), chemokine (CXCL1) and adhesion molecule (ICAM-1) expression levels. It has been reported that chemical allergens form two groups: those with the ability to cause ACD and a smaller group with the ability to cause sensitization of the respiratory tract and occupational asthma or rhinitis (Kimber et al., 2010). These different types of chemicals have been shown to polarize the immune response to either a Th1 or a Th2 phenotype. Prolonged (2 week) exposure to either contact or respiratory allergens results in cytokine production by LNC that is characteristic of each group of allergens (Dearman and Kimber, 1999). The purpose of the current series of experiments was to determine whether different classes of chemical, including a respiratory allergen, exhibited specific patterns of cutaneous gene expression following single dose exposure. Indeed, it was established previously that exposure to respiratory allergen TMA resulted in delayed LC migration (17h) in comparison with contact allergens (4h) via cutaneous production of IL-10 (Cumberbatch et al., 2005). Mice were exposed to TMA, DNTB or SLS, and time dependent changes in skin gene expression were examined. It was demonstrated before that exposure to TMA and SLS resulted in LC migration that was dependent upon TNF-α signalling (Figure 6.10). Treatment with TMA was without effect on expression of majority of markers throughout the time course (TNF-α, IL-1α, IL-33, IL-18, ICAM-1). However, a marked up-regulation of Cxcl1 was recorded that peaked at 16h with a 15-fold increase in comparison with naive tissue. Another up-regulated gene was Il-1β; its expression did not increase until 24h after initiation of exposure and was approximately 10 times higher than that of untreated (naive) controls at this time point (Figure 6.16). It is of interest that TMA induced LC migration takes place 17h following exposure.
(Cumberbatch et al., 2005), as opposed to 4h that is sufficient for other stimuli reported in the current investigations. Therefore, delayed Cxcl1 and Il-1β expression were in agreement with LC migration data.

Figure 6.16 TMA-induced skin cytokine profile.

Young (6-8 weeks) BALB/c strain mice were treated with 25μl 25% TMA on the dorsum of both ears. After 2, 4, 6, 8, 16 or 24h animals (n=1 per group; 2 ears) were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer, mRNA was extracted, and then cDNA was prepared and utilized in a qPCR reactions using TaqMan primers. Expression of Tnf-α, Il-1β, Cxcl1, Il-1α, Icam-1, Il-18 and Il-33 was measured relative to the Hprt1 gene. Results are presented as a fold change in the gene expression relative to the untreated (naive) animals (mean ± range) and data from a single experimental animal (2 ears per group).

Sodium lauryl sulphate is an irritant which is capable of inducing LC migration, but does not elicit a specific adaptive immune response (Ikarashi et al., 1993). Application of SLS resulted in more rapid (2h post-treatment), but transient, increase in various markers than did the allergens. Thus, somewhat elevated levels of Il-1β, Tnf-α and Icam-1 were recorded, but the most profound changes were in the expression of Cxcl1,
which displayed 8 and 1.5-fold increase compared to the naive tissue, respectively, at 2h and 4h. It has been shown previously that LC migration in response to SLS was dependent on two cytokine signals TNF-α and IL-1α (Cumberbatch et al., 2002a). Interestingly exposure, to SLS did not result in up-regulation, or down-regulation of IL-1α, as observed following exposure to contact allergens. Instead IL-1α expression was the same as that observed in the naive tissue (Figure 6.17).

![Graph showing cytokine expression](image)

**Figure 6.17 SLS-induced skin cytokine profile.**

Young (6-8 weeks) BALB/c strain mice were treated with 25μl of 10% SLS in DMF on the dorsum of both ears. After 2, 4, 8 or 24h animals (n=1 per group) were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer, mRNA was extracted, and then cDNA was prepared and utilized in a qPCR reactions using TaqMan primers. Expression of Tnf-α, Il-1β, Cxcl1, Il-1α, Icam-1, Il-18, Il-17F and Il-33 was measured relative to the Hprt1 gene. Results are presented as a fold change in the gene expression relative to the untreated (naive) animals (mean ± range) and data from a single experimental animal (2 ears per group).
Dinitrothiobenzene belongs to the same chemical family as DNCB and DNFB and yet application of DNTB to the skin did not result in LC migration (Schwarzenberger and Udey, 1996). Furthermore, DNTB was demonstrated to render animals that were treated previously with DNTB resistant to subsequent attempts to induce DNFB-mediated CHS (Riemann et al., 2005). Similar to the respiratory allergen TMA, following application of DNTB there was little change in the expression of the most measured markers (Tnf-α, Il-1α, Il-33, Il-18, Icam-1) during the time course. However, exposure of animals to DNTB resulted in the increased expression of Cxcl1 and Il-1β that both peaked 8h after treatment (25 and 5-fold increase compared to naive tissue, respectively) (Figure 6.18).

![Graph showing cytokine expression](image)

**Figure 6.18 DNTB-induced skin cytokine profile.**

Young (6-8 weeks) BALB/c strain mice were treated with 25μl of 1% DNTB on the dorsum of both ears. After 2, 4, 8 or 24h animals (n=1 per group) were sacrificed and ears were excised and immersed in RNAlater. Subsequently, tissue was homogenised in lysis buffer, mRNA was extracted, and then cDNA was prepared and utilized in a qPCR reactions using TaqMan primers. Expression of Tnf-α, Il-1β, Cxcl1, Il-1α, Icam-1, Il-18, Il-17F and Il-33 was measured relative to the Hprt1 gene. Results are presented as a fold change in the gene expression relative to the untreated (naive) animals (mean ± range) and data from a single experimental animal (2 ears per group).
6.3 Discussion

Following exposure to biological, chemical or physical stimuli (Byrne et al., 2001; Ghaznawie et al., 1999; Lee et al., 2012) a proportion of LC has been shown to undergo changes, that eventually lead them to migrate away from the epidermis via afferent lymphatics to the LN (Cumberbatch et al., 2000a). In this chapter, the impact of different classes of chemical (contact and respiratory allergens, as well as an irritant) was assessed on the mechanism of LC migration and cutaneous cytokine production.

Previous data from our laboratory have shown that in naive male BALB/c strain mice, resting LC frequencies of 800-1000 cells per mm$^2$ and of 1200 cells per mm$^2$ in female naïve mice were recorded (PhD thesis Eaton, 2012). Therefore, the frequencies of LC observed in the current studies were in agreement with the previously presented data. Langerhans’ cell numbers in C57BL/6 strain mice were considerably lower than those observed in BALB/c strain mice and, regardless of gender of the mice, presented at approximately 600-800 cells per mm$^2$ which also was in accordance with the previous data from our laboratory (PhD thesis Eaton, 2012). Langerhans’ cell migration following exposure to allergen has been reported to be between 20% and 30% of cells in mice and in humans (Antonopoulos et al., 2008; Cumberbatch et al., 2001; Griffiths et al., 2001), which coincides with the proportion of cells exhibiting decreased E-cadherin expression following exposure to allergen (Schwarzenberger and Udey, 1996). However, the exact mechanism that determines which cells will leave the epidermis and which will stay, is not known. In accordance with the published data, in the current study 4h exposure to oxazolone and DNCB resulted in LC mobilisation. Increase in the time of exposure to allergen (up to 24h) did not induce further LC mobilisation and the proportion of migrating cells remained between 20% and 30%; the proportion of LC which is apparently the number of cells that are able to respond rapidly at any one time to migratory stimuli.

Similarly, increasing or decreasing the concentration of oxazolone and DNCB, respectively, did not change the proportion of migrating LC. In addition to triggering LC migration, exposure to allergen resulted in changes to LC morphology. In the steady state LC had relatively small cell bodies and visible dendrites that were not particularly pronounced. However, following treatment with allergens, the cell bodies of cells that
did not migrate became larger and dendrites were more visible, particularly in the animals treated with DNCB and DNFB, and high concentrations of oxazolone. In the current investigations, LC were visualised by staining with FITC-conjugated MHC II antibody, therefore, brighter appearance of LC could be attributable to a higher expression of MHC II molecules. It has been established that \textit{in vivo} (DNCB, oxazolone, DNFB) and \textit{in vitro} (DNFB) exposure of LC to contact allergens resulted in decreased cell surface MHC II expression, but increased overall intracellular levels of the molecule (Becker et al., 1992). Therefore, the observed increase in brightness of MHC II expression following exposure to allergens was likely to be due to increased intracellular expression. In the protocol used in the current study, prior to incubation with the detection antibody, epidermal sheets were fixed with acetone, which acts as a protein denaturing agent, therefore disturbing the integrity of cells and allowing anti-MHC II antibody access inside the LC. Following exposure to lower concentrations of DNCB (0.25%), LC presented a less activated phenotype, more resembling that observed after oxazolone treatment. Divergent phenotypes observed following exposure to various concentrations of allergens suggested that with decreasing the concentration of allergen, the production of inflammatory mediators responsible for the phenotypic changes is diminished. However, currently the direct factors responsible for these morphological changes are not known. Indeed, the origins of changes to LC morphology following exposure to allergens in general are not well characterized. Up-regulation of MHC II by LC following exposure to allergen has been established (Becker et al., 1992), however, changes to the LC cytoskeleton that would account for the increased surface area of the cells, or more prominent dendrites have not been described in the literature. Vimentin is an intermediate filament protein, like keratin, it is a part of the cytoskeleton and participates in cellular adhesion and migration. Mice that lack vimentin have impaired wound healing and disturbed leukocyte homing to the LN (Ivaska et al., 2007). An attempt was made by Bacci and colleagues (1996) to investigate the effects of UV radiation and TNF-α on vimentin expression by LC. It was found that both stimulants significantly decreased vimentin expression, as assessed by a decreased signal from anti-vimentin antibody. In addition, exposure to vinblastine, an alkaloid that disrupts microtubules, resulted in down-regulation of vimentin expression by LC. Vinblastine also triggered LC migration. These data suggested that stimuli that cause LC migration (UV radiation and TNF-α) disrupt LC cytoskeleton, and conversely, compounds that impact on cytoskeleton (vinblastine) result in LC mobilisation (Bacci et
Therefore, it could be concluded that treatment with allergens: oxazolone and to larger extent with dinitrohalobenzenes (DNCB and DNFB) disrupts the cytoskeleton causing the characteristic ‘flattened’ LC phenotype, although the specific mechanism is not fully understood. Despite these indications of divergent phenotypes driven by different allergens, the extent of migration triggered by both doses of DNCB and oxazolone was similar. It was reported previously that intradermal injection of homologous LF inhibited DC accumulation in the LN following exposure to oxazolone. Further work demonstrated that LF also inhibited LC migration in response to exogenous IL-1β, but not TNF-α, suggesting that LF exerted its action via inhibition of TNF-α signalling (Cumberbatch et al., 2000b). Moreover, it was demonstrated that topical application of homologous LF had similar effects in humans. Indeed, exposure to LF was reported to inhibit LC migration in response to DPC, IL-1β, but not that triggered by exogenously supplied TNF-α. Furthermore, Almond et al. (2013) demonstrated that topical application of heterologous (human) LF of both native and recombinant origin, inhibited LC migration in response to oxazolone in mice. In dose response experiments it was found that the inhibitory effects of LF were retained even at the low dose of 0.05μg. The standard amount utilized in the current and previous experiments in mice was 0.5μg and in investigations using homologous murine LF the application contained 0.75μg (Cumberbatch et al., 2000b). In experiments in humans, 50μl of a 0.04% LF solution (equating to 0.02μg of LF per treatment) was used (Griffiths et al., 2001), suggesting that LF is indeed active in vivo even at a very low concentration. The amount of LF used in experiments in human was based on the quantity of that protein in the human tears, which has been established to be 2mg/ml and consisted 25% of total amount of protein present in tears (Kijlstra et al., 1983). The amount of LF used in the current study was equivalent of 6.25 pM and dose response experiment established inhibitory role of LF even at 625 fM dose. Biological activity of proteins at such low concentrations is characteristic for cytokines. Indeed, cytokines utilized in the studies described herein were shown to be active at pico- and femtomolar concentrations. In the Chapter 5 0.1ng (~5.7 fM) of IL-1α and β were able to trigger TNF-α expression by PAM cells and in vivo effects were observed following injection of 50ng (~2.8 pM) of IL-1α and β (Chapter 3).

The kinetics of the effect of LF also was examined. Whilst the effect of oxazolone on LC migration was relatively similar at all time points, the inhibitory effect of LF was
transient. Significant inhibition of migration by LF was observed when mice were exposed to oxazolone for 4h, however, inhibitory effect was diminished after 6h and it was completely lost after 24h. One of the reasons for the inability of LF to exert its inhibitory potential after more than 4h might be the fact that allergens have the ability to persist in the skin for up to 14 days following first application (Saint-Mezard et al., 2003). Indeed, it was demonstrated in the current studies, in the Chapter 3, that 120h following initiation of oxazolone treatment the level of Tnf-α and Il-1β mRNA begun to increase, suggesting that the local effects of topical oxazolone exposure, if not oxazolone itself, were persistent. However, under the conditions examined, LF did not modulate oxazolone-induced cutaneous cytokine expression or LN cell proliferation (Chapter 3). Therefore, it was not entirely surprising that there was no long-lasting effect of LF on LC migration. In the study reported by Cumberbatch et.al (2000) LF inhibited oxazolone-triggered DC accumulation in the draining LN 18h following initial exposure (Cumberbatch et al., 2000b). The different time point examined in the study by Cumberbatch et al. (2002) is consistent with the arrival of dermal DC into the LN which starts at 8h following exposure to allergen (Allan et al., 2006). In the study by Griffiths et.al (2001) LF was found to exert its inhibitory potential on LC migration measured 17h following treatment with DPC (Griffiths et al., 2001). The possible explanation for the discrepancy between current study and that reported by Griffiths et al. (2001) could be the differences between LF metabolism in human and mice or the different allergen (DPC versus oxazolone). Moreover, LF utilized in the studies described in this thesis was heterologous, which could have impacted on the rate of its turnover. Furthermore, longer inhibitory effect of LF in humans might be due to the fact that it is believed that LC mobilisation in the human skin is a slower process than that observed in the murine equivalent, due to the thicker epidermis in human subjects (personal communication, Dr. Rebecca Dearman).

Lactoferrin is produced as a glycosylated protein (Spik et al., 1988) and there have been reports that attributed opposing functions, stimulatory and inhibitory to glycan and protein moieties of LF, respectively (Ando et al., 2010). However, the ability to inhibit oxazolone and DPC-induced LC migration is thought to be attributable to the protein part of LF due to the following information. Firstly, recombinant human LF, produced in Aspergillus niger var. awamori, was demonstrated to have an inhibitory effect in human skin (Griffiths et al., 2001), and it was shown that this recombinant form of LF
had the same amino acid sequence, but a differential glycosylation pattern (Ward et al., 1997). Furthermore, a native human LF, human recombinant LF produced in *Aspergillus* and in rice, were all demonstrated to inhibit oxazolone induced LC migration in mice, and similarly all of those proteins share identical amino acid sequence, but a differential glyco-profile (Almond et al., 2013). And lastly, as demonstrated by Almond et al. (2013) and in the current investigations, human LF inhibited oxazolone-mediated LC migration in mice, and murine and human LF share ~70% amino acid homology (Baker, 1994). Therefore, the ability of the commercially available LF peptide, N-lobe, to inhibit oxazolone-induced LC migration was examined. There is 67% homology between mouse and human within corresponding 231-245 residues of LF. It was hypothesised that if the protein proportion of LF was responsible for inhibitory effect of LF it could be due to the N-lobe, which was demonstrated previously also to have immunosuppressive properties (Nibbering et al., 2001; van der Does et al., 2012). To assess the ability of N-lobe to inhibit oxazolone-induced LC migration the same amount of N-lobe and LF was used, as well as N-lobe in excess. Prior application of the N-lobe failed to compromise oxazolone-induced LC migration, suggesting that this part of LF was not responsible for inhibition of LC mobilisation following exposure to oxazolone. It was reported previously, that in mice intraperitoneal administration of the N-lobe (10μg and 100μg/mouse) was capable of approximately ~50% suppression of DTH reaction of footpad swelling following challenge with SRBC, as well as inhibiting to a similar extent the frequency of SRBC-positive plaque forming cells (Siemion et al., 1995). Thus, Siemion et al. (1995) reported that the N-lobe was able to suppress adaptive immune response at both the humoral and cellular levels. However, in the current investigations, the role of N-lobe in modifying innate immune responses was assessed. The lack of effect of the N-lobe under these conditions suggested that this peptide might interact primarily with T cells, which are unlikely to be involved in the LC migration initiated following primary exposure to allergen. These results implied also that another part of LF is responsible for its inhibition of LC migration.

The immunomodulatory role of LF was investigated primarily utilising oxazolone. Moreover, oxazolone has been used previously to elucidate which cytokine signals were required for LC migration, namely TNF-α and IL-1β. Another popular contact allergen, DNCB, has been used widely in our laboratory, primarily for the examination of
divergent immune responses, in comparison with the respiratory allergen TMA. Prolonged exposure to these two allergens results in polarisation of the immune response towards Th1/Tc1 and Th2 for DNCB and TMA, respectively (Dearman and Kimber, 1999). It had been shown previously that exposure of mice to DNCB resulted in LC migration (Cumberbatch et al., 2005). It was decided to examine whether LF was able to inhibit DNCB-induced migration. As reported before, LF inhibited oxazolone induced LC migration (measured at 4h), but was without effect on DNCB-triggered mobilisation. An examination of the kinetics of the responses revealed that whilst there was no inhibition of oxazolone-induced migration 6h following primary exposure, there was a significant inhibition (in a single experiment) of DNCB-triggered migration. This result was somewhat intriguing considering that there was no inhibition of migration in the presence of LF 4h or 24h after initial exposure to DNCB. Therefore, it was speculated that following an initial loss of LC (at 4h), the effect of LF was to recruit LC into the epidermis at the 6h time point. Indeed, it has been demonstrated previously that LF impacted on cell recruitment. Thus, intra-peritoneal injection of TLF in mice resulted in a rapid (4h after injection) accumulation of neutrophils and subsequent recruitment of monocytes 24h following injection of TLF (Yang et al., 2009). However, LC depleted by heat began repopulating epidermis 3 days after the initiation of treatment (Ghaznawie et al., 1999), suggesting that LC recruitment is not always a rapid process. Conversely, in a study by Cumberbatch et al. (1999a) it was demonstrated that 300ng of TNF-α induced LC migration measured at 30 minutes following injection of cytokine, however, LC frequency after 4h was no different than that found in naive animals, suggesting that between 30 minutes and 4h LC recruitment and replenishment took place. In the current study, however, it is also plausible that the observed inhibitory effect of LF on DNCB-induced migration at 6h was not biologically relevant, given that statistical significance was observed in a single experiment.

The concentrations of oxazolone and DNCB employed in the current investigations were chosen on the basis of their ability to elicit the same level of LC migration, as well as their ability to yield a similar level of T cell proliferation in the LN (Cumberbatch et al., 2005; Cumberbatch et al., 2002b; Hilton et al., 1995). Therefore, it was considered that oxazolone and DNCB exhibited the same level of “potency”. However, despite these assumptions it was hypothesised that DNCB still could have provided a stronger signal than oxazolone, which was not able to be inhibited by LF. Therefore, a lower
concentration of DNCB (0.25%) was selected. DNCB-induced LC migration was not inhibited by LF at any concentration of DNCB tested, implying that there must be a difference in the quality of immune response caused by oxazolone and DNCB.

In the work conducted previously by our laboratory a paradigm of LC and DC migration was established, whereby at least two non-redundant signals from TNF-α and IL-1β were required for LC migration from the epidermis and DC accumulation in the LN (Cumberbatch et al., 1997a). Results presented in the studies described herein challenged that hypothesis. Therefore, whilst oxazolone-induced migration was effectively abrogated by pre-treatment with either anti-TNF-α or anti-IL-1β antibody, DNCB-triggered LC mobilisation was not inhibited by anti-TNF-α antibody and was only partially inhibited by anti-IL-1β antibody. These data suggested that DNCB was capable of inducing LC migration in the absence of TNF-α signalling, and were consistent with the lack of inhibition of DNCB-induced LC migration by LF. Further investigations of DNCB migration in the complete absence of TNF-α signalling confirmed this hypothesis. These experiments were conducted using mice that lacked TNFRII, the sole TNF-α receptor present on LC (Wang et al., 1996), making LC from these animals unresponsive to TNF-α signal. Importantly, following exposure to various concentrations of DNCB (0.25%, 0.5%, and 1%), despite the complete lack of TNF-α signalling, LC migration was still observed. In addition to triggering significant levels of LC mobilisation in WT and KO animals, DNCB also induced significant changes in LC morphology. From the experience in our laboratory the morphological changes to LC observed following exposure of the skin to DNCB were unique among allergens. It was speculated, that one of the reasons for these DNCB-induced, pronounced, phenotypic changes to LC were different and/or higher levels of ROS, cytokines and chemokines, or all of these together. Indeed, these suspicions were confirmed when allergen-triggered changes to the cutaneous gene expression were quantified.

The lack of dependence on TNF-α of DNCB-induced LC migration was intriguing. Members of the dinitrohalobenzene family, namely DNCB and DNFB, were found to trigger LC migration in the absence of TNFRII. It was found that LC mobilisation triggered by other tested chemicals TMA, SLS and PPD was dependent on TNF-α signalling, suggesting that TNF-α independence of dinitrohalobenzenes is very much more the exception rather than the rule (Eaton et al., 2014). Albeit surprising in the context of contact allergens, it was not the first time that LC migration was observed in
the absence of TNF-α signalling. Byrne et al. (2001) reported that cutaneous West Nile virus infection was able to trigger LC mobilisation in TNF-α deficient mice. However, whilst West Nile virus-induced LC migration was independent of TNF-α, LC migration from epidermis and accumulation in the LN was significantly inhibited by treatment with anti-IL-1β antibody (Byrne et al., 2001), again consistent with the pattern for dinitrohalobenzenes reported herein.

Thus, it has been established that DNCB and oxazolone exhibited differential cytokine requirements with regards to LC migration. To gain more detailed understanding of the processes that might be responsible for the divergence between these two allergens, the cutaneous gene expression profiles triggered by allergen exposure were investigated over the period of 24h. As previously described, qPCR was utilized to investigate the changes in gene expression in comparison with untreated samples. It has been established previously using PCR and liquid hybridization (whereby PCR products underwent electrophoresis and subsequently were incubated with complementary primers, tagged with radioactive phosphorus) that shortly (within 90 minutes) after exposure to 3% TNCB expression of Tnf-α, Il-1β and Cxcl10 was observed (Enk and Katz, 1992a). TNCB is widely used in the investigations of ACD, however, it is impossible to utilize this chemical in our laboratory due to safety risks, as TNCB is an explosive (Parihar et al., 1967). In the current investigations substantial induction of Tnf-α expression was not observed. The discrepancy between two studies could stem from the fact that different stimulants and detection methods were utilized. However, rapid expression of IL-1β was demonstrated in the study reported herein. There were marked differences observed between cytokine levels induced by oxazolone and DNCB. Thus, it was established that DNCB induced significantly higher cutaneous expression of Tnf-α, Il-1β, Cxcl1, Icam-1 and Il-17F, suggesting that these higher cytokine signals might be responsible for the lack of DNCB dependence upon TNF-α. Consistent with the gene expression data, it was found that DNCB triggered higher levels of IL-1β protein release. To account for that fact that different concentrations of oxazolone and DNCB were used (despite triggering the same extent of LC migration), the ability of high and low concentrations of both oxazolone and DNCB to induce cytokine production and release was considered. It was found that although there were no differences in the release of TNF-α and IL-1α there was a significant divergence in the level of IL-1β release. Interestingly, it was observed that exposure to a high (3%)
concentration of oxazolone resulted in a similar level of IL-1β release as that triggered by both concentrations of DNCB. This result suggested two things. First, that IL-1β might be the mediator of TNF-α independence of DNCB, due to its high levels in DNCB-treated skin, and a close resemblance of LC morphology between DNCB and IL-1β exposure. Secondly, it could be speculated that if IL-1β was responsible for TNF-α independent DNCB-induced LC migration, therefore, a high (3%) concentration of oxazolone, that induced similar vigorous IL-1β production, could also trigger TNF-α independent LC migration. To test this hypothesis, LC migration was assessed following exposure to 0.5% and 3% oxazolone in the presence and absence of LF. It was found that pre-treatment with LF significantly inhibited oxazolone induced LC migration (using the standard, low dose of oxazolone), which was demonstrated to be a result of inhibition of de novo TNF-α production (Cumberbatch et al., 2000b). The dose effect of oxazolone could not be reconciled on the basis of differential TNF-α expression, although it should be noted that marked allergen-induced TNF-α release was not observed during these experiments. Therefore, it was hypothesised, that the failure of LF to inhibit LC migration provoked by the high concentration (3%) of oxazolone was due to higher IL-1β levels present in the skin. These results implied that when a high concentration of IL-1β is present, TNF-α signalling might be redundant for LC mobilisation.

The C57BL/6 TNFRII KO strain mice could not be used to investigate the effect of 3% oxazolone on TNF-α independent LC migration due to the inability of C57BL/6 strain mice to respond robustly to oxazolone with LC migration (PhD thesis Eaton, 2012). There have been numerous reports demonstrating differences between BALB/c and C57BL/6 strain mice. Overall, it is considered that BALB/c and C57BL/6 strain mice have a higher propensity to exhibit Th2 and Th1 driven conditions, respectively (Watanabe et al., 2004). Oxazolone was identified previously as a contact allergen on the basis of its failure to trigger IgE production following an appropriate dosing regimen involving 2 exposures over a 14 day period (Dearman and Kimber, 1992). However, in another study utilising Brown Norway rats, oxazolone was characterized as a potential respiratory allergen capable of inducing IgE production and gene expression comparable to that triggered by TMA (Kuper et al., 2011). Furthermore, oxazolone was employed for development of colitis in C57BL/10 mice, which was driven by Th2 response and was dependent on NK-cell derived IL-13 (Heller et al., 2002). On the
contrary, another report established that oxazolone was unable to induce IgE production and demonstrated that intranasal sensitization and challenge with oxazolone did not result in allergic rhinitis (Farraj et al., 2004). There are no reports regarding oxazolone allergy in humans, due to lack of significant exposure. Although there are anecdotal reports of ACD to oxazolone among laboratory workers (personal communication, Prof. Ian Kimber), however, it is difficult to assess what properties oxazolone-induced sensitivity would have in humans. Nonetheless, despite some reports suggesting that oxazolone could be considered as a respiratory allergen, prevalent consensus exists that oxazolone is in fact a contact allergen.

Despite classification of oxazolone as a contact sensitizer, reports suggesting that oxazolone is a respiratory sensitizer, and therefore would induce Th2-skewed immune response, could partially explain why oxazolone is unable to induce sufficient LC migration in C57BL/6 strain mice (PhD thesis Eaton, 2012), which are known to preferentially mount Th1-dependent responses (Watanabe et al., 2004). In their study Watanabe et al. (2004) investigated responses of macrophages derived from both mouse strains and found that C57BL/6 strain mice produced higher levels of TNF-α and IL-12 in response to LPS than BALB/c strain mice, which in turn can lead to Th1 polarized response in C57BL/6 strain mice (Watanabe et al., 2004). Conversely, oxazolone readily triggered LC mobilisation in BALB/c strain mice, which were demonstrated to be more prone to Th2 polarized responses (Watanabe et al., 2004). These two mouse strains were demonstrated to differ with regards to TLR expression by their respective DC, which explained their divergent susceptibility to Listeria monocytogenes infection (Liu et al., 2002), and could potentially be the reason for differential responses to oxazolone. Naive DC isolated from spleens of C57BL/6 and BALB/c strain mice preferentially expressed TLR9 and TLR 2, 4 and 6, respectively (Liu et al., 2002). TLR 2 and 4 were shown previously to be required for the development of CHS reactions in response to TNCB (Martin et al., 2008). Therefore, the higher degree of expression of these TLR receptors on DC from BALB/c strain mice could explain the ability of LC from these mice to readily migrate from the epidermis following exposure to oxazolone.

Another reason for the divergence between mice strains with respect to their response to oxazolone might be the fact that C57BL/6 strain mice have naturally disrupted gene encoding phospholipase A2 (PLA2) (Kennedy et al., 1995). Indeed, PLA2 was demonstrated to be expressed in the skin of both mouse and human, and was implicated
in both maintaining the barrier function of epidermis via its involvement in fatty acid metabolism, as well as in the inflammatory response due to participation in synthesis of arachidonic acid (Ilic et al. 2014). In fact, it was reported that overexpression of human PLA2 in mice resulted in a phenotype that corresponds with ACD (Sato et al., 2009). Therefore, differences with respect to PLA2 expression might be another reason for differential responses of BALB/c and C57BL/6 strain mice to oxazolone. Moreover, PLA2 potentially might be involved in a signalling cascade triggered by oxazolone that leads to LC mobilisation. Lastly, as oxazolone-induced LC migration is dependent on TNF-α there is a possibility that there are differences between the two mice strains with respect to their ability to produce and/or respond to TNF-α in the skin. However, data published by Friedrich et al. (2014) showed that in the steady state the amount of TNF-α present in the skin of BALB/c and C57BL/6 strain mice was comparable, ~12pg/mg of protein. Significant increase in TNF-α level was observed in C57BL/6 but not in BALB/c strain mice following exposure to UV light. However, detected levels of TNF-α were below the limit of accurate detection of the ELISA kit that was utilized in the study by Friedrich et al. (2014).

To summarise, it was demonstrated, in the current investigations that under conditions where high levels of IL-1β were present LC migration could take place in the absence of TNF-α signalling. Interestingly, Haley et al. (2007) demonstrated that mice that lack MyD88 (an adaptor molecule for IL-1R1) still display the same level of LC migration in the steady state, as measured by LC accumulation in the draining LN; as well as following exposure to TRITC. Tetramethylrhodamine has been used, similarly to FITC, as a stimulator of LC migration (Haley et al., 2012). It could be argued that in the study by Haley et al. (2012), the trigger that stimulated LC migration was different to that utilized in the current investigations, therefore partially accounting for differences. Moreover, in the current study it was shown that presence of IL-1β was required, but it is not known if this cytokine acts directly upon LC or if it induces the production of another mediator, e.g. CXCL1. While the study presented herein was not in complete agreement with evidence proposed by Haley et al. (2010), it was consistent with the report by Antonopoulos et al. (2001) in which a critical role for caspase 1 was demonstrated, both in the events of LC migration, and CHS measured by challenge-induced changes in ear swelling (Antonopoulos et al., 2001). Caspase 1 is a cysteine
protease that participates in the conversion pro-IL-1β into its active form (Thornberry et al., 1992).

One of the difficulties in interpreting the results from the current investigations in the context of previously published data is that it is relatively rare for previous publications to have employed both of these allergens. One such study that utilized both oxazolone and DNCB was conducted by Traidl et al. (1999), wherein the dependence of CHS reaction on IL-4 signalling triggered by both allergens was tested. They found that the CHS reaction, as measured by challenge-induced changes in ear swelling, triggered by DNCB was attenuated in IL-4 KO mice. Interestingly, when ear swelling was triggered by oxazolone, there was no difference in the hypersensitivity manifestation between WT and KO animals (Traidl et al., 1999), suggesting a differential reliance of these two allergens on IL-4 signalling. In previous work, in two independent studies, another difference between the two allergens was identified. The first study demonstrated that the extent of the CHS reaction to oxazolone was similar in WT and IL-1β deficient mice (Zheng et al., 1995). In the second study the same experiment was conducted by Shornick et al. (1996), but on this occasion TNCB, a chemical different from DNCB by one nitro group, was utilized. It was found that when low doses of TNCB (0.03%, 0.1%, 0.3%) were selected for sensitization, there were visible differences between WT and IL-1β KO animals; namely, the CHS reaction was attenuated in the absence of IL-1β. However, when the concentration of the sensitizing dose was increased to 1% or 3% the divergence that was observed between IL-1β KO and WT mice was no longer present (Shornick et al., 1996). That is, low dose of sensitizer was dependent upon IL-1β, whereas, high dose was independent. These results from IL-1β deficient mice are of particular interest, as evidence from the current study suggests that one of the differences between oxazolone and DNCB might be their differential ability to induce IL-1β production in the skin. While oxazolone induced LC mobilisation was dependent on IL-1β, data presented by Zheng et al. (1995) proposed that IL-1β was not essential for the development of CHS. However, it has to be noted that concentrations used in that study were very high (4% and 1% of oxazolone for sensitization and elicitation, respectively). Therefore, it is possible that whilst in the experiments reported herein inhibition of oxazolone-induced LC migration was observed following anti-IL-1β injection, if the higher concentration of oxazolone had been used, the inhibitory effect of anti-IL-1β antibody might have been overcome. Conversely, the study by Shornick et
al. (1996) reported a necessity for IL-1β signalling to achieve an optimum CHS reaction in response to TNCB. However, the need for IL-1β was overcome by the use of a higher concentration of allergen, thereby allowing IL-1β KO mice to mount the same level of CHS measured by footpad swelling. The approach taken by Shornick et al. (1996) was similar to that used in the investigations described herein, whereby it was demonstrated that the requirement for TNF-α signalling was no longer apparent when a higher concentration of IL-1β was present. The data presented in this chapter, as well as that reported by Shornick et al. (1996), suggest that there might be compensatory mechanisms in place within the immune system that ascertain, that if the danger signal is vigorous enough, there will be an appropriate response, even in the absence of one of the key cytokines: IL-1β or TNF-α. These results also further stress the need to critically assess the data derived from chemical allergens with respect to both the identity and the dose of chemical allergen. It is clear that despite many similarities, major differences exist in the quality of induced immune response.

It is clear from this study and from the literature that IL-1β and TNF-α play key, albeit still not fully elucidated, roles in the mobilisation of LC and in CHS. An attempt to investigate the exact roles of TNF-α and IL-1 in the events of CHS was made by (Nakae et al., 2003). They found that IL-1 and TNF-α had distinct roles in the sensitization and elicitation phases of CHS. It was found that there was a comparable suppression of CHS responses to TNCB and oxazolone in both C57BL/6 and BALB/c strain mice that lacked either IL-1α/β or TNF-α in comparison to WT counterparts. However, when triple KO mice were utilized the suppression was even greater but not complete. Similar observations were made when the presence of FITC positive cells was assessed in the draining LN, representing presumably antigen-bearing DC. Thus, IL-1α/β or TNF-α KO mice displayed decreased levels of CD11c⁺FITC⁺ cells (approximately 26%) in comparison to WT (44%), but when triple KO mice were utilized migration was almost completely absent and antigen bearing DC levels were around 3%; therefore suggesting the existence of compensatory mechanisms in the case of absence of one of the cytokines. Additionally, there were differences between cytokines in relation to their role in the T cell activation. When LN cells from sensitized animals were cultured with haptenated spleen cells, a lower level of activation was observed in cells derived from IL-1α/β KO mice, whereas cells isolated from TNF-α KO mice displayed levels of activation comparable to those observed in WT animals (measured by radiolabelled
thymidine incorporation). These results suggested that TNF-α was not required for the effective T cell priming in the sensitization phase of CHS. However, when sensitized LN T cells from WT mice were transferred into KO animals it was found that all three types of KO mice displayed decreased ear swelling on challenge, suggesting that all three cytokines were essential for the elicitation phase of CHS. Administration of exogenous recombinant TNF-α to IL-1 KO mice rescued CHS, whilst delivery of recombinant IL-1α/β to TNF-α KO mice did not modify the suppressed phenotype (Nakae et al., 2003).

The role of CXCL10 chemokine in the elicitation phase of CHS was also investigated (Nakae et al., 2003). It was found that IL-1 induced TNF-α, which in turn caused the production of CXCL10. Thus, IL-1α/β induced CXCL10, and this induction was reduced by anti-TNF-α antibody. Moreover, diminished ear swelling in TNF-α KO mice was rescued by delivery of exogenous CXCL10 at the time of elicitation, suggesting that one of the crucial roles of TNF-α was to induce the production of CXCL10 (Nakae et al., 2003). As CXCL10 seemed to be a ‘proxy’ for TNF-α, it was decided to examine the induction of Cxcl10 mRNA in oxazolone and DNCB treated mice. It was found, however, that there were no differences in the level of Cxcl10 induction between these two allergens (data not shown), suggesting once again that the ability of oxazolone and DNCB to stimulate TNF-α production was similar. It was shown previously that stimulation of human oral keratinocyte cell line RT7 with TLR3 and TLR5 ligands resulted in up-regulation of CXCL10 mRNA and protein after treatment for 24h and 48h, respectively (Fukui et al., 2013). These results suggested that CXCL10 might be regulated at the level of transcription, implying that the lack of divergence between allergen-induced Cxcl10 mRNA expression observed herein correlated with the lack of differences in the protein levels of CXCL10.

Differences between oxazolone and DNCB were also investigated by Ku et al. (2009). They found that there were differences between oxazolone and DNCB-induced CXCL9 and CXCL10 gene expression in mouse skin following a 3 day exposure protocol, with much higher levels stimulated by exposure to oxazolone. On the other hand, no differences were reported in levels of IL-1β transcripts (Ku et al., 2009). Expression of CXCL9 and CXCL10 was also analyzed in the current investigations; however, there was no difference in the level of induction between two allergens (data not shown). Similarly, results regarding IL-1β expression reported by Ku et al. (2009) were not in
agreement with those demonstrated in the current study. These discrepancies were likely to be caused by a different mouse strains utilized by Ku et al. (2009) and the current study, which were CBA/N and BALB/c strain mice, respectively. In addition, the exposure protocol utilized by Ku et al. (2009) and in the current studies was different. In the studies described herein mice were exposed to allergen once for various times, whereas in the study by Ku et al. (2009) animals were exposed to allergens 3 times on day 0, 1 and 2 and samples were collected 24h after last exposure. Additionally, the dose of oxazolone utilized in the experiments described here was lower (0.5%) than that used by Ku et al. (2009) (1%), and as mentioned in the current studies and in the investigations by Shornick et al. (1996), the dose of allergen exposure can dramatically change the quality of immune response to allergens.

Langerhans’ cell migration in response to contact allergens was believed previously to depend upon receipt of two signals by LC, TNF-α and IL-1β. Contact allergens were considered to be one group of chemicals that have the ability to cause ACD but do not generally display respiratory sensitising potential. Contact allergens are distinct from a smaller group of chemicals, respiratory allergens that display a different clinical profile and cause sensitization of the respiratory tract. These two groups of allergens (contact and respiratory) exhibit differences with regards to the quality of immune response that they trigger; namely Th1/Tc1 and Th2, respectively (Dearman and Kimber, 1992). The study presented herein has exposed that there is a divergence within, the previously considered to be uniform, contact allergen group that is exemplified by differential effects on LC migration. These differences are probably due to a divergent pattern of induction of gene expression elicited by chemicals that in turn might be a consequence of the utilization of differential signalling pathways. The exact mode of action of oxazolone and DNCB is still not known. One of the only allergens for which the exact mechanism has been fully established is nickel. It was recently discovered, that in the skin, it directly binds to TLR4 receptor within histidine residues 456 and 458, therefore, inducing signalling cascade that results in sensitization (Schmidt et al., 2010).

The pattern of gene expression of some other chemicals that were employed in the current study was investigated to ascertain if any conclusions could be drawn between the pattern of cutaneous gene expression and ability to result in LC mobilisation. All of the chemicals used, with the exception of DNTB, were demonstrated previously to trigger LC migration. However, a recent study challenged the notion that DNTB does
not trigger LC migration. Gomez de Aguero et al., (2012) measured the number of epidermal LC in the draining LN following exposure to DNTB. Mice used in their study expressed enhanced green fluorescent protein (EGFP) under control of the langerin promoter. Furthermore, DC were visualised by exposure of the skin to TRITC. Lymph node cells were further stained with anti-CD103 and anti-CD207 antibodies to allow for LC identification that would be EGFP, TRITC and CD207 positive and CD103 negative. By employing this approach de Aguero et al. (2012) demonstrated the presence of low number of LC (1% of TRITC\(^+\) cells) in the draining LN 24h following exposure to DNTB that increased 72h after (3% of TRITC\(^+\) cells), which corresponded with the peak LC migration to the draining LN. Moreover, they reported that LC were responsible for DNTB-induced protection against CHS after treatment with DNFB (Gomez de Agüero et al., 2012). The main differences observed between chemicals were with regards to \(\text{Il-1}\beta\) and \(\text{Cxcl1}\) expression. Levels of \(\text{Il-1}\beta\) and \(\text{Cxcl1}\) peaked rapidly after exposure to SLS, which corresponds with the pattern of expression of these genes observed following exposure to oxazolone and DNCB; with somewhat later peaks recorded for DNTB and TMA.

Cytokine expression data in combination with the knowledge about LC migration allows for speculation regarding a connection between the two processes. Indeed, early \(\text{Il-1}\beta\) and \(\text{Cxcl1}\) induction correlates with LC migration observed 4h after exposure to SLS. However, it is not known if LC mobilisation could be observed sooner. TMA triggered LC migration occurs 17h after exposure (Cumberbatch et al., 2005) and this delayed migration correlated with delayed cytokine expression data. Intriguingly, there was a clear division between the tempo of \(\text{Cxcl1}\) and \(\text{Il-1}\beta\) signals for TMA which peaked after 16h and 24h, respectively. Putting together the information regarding LC migration and the kinetics of induction of cytokine production, it could be speculated that LC migration following exposure to DNTB could potentially be expected approximately 8h after initiation of treatment due to the induction of \(\text{Cxcl1}\) and \(\text{Il-1}\beta\). The role of IL-1\(\beta\) was demonstrated previously to be crucial in mounting a complete CHS in response to TNCB (Shornick et al., 2001; Shornick et al., 1996), most likely due to the more recently discovered role of inflammasome as a master switch between establishing tolerance and sensitization (Watanabe et al., 2008). The results obtained in the current investigations allow speculation regarding the role of CXCL1 in the sensitization process. Indeed, it has been demonstrated herein that CXCL1 was
abundantly expressed following exposure to chemicals that induce LC migration and DNTB. Previous reports suggested a CXCL1-dependent role of neutrophils in the elicitation of CHS in response to DNFB (Dilulio et al., 1999). Neutrophils were also required for establishment of ACD in response to OVA via their production of eikosanoid, LTB4 (Oyoshi et al., 2012). Moreover, it was demonstrated that LTB4 production was dependent on CXCR2 dependent cytokines, namely CXCL1 and CXCL5 (Grespan et al., 2008). Overall, evidence from published studies, as well as from the current investigations, suggests that CXCL1 plays a crucial role in response to antigen. It would be interesting to establish what effect CXCL1 has on LC migration, as it was demonstrated in the previous chapter that LF inhibited IL-1α/β induced CXCL1 mRNA expression.

The conclusion from the experiments presented in this chapter is that there are differences between allergens that stem from their differential ability to induce cytokine and chemokine production in the skin, that in turn might result in divergent quality of immune response, i.e. either differential T cell polarisation or ability of DC to migrate in the absence of previously considered essential signal. The idea that there would be a divergence among allergens should not be altogether surprising. There are over 4000 chemical contact allergens identified that belong to many classes of chemicals (Krob et al., 2004). Within contact allergens there is a well established hierarchy in terms of their potency as assessed by guinea pig maximization test and local lymph node assay (Kimber et al., 1994). The response to chemicals that is manifested by hypersensitivity reactions originates in the ability of immune system to recognize and respond to danger. It is therefore a difficult task to identify which components of immune system are ‘tricked’ by sensitizing chemicals into sensing danger. So far several receptors, previously considered a cornerstone of anti-bacterial protection, have been implicated in CHS, including TLR4 and TLR2 (Martin et al., 2008; Schmidt et al., 2010). The induction of adaptive immunity as a result of exposure to chemical contact allergens is a complex process, which despite intensive research, is not fully understood. In this chapter, it has been established that one of the first events of CHS, LC mobilisation, can occur in the absence of TNF-α signalling, when sufficiently high concentrations of IL-1β is present. These investigations highlight the fact that whilst a lot of information might be transferable in terms of knowledge about haptens, a caution should be employed when analysing results from studies utilising different chemical allergens.
7 General discussion

7.1 Background

Skin is the largest organ of the body. It is the physical barrier separating the organism from the outside world. The surface of the skin is home to a diverse population of symbiotic microbiota that can aid the organism by inhibiting growth of pathogenic organisms and processing the protein and fatty acids of the skin (Cogen et al., 2008; Grice et al., 2008). Another line of defence of the skin are the versatile populations of innate and adaptive immune cells which can respond to external insults and danger signals (Nestle et al., 2009). Skin pathologies are common conditions, with 15% of population visiting their General Practitioner with skin complaints each year (Draper, 2009). Skin diseases can be greatly debilitating, despite being mostly non-life threatening ailments (Katugampola and Finlay, 2007). One of the most common skin pathologies is contact dermatitis. In the United States of America it accounts for 95% of all reported occupational diseases (Taylor and Amado, 2014). Non-specific contact dermatitis occurs in response to irritants, while skin exposure to chemical allergens may result in ACD, which has been the subject of this PhD thesis. In addition to being a prevalent skin disease ACD is also an attractive model for the investigation of cutaneous immunity.

Despite recent reports questioning the role of LC during the acquisition of skin sensitization, LC are a crucial component of the skin immune and surveillance system. It has been demonstrated previously in the mouse that LF inhibited oxazolone and IL-1β induced LC migration and DC accumulation in the LN (Almond et al., 2013; Cumberbatch et al., 2000b). These observations regarding LC mobilization were confirmed in humans utilizing the contact allergen DPC. Moreover, it was established that LF alleviated irritant reactions associated with DPC treatment by decreasing the extent of erythema (Cumberbatch et al., 2003; Griffiths et al., 2001). Lactoferrin is present in breast milk and indeed, human breast milk has been shown to have beneficial effects on two cases of ulceration in infantile haemangioma (Laws et al., 2012) and was reported to be as effective as steroid (1% hydrocortisone) ointment in treating diaper rash in infants (Farahani et al., 2013). Human milk is the source of immunoglobulins, cytokines, proteins and immune cells (macrophages [65%], neutrophils [25%]) (Chirico
et al., 2008), and any of these components could have contributed to the beneficial outcomes observed following treatment with milk. However, it cannot be excluded that LF contributed to the reported positive effects of human milk on infant skin conditions. Preliminary investigations of mechanisms governing the processes of LC and DC migration in mice (Cumberbatch et al., 2000b), and LC migration in humans (Griffiths et al., 2001), indicated that the most likely mode of action of LF was via inhibition of de novo production of TNF-α production. The probable source of TNF-α was thought to be keratinocytes (Cumberbatch et al., 2000a), the most abundant cell type of the epidermis (Gutowska-Owsiak and Ogg, 2012).

Therefore, an important question to be addressed in this body of work was what are the molecular mechanisms through which LF exerts immunomodulatory activity in the skin? While working towards this – and as part of those investigations – it became clear that LC migration induced by different contact allergens might display variable requirements for cytokine TNF-α signalling. As a consequence, this phenomenon was investigated and comparisons drawn between migration induced by oxazolone and DNCB.

7.2 Main findings

7.2.1 Lactoferrin has a dual immunomodulatory potential

Lactoferrin is a fascinating protein that has a therapeutic potential. As described in the introduction, there are many studies reporting stimulatory, as well as inhibitory, actions of LF within the immune system. Indeed, it has been described as the double edged sword of the immune system (Ando et al., 2010). Lactoferrin, in addition to its presence in the breast milk, is also a component of secondary granules in the majority of circulating neutrophils (Baggiolini et al., 1970; Esaguy et al., 1989). As such, it is implicated in the immune response. Moreover, it can be involved at the very early stages of infection, as neutrophils are the first cells to be recruited to the source of inflammation (Kolaczkowska and Kubes, 2013). In that respect LF was described as an alarmin, a molecule that has the ability to mobilize and activate DC (Yang et al., 2009). The concept of the organism responding to self damage was suggested 20 years ago by Matzinger, who shifted the paradigm of the immune system from the protector of self
from non-self to the defender of self from danger, regardless of its source (Matzinger, 1994).

In the study described herein and in other publications, LF has been shown to interact with surface nucleolin. Among its other roles, surface nucleolin has been described as the receptor for apoptotic cells (Hirano et al., 2005) and oxidized red blood cells (Miki et al., 2007) via recognition of oxidized carbohydrate chains (Miki et al., 2014; Miki et al., 2012). As such, nucleolin is a scavenger receptor implicated in eliminating danger signals. It was observed in the investigations described herein that following exposure to LF 72h THP-1 macrophages released TNF-α and IL-8 in a nucleolin-dependent fashion. It has not been reported whether recognition of other substrates by surface nucleolin is associated with inflammatory cytokine release. Rather, it was found that phagocytosis of apoptotic cells had an anti-inflammatory potential via the release of TGF-β and PGE₂ by macrophages (Fadok et al., 1998). Further, apoptotic cells were demonstrated to increase IL-10 and decrease TNF-α secretion by PBMC following 16h incubation with LPS. Anti-CD36 antibody (CD36 is a phagocytic receptor) was found to mimic the suppressive effect of apoptotic cells, whereas blocking of phagocytosis decreased the inhibitory effects of apoptotic cells (Voll et al., 1997). Lactoferrin was recognized by the same receptor as apoptotic cells; however, as described herein, its effects were found to be immunostimulatory when incubated with 72h THP-1 macrophages. Conversely, administration of LF in vivo inhibited oxazolone-triggered LC mobilization potentially via decrease of de novo TNF-α production. It is possible that the fact that LF is found in the secondary neutrophil granules influences the way it is recognized by the immune system and the way that cells respond to it. Indeed, apoptotic cells release variety of ‘find me’ factors, including ATP and uridine-5’-triphosphate (UTP) (Elliott et al., 2009), CX3CL1 (Truman et al., 2008) and LF (Bournazou et al., 2009), that influence the movement of various types of cells (Willems et al., 2014). Hence, in the in vivo model of oxazolone induced migration, the presence of LF could be perceived as though neutrophils undergoing apoptosis are present and thus the anti-inflammatory pathway is activated. In contrast, in the in vitro THP-1 model the concentration of LF could have been too high to mimic apoptotic cells and was recognized as a danger signal, to which macrophages responded with the release of inflammatory mediators. Therefore, it could be speculated that function of LF depends on both the context and concentration. Indeed, when high, pathologically
relevant, concentrations of LF (10-100μg/ml) are present, DC activation was demonstrated (de la Rosa et al., 2008; Spadaro et al., 2008). Therefore, high LF concentrations, which are symptomatic of neutrophil degranulation, aid the adaptive immune response. At higher concentrations another ‘find me’ signal, ATP, is considered to be pro-inflammatory (Kono and Rock, 2008), therefore, it is possible that LF could work in a similar manner. High concentrations of LF could indicate to DC that the situation is no longer suitable for phagocyte recruitment and instead initiation of inflammation is required.

Expression of surface nucleolin by LC or keratinocytes has not been reported in the literature to date. Consequently, it is difficult to speculate whether the effects of LF in vivo are mediated via this receptor. A receptor for LF has been identified on activated human PBMC including γδ T cells (Mincheva-Nilsson et al., 1997), which is consistent with the data reported herein, that LF inhibited IL-1β induced IL-17F expression in the skin, the latter cytokine probably being derived from γδ T cells (Gray et al., 2011; PhD thesis Hayes, 2012; Nielsen et al., 2014).

7.2.2 Keratinocytes might not be the source of TNF-α during the process of LC migration

Keratinocytes are the main cell population in the epidermis and have been demonstrated to be a rich source of cytokines and chemokines (Gröne, 2002). It has been speculated that during skin sensitization IL-1β and TNF-α cytokine signals that are required for LC migration derive from LC and keratinocytes, respectively (Enk et al., 1993; Enk and Katz, 1992a). Therefore, to determine whether LF could regulate TNF-α production by keratinocytes, in vitro keratinocytes from three different sources were utilized to measure the impact of LF on IL-1α and IL-1β induced gene expression. Keratinocytes did not readily secrete TNF-α (PAM and HaCaT cells) or secreted only low amounts of the cytokine (primary keratinocytes), suggesting that they might not be the primary source of this cytokine in vivo. Tumour necrosis factor α was detected at higher levels in skin explants following in vivo exposure to oxazolone, however, the cytokine was most likely produced due to the trauma of explant preparation rather than in response to allergen. Indeed, treatment with oxazolone did not result in TNF-α release above the levels found in the naive ear explant samples. The expression of TNF-α mRNA by
keratinocytes was induced by IL-1α and IL-1β treatment, but was not modulated by LF. The data reported here suggested one of two things. One possibility is that the amount of TNF-α released by keratinocytes was very low and below the level of detection. Alternatively, it is plausible that following allergen exposure TNF-α is not produced primarily by keratinocytes, but rather by cutaneous mast cells and/or γδ T cells.

Published data has demonstrated mast cells to be the major source of reactive TNF-α in the skin and this cytokine was released following skin exposure to DNCB in human subjects (Walsh et al., 1991). Lactoferrin also has been shown to be taken up by skin mast cells (He et al., 2003). Moreover, it was reported that LF inhibited the activity of mast cell-derived cathepsin G and inhibited IgE stimulated histamine release by 50% (at a range of 6-600μg) (He et al., 2003). Another piece of evidence pointing to mast cells as the main source of TNF-α in the skin came from the study by Heib et al. (2007), whereby it was demonstrated that in mast cell-deficient mice there was no expression of TNF-α mRNA in the skin, and that cutaneous IL-1β mRNA levels also were decreased following treatment of these animals with imiquimod (TLR7 ligand). Conversely, in the WT counterparts TNF-α and IL-1β expression was induced following exposure to imiquimod (Heib et al., 2007). Moreover, mast cell-derived TNF-α was shown to be required for rapid (24h) DC migration to the LN, following topical exposure of mice to FITC (Suto et al., 2006). Additionally, it has been shown in DT conditional mast cell KO mice that the DNFB induced CHS reaction, as measured by the increase in the ear swelling, was decreased in the absence of mast cells. Also, mast cell-bound TNF-α was found to be necessary for bone marrow derived DC maturation (Otsuka et al., 2011). Additionally, mast cells isolated from human skin were found to store pre-formed TNF-α and IL-8 that could be detected in the culture supernatants within 30 minutes of treatment with IgE, neutropeptide substance P or compound 48/80 (Gibbs et al., 2001).

Due to these several lines of evidence suggesting that mast cells were a major source of TNF-α in the skin, an effort was made to use mast cells in the current investigations of the immunomodulatory potential of LF. The mastocytoma cell line P815 was acquired and attempts were made to stimulate TNF-α release. There were published reports which suggested that P815 cells line produced TNF-α in vitro following exposure to PMA but not LPS, calcium ionophore or IgE (Ohno et al., 1990). Indeed, in the current investigations PMA induced TNF-α production by P815 cells but the level of induction was deemed to be too low and too close to the accurate limit of ELISA detection.
(~50pg/ml) to be further modulated with LF (data not shown). In agreement with results by Ohno et al. (1990), no TNF-α production was observed following IgE treatment. Furthermore, there was no induction of TNF-α expression after treatment with IL-1α or IL-1β.

It could be speculated that primary mast cells or bone marrow derived mast cells would have been more efficient in their production of TNF-α, and therefore could have been a better system in which potential of LF was investigated. As previously mentioned, TNF-α is able to remain in a membrane bound form. Indeed, it was shown that direct contact between mast cells and DC was required for DC maturation (Otsuka et al., 2011). It is possible that TNF-α produced by P815 cell line was bound to the cell membrane and not released into the culture medium, and could have been detected using whole cell lysates in the ELISA analysis. Nevertheless, such experiments were not conducted.

However, the suggestion that the TNF-α is pivotal in the events of LC migration might come from mast cell stores is contradicted by time course experiments using exogenous cytokine. Injection of recombinant TNF-α resulted in IL-1β dependent LC migration within 30 minutes, suggesting that IL-1β was already present in the cell and did not have to be synthesized. Whereas injection of recombinant IL-1β resulted in TNF-α-dependent LC migration after 2h, suggesting that TNF-α may be produced de novo (Cumberbatch et al., 1997a). Degranulation of mast cells, as observed by decreased chymase staining in the skin, was one of the first changes observed after exposure to allergen. In the human skin it was reported to take place as soon 1h after DNCB application (Waldorf et al., 1991). However, it is possible that despite the rapid release of mast cell contents, the 2h delay with respect to the initiation of LC migration observed by Cumberbatch et al. (1997) was a result of the diffusion of TNF-α from the dermis to the epidermis. Alternatively, release of TNF-α by mast cells in response to exogenous IL-1β could be secondary to stimulation with a mediator induced by IL-1β and not by IL-1β itself. Equally, there is some evidence which suggested that keratinocytes were the source of TNF-α. In preliminary experiments, although keratinocytes did not secrete TNF-α, high levels of TNF-α mRNA induction were detected following stimulation in vitro with cytokines relevant in the events of LC migration.
Murine epidermal γδ T cells have been shown to express TNF-α following activation with a mitogen, concanavalin A (Matsue et al., 1993b). Similarly, human dermal γδ T cells secreted large quantities of TNF-α following incubation with PMA and ionomycin (Ebert, 2006). In the epidermis DETC cells are in close proximity to LC and through production of TNF-α could efficiently activate adjacent LC. As mentioned previously, TNF-α is found in both soluble and membrane bound forms. It has been found that membrane bound form of TNF-α binds TNFRII more strongly than does the soluble cytokine, and it has been suggested that full activation of TNFRII is achieved only via interaction with membrane bound TNF-α (Faustman and Davis, 2010; Grell et al., 1995). Langerhans’ cells were shown to express only TNFRII (Larregina et al., 1996). Therefore, DETC would seem like a plausible source of TNF-α in the skin with regard to the activation of LC.

7.2.3 Divergence in the contact allergen family revealed a crucial role of IL-1β

It has been shown before that chemical allergens and irritants induce various signals that impact on LC mobilization. One of the types of materials is an irritant, SLS, a surfactant commonly used in cleaning and hygiene products. Despite its inability to trigger a specific immune response in the draining LN following skin exposure, treatment with SLS results in LC migration. It was found using neutralizing anti-cytokine antibody that in common with oxazolone, SLS-induced LC mobilization was dependent upon a signal from TNF-α (Cumberbatch et al., 2002a). However, the requirement for the second signal was different. Irritants were found to require a second stimulus from IL-1α (Cumberbatch et al., 2002a), and not IL-1β, which was shown previously to be required for allergen induced LC migration (Cumberbatch et al., 1997a). Cumberbatch and colleagues have shown that SLS induced LC migration and DC accumulation in the LN was not inhibited with anti-IL-1β antibody, whilst administration of anti-IL-1α antibody effectively inhibited SLS-induced LC mobilization and DC accumulation. Conversely, oxazolone induced changes in LC and DC populations were inhibited by anti-IL-1β, but not anti-IL-1α, antibody (Cumberbatch et al., 2002a).

Another example of differential cytokine production in response to another chemical that stimulates LC migration, is that observed following treatment with the respiratory allergen, TMA, which has been shown to trigger delayed LC migration in comparison
with DNCB, i.e. after 17h compared with 4h, respectively (Cumberbatch et al., 2005). Delayed LC mobilization was shown to depend on the rapid up-regulation of cutaneous IL-10 production. That in turn inhibited IL-1β production, such that levels were considerably lower than those observed after treatment with DNCB. Delivery of anti-IL-10 antibody prior to exposure to TMA resulted in LC migration at an earlier time point (4h) and increased IL-1β production, suggesting that the presence of IL-1β is crucial for TMA induced LC migration (Cumberbatch et al., 2005).

Those examples highlight the diversity between cytokine signals triggered by chemicals that result in LC mobilization. In the investigations reported here, divergent reliance of LC mobilization on TNF-α signalling of oxazolone and DNCB was demonstrated. Those observations lead to identification of differential levels of IL-1β production triggered by oxazolone and DNCB, which in turn allowed the conclusion that in the presence of high concentrations of IL-1β, TNF-α signalling can become redundant for LC migration.

This fascinating discovery revealed diversity among contact allergens (with respect to LC mobilization) and provided insights of the mechanism through which DNCB induces LC migration. Indeed, it was shown that exposure of mouse skin to DNCB resulted in a unique LC morphology that was not observed after oxazolone treatment. This fact alone implied that there were differences between oxazolone and DNCB with respect to their impact on LC. Current investigations highlighted the need for a careful consideration of contact allergens, their properties, and application concentrations in experimental studies of skin sensitization. Allergic contact dermatitis induced by chemical allergens is a widely used experimental system of elucidating adaptive immune responses. It is known that the type of chemical utilized can determine the quality of adaptive immune response, i.e. Th1 and Th2 cell activation observed for contact and respiratory allergens, respectively. A major obstacle that has been highlighted throughout this thesis is the fact that aspects of the mechanisms of action of the contact allergens utilized in the studies described herein are still not elucidated, i.e. it is not known which receptors are utilized by contact allergens, as well as the lack of certainty regarding the cellular sources of cytokines that are involved in the mobilization of LC and the acquisition of skin sensitization. However, experiments presented within these investigations have given insights into which cytokines and chemokines might be particularly important.
The data from current studies, together with published reports, point to IL-1β as a key cytokine during skin sensitization. Indeed, the inflammasome has been described as a master switch of sensitization on which the chemical sensitizing ability lies (Watanabe et al., 2008).

There are two possible reasons for the divergence of cytokine requirements between oxazolone and DNCB. Firstly, as it has been mentioned before with regards to different cutaneous DC subsets, there can be a certain level of overlap of function between various DC populations. Similarly, it is possible that in the presence of high levels of IL-1β, or a potent stimulus which triggers high levels of IL-1β production, absence of TNF-α is not an obstacle in the mobilization of LC. Secondly, chemical contact allergens are thought to be misrecognized by the immune system as infectious organisms. There is evidence that pathogens may exhibit different patterns of cytokine requirements for LC migration. It has been shown that infection of the skin with a West Nile virus resulted in migration of LC that was independent of TNF-α signalling but still reliant on IL-1β (Byrne et al., 2001). Conversely, LC cells from mice unresponsive to IL-1β (MyD88 KO) exhibited unaltered mobilization in response to *C. albicans* infection (Haley et al., 2012). Migration of LC exposed to staphylococcal enterotoxin-A was found to depend on signals from both IL-1α and TNF-α (Shankar et al., 1996). It is possible that oxazolone and DNCB with their mechanisms of action resemble different pathogens that exert divergent effects on cutaneous cytokine release and subsequently differently affect migration of LC.

### 7.3 Future Aims

The aim of this PhD thesis was to investigate the molecular mechanisms of skin sensitization and its immunomodulation by LF. Some progress has been made towards that goal; however, there are still many questions that remain unanswered. One of the main uncertainties highlighted by these investigations is the cellular source – or sources – of TNF-α in the skin following exposure to allergen. It would be particularly important to use IHC techniques to detect TNF-α protein. That would allow for preserving the integrity of the tissue with respect to the relationship between different cell types and the identification of the exact cellular source or sources of this cytokine. Furthermore, IHC could be employed to investigate the fate of LF in the skin.
Additionally, in the follow up studies it would be of interest to identify whether LF impacts on the CHS reaction as measured by changes in challenge-induced ear thickness and cutaneous cellular infiltrate, and thus assessing its potential as a treatment for ACD. Studies in human subjects have shown that LF has the ability to decrease the erythema triggered by initial exposure to DPC (Griffiths et al., 2001). Moreover, due to its impact on IL-17F expression, LF might potentially therapeutic potential for psoriasis, as IL-17 is considered to be an important cytokine in the pathogenesis of that disease (Golden et al., 2013). Internet searches revealed that there are treatments available directed at psoriasis patients, based on claims of LF activity, e.g. equilac, available in cream and capsule forms, which contains LF from mare’s milk (Ecopharma, 2014). Indeed, a recent publication reported improvement in psoriatic plaques following topical treatment with bovine LF (Saraceno et al., 2014). However, no mechanistic information is available to provide clues as to how exactly LF improved psoriatic plaques. Lactoferrin has been shown to have a beneficial effect on the management of acne, whereby oral delivery of bovine LF resulted in a decreased number of inflammatory acne lesions in an open label (Mueller et al., 2011) and double blind (Kim et al., 2010) trial. Inflammation of the skin, despite its low mortality rate, has a huge impact on the wellbeing status of patients and can be gravely debilitating. The ability of LF to mitigate cutaneous inflammation would be greatly beneficial.

The current investigations have highlighted further the differences between contact allergens and revealed a novel TNF-α independent mechanism of LC mobilization. It would have been of interest to conduct LC mobilization experiments utilising a wider range of contact allergens, possibly employing IL-1β KO animals, to definitively assess the role of this cytokine in the process of LC migration in response to allergens. Similarly, the role of TNF-α, and not only of TNFRII, could be assessed using TNF-α KO mice. Importantly, TNFRII KO mice not only lack TNFRII expression by LC but also by other DC subsets and most likely γδ T cells. TNFRI is constitutively expressed by nearly all cells, whereas TNFRII is an inducible receptor expressed by the cells of myeloid lineage, peripheral T cells and macrophages. However, the most definitive answers could be gained by deploying mice lacking both TNF-α and IL-1β to determine the central roles of these two cytokines. It is plausible that in the situation when either of these two cytokines was absent, compensatory mechanisms would be able to restore
LC mobilization. Additionally, it should be determined how LNC proliferation and cytokine production is affected in the absence of TNF-α and/or IL-1β cytokine signals.

7.4 Conclusions

![Figure 7.1 Mechanism of the immunomodulatory action of LF.](image)

Following exposure of the skin to allergen, skin resident cells release a variety of factors, including, but not restricted to, TNF-α, IL-1α, IL-1β, IL-18, CXCL1, IL-17 and ATP to create an inflammatory environment in the skin that facilitates LC migration. Lactoferrin has been found to inhibit LC migration via inhibition of TNF-α production. The current investigations have shown that LF inhibits expression of CXCL1 and IL-17F stimulated by IL-1, but not TNF-α. It is possible that the effect of LF on TNF-α production is secondary to its influence on CXCL1 and IL-17F.
The effect of the concentration of antigen on the dependency of various cell types and cytokines to induce immune response has been considered previously. It has been said by Paracelsus in 17th century that the dose makes the poison. It is important not only in the consideration of toxicology, but can be extrapolated to immunology. That simple notion should be regarded especially now when there are so many known variables in the system of cutaneous immune network. The advent of ever more accurate techniques of detection of cells, as well as new methods for their in vivo visualization, e.g. Kaede protein (Tomura and Kabashima, 2013) or intravital multiphoton imaging (Kabashima and Egawa, 2014) makes immunology an exciting and ever expanding field. This PhD thesis has established that immunomodulatory effects of LF in the skin might be mediated by the inhibition of CXCL1 and IL-17F signalling. Moreover, it was found that in certain circumstances LC migration is possible in the absence of TNF-α signalling, which is potentially mediated by the presence of high levels of IL-1β. This knowledge revealed a novel mechanism of LC mobilization and further highlighted the importance of IL-1β cytokine in the cutaneous immunity.


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