EPIDERMAL CYTOKINES: HOMEOSTASIS AND THE
REGULATION OF LANGERHANS CELL MIGRATION AND
CUTANEOUS IMMUNITY

By

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ABSTRACT

Langerhans cells (LC) are bone marrow derived professional antigen presenting cells that migrate rapidly from epidermis to lymph node following epicutaneous application of allergen. A number of cytokines, most notably interleukin (IL)-1β and tumour necrosis factor-α (TNF-α) have been shown to be important in initiation of LC migration.

IL-1β requires cleavage by the cysteine protease caspase-1 to release its biologically active form. In view of the role of caspase-1 in regulating the processing and release of IL-1β, caspase-1 deficient (-/-) mice and specific caspase inhibitors were used to define the role of caspase-1 in LC migration and induction of murine contact hypersensitivity (CHS). Hapten-induced migration was impaired and CHS was suppressed in caspase-1 -/- mice. In parallel, YVAD, a caspase-1 inhibitor, prevented LC migration both in vivo and in vitro and suppressed CHS in vivo.

The potential influence of IL-18, a cytokine structurally similar to IL-1β, on LC mobilisation and CHS was next examined. Intradermal injection of rMuIL-18 caused significant LC migration in BALB/c mice. Furthermore, allergen-induced LC migration was absent in IL-18 -/- mice. CHS was suppressed in IL-18 -/- mice, but could be restored by intradermal IL-18 pre-treatment prior to sensitisation. Parallel studies showed that IL-18 acts proximally to IL-1β and TNF-α. In addition, it was shown that IL-18 was not involved in irritant contact dermatitis (ICD) and thus, this cytokine is a differentiating signal between CHS and ICD.

The numerous control mechanisms of the IL-1 system within the epidermis suggest that a co-ordinated regulation is essential in the cutaneous microenvironment. Mice over-
expressing either IL-1α or sIL-1 receptor antagonist in basal keratinocytes and a murine keratinocyte line were used to examine whether such homeostatic mechanisms occur both in vitro and in vivo in the epidermis. The data revealed from these studies suggest that there is a complex regulatory mechanism in which not only increased levels of IL-1 agonist lead to increases in antagonist production, but also that enhanced IL-1 receptor antagonist release results in increased production of agonist.

The mobilisation of LC and their directed migration from the epidermis to draining lymph nodes are processes of pivotal importance in the generation of cutaneous immune responses. The data presented in this thesis provided important information on the role of the IL-1 system in the initiation and regulation of skin immune responses.
PUBLICATIONS ARISING FROM THIS WORK

At the time of submission, 2 manuscripts based on the work in this thesis had been published and 2 manuscripts were submitted for publication, as detailed below.

Published manuscripts:


Submitted manuscripts:
Antonopoulos C, Cumberbatch M, Dearman RJ, Mee JB, Wei X, Liew FY, Kimber I and Groves RW. IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. Submitted in *J Immunol.*

Mee JB, Antonopoulos C, Poole S, Kupper TS and Groves RW. Counter-regulation of Interleukin-alpha and Interleukin-1 receptor antagonist in murine keratinocytes. Submitted in *J Invest Dermatol.*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACD</td>
<td>Allergic contact dermatitis</td>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>AOO</td>
<td>Acetone olive oil</td>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
<td>IL-1R-AcP</td>
<td>Interleukin-1 receptor accessory protein</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
<td>IL-1RI</td>
<td>Type I interleukin-1 receptor</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte-associated antigen</td>
<td>IL-1RII</td>
<td>Type II interleukin-1 receptor</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
<td>IP-10</td>
<td>Interferon gamma inducible protein 10</td>
</tr>
<tr>
<td>DDCs</td>
<td>Dermal dendritic cells</td>
<td>IRAK</td>
<td>IL-1 receptor-associated protein</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double-distilled water</td>
<td>K14</td>
<td>Keratin 14</td>
</tr>
<tr>
<td>DETC</td>
<td>Dendritic epidermal T cell</td>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
<td>kDa</td>
<td>KiloDaltons</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>DNFβ</td>
<td>2, 4-dinitrofluorobenzene</td>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetate acid</td>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Gro</td>
<td>Growth-related protein</td>
<td>OX</td>
<td>Oxazolone</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1 beta converting enzyme</td>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ICD</td>
<td>Irritant contact dermatitis</td>
<td>PMNs</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td>icIL-1ra</td>
<td>Intracellular interleukin-1 receptor antagonist</td>
<td>R10</td>
<td>RPMI with 10% FCS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
<td>RANTES</td>
<td>Regulated upon activation, normal T expressed and secreted</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
<td>RPMI</td>
<td>Roswell Park Memorial Institute: (medium)</td>
</tr>
<tr>
<td>IGIF</td>
<td>Interferon gamma inducing factor</td>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin</td>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<td></td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
<td></td>
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<tr>
<td>sIL-1R</td>
<td>Soluble interleukin-1 receptor</td>
<td></td>
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</tr>
<tr>
<td>sIL-1ra</td>
<td>Secreted interleukin-1 receptor antagonist</td>
<td></td>
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</tr>
<tr>
<td>SLS</td>
<td>Sodium lauryl sulphate</td>
<td></td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
<td></td>
<td></td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNCB</td>
<td>Trinitrochlorobenzene</td>
<td></td>
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</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
<td></td>
<td></td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
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CHAPTER 1

INTRODUCTION
1.1 THE SKIN

The skin is the largest organ in the body, in adults accounting for about 16% of total body weight. As our major interface with the environment, the skin, composed of specialised epithelial and connective tissue cells, has many protective and synthetic functions. These are subserved by three layers; epidermis, dermis and hypodermis (Haake and Holbrook, 1999) (Fig. 1.1)

![Image of skin layers](image)

**Figure 1.1: The three layers of the skin.** The outermost layer of the skin is the epidermis. It is highly cellular, avascular, lacks nerves and sits on a basement membrane. Below the epidermis is the dermis and underlying this there is a layer of fat-producing cells known as the hypodermis. The dermis contains many cell types including blood and lymphatic vessels, sweat glands, hair follicles, sebaceous glands and nerve endings (Modified from Haake and Holbrook, 1999).

1.1.1 The structure of the epidermis and its cellular components

The epidermis forms the external surface of the skin and is largely composed of keratinocytes that differentiate to form 4 layers, the stratum basale (basal layer), stratum
spinosum (spinous or prickle cell layer), stratum granulosum (granular layer), and stratum corneum (surface layer) (Fuchs, 1990; Stanley, 1993). Other cells in epidermis include Langerhans cells (LC), melanocytes and Merkel cells.

**Keratinocytes**

Approximately 90-95% of epidermal cells are keratinocytes, which arise from the superficial ectoderm of the implanted embryo during the first weeks of development. The basal keratinocyte is a mitotically active cell, which differentiates terminally from the basal to cornified cells (Maestrini *et al.*, 1996). This process typically takes approximately 28 days. As it migrates upward, the keratinocyte progressively synthesises keratin proteins, which provide structural integrity to the epithelial layer. Keratins are the most diverse of the intermediate filaments and their molecular mass ranges between 40 and 70 kDa (Moll *et al.*, 1982). The human keratins are organised into two families based on their size and charge (Smack *et al.*, 1994), the acidic, type I (K10-20) and basic-neutral, type II (K1-K9).

Not only do keratinocytes undergo a defined differentiation program in the epidermis, they may also become activated in response to injury or in certain pathologic conditions such as psoriasis, resulting in expression of new keratin molecules (Kupper and Groves, 1995). The activation process is influenced by cytokines and growth factors, such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), transforming growth factor-α (TGF-α), TGF-β and interferon-γ (IFN-γ). Activated keratinocytes express K6, K16 and K17, which are distinct from the keratins of the healthy epidermis (Murphy *et al.*, 2000).
In addition to providing structural integrity to the epidermis, keratinocytes also play an active role in host defense against infection. Thus, following a variety of stimuli, keratinocytes can secrete proinflammatory cytokines that activate both epidermal and dermal cells and induce production of chemokines that attract leukocytes from the circulation to the inflammatory focus (Tomic-Canic et al., 1998). Potentially, abnormalities in this defense function of keratinocytes can lead to imbalance in the skin cytokine network and thereby result in inflammatory and autoimmune disease (Nickoloff and Turka, 1993).

**Langerhans cells**

The Langerhans cell (LC) was identified by Paul Langerhans, at that time a medical student, in 1868. LC are dendritic cells that reside in the suprabasal layer of the epidermis, forming a semi-contiguous network (Kimber et al., 2000; Banchereau and Steinman, 1998; Steinman et al., 1995). LC are not unique to epidermis but are found in other squamous epithelia, including the oral cavity, esophagus and vagina, in lymphoid organs, such as the spleen, the thymus and lymph node and in normal dermis (Katz et al., 1979). They represent 2-5% of the total epidermal cell population and attach to keratinocytes through homotypic E-cadherin mediated adhesion (Udey, 1997). They derive from blood-borne precursors which themselves come from CD34+ve progenitor cells in the bone marrow (Strunk et al., 1997) and reside in the epidermis for a time, during which they process and present foreign antigens acquired locally (Katz et al., 1979; Williams and Kupper, 1996). LC will be discussed in more detail in the next section.
Melanocytes

Melanocytes are cells in low abundance in epidermis that produce the pigment melanin for skin pigmentation, which is partially protective against UV radiation. These cells are derived from the neural crest and migrate to the basal layer of the epidermis during fetal development, where they become interspersed among keratinocytes by 8-10 weeks of gestation in the cephalic skin and by the 4th month of gestation in the caudal regions (Holbrook, 1998). Melanosomes (pigment containing granules produced within melanocytes) are present in melanocyte dendrites and are transferred to surrounding keratinocytes (Gordon et al., 1989). The melanosomes tend to form a cap over the keratinocyte nucleus in order to protect it from UV radiation.

Like keratinocytes, melanocytes are also involved in regulation of the skin cytokine network. In addition to the proinflammatory cytokines IL-1, TNF-α and IL-6 (Swope et al., 1994; Kruger-Krasagakes et al., 1995), melanocytes can also release the CXC chemokines, IL-8 and GRO-α (Mattei et al., 1994). Furthermore, melanocytes appear to be a significant source of IL-10 in the epidermis and these cells are also capable of producing TGF-β (Kruger-Krasagakes et al., 1994; Mattei et al., 1994). It has been shown that normal human melanocytes can function as phagocytes against microorganisms and that melanosomes are lysosomal structures, suggesting a role of these cells in host defense (Le Poole et al., 1993b).

Merkel cells

Merkel cells are neuroendocrine cells which form a small percentage of the cells in the basal layer of the epidermis (Moll et al., 1996). Merkel cells are joined to keratinocytes by desmosomal junctions and are concentrated in the epidermal ridges of developing
epidermis and the outer root sheath of the hair follicle. Epidermal Merkel cells produce nerve growth factor and dermal Merkel cells express nerve growth factor receptors before connecting to nerves (Narisawa et al., 1992). They have contact with small unmyelinated nerve endings and most likely function as slow mechanoreceptors (Kim and Holbrook, 1995).

1.1.2 The structure of the dermis and its cellular components

The dermis is a thick, supple and sturdy layer of connective tissue that makes up about 90 percent of the skin's thickness. It is separated from the epidermis by the basement membrane and this junction consists of interlocking rete ridges and dermal papillae. The dermis is divided into 2 layers, the papillary dermis and the reticular dermis (Haake and Holbrooke, 1999). It is filled with a dense network of collagen and elastin fibers that support lymph and other vessels, nerves, muscle cells, sweat and sebaceous glands and hair follicles (Kadler et al., 1996).

Fibroblasts

The primary cell type of the dermis is the fibroblast, a mesenchymal-derived cell that migrates through the tissues and is responsible for the synthesis and degradation of fibrous and non-fibrous connective tissue matrix proteins and a number of soluble factors. The fibroblast produces the collagens and elastins that make skin durable, but has not been traditionally associated with immune responses. However, it is now widely accepted that fibroblasts play an important amplification role in response to cytokines originating from keratinocytes. A number of cytokines have been shown to be secreted secondarily to IL-1 and TNF-α stimulation in vitro, such as IL-8 (Schroder et al., 1990), monocyte chemotactic protein-1 (Larsen et al., 1989) and granulocyte-
macrophage colony stimulating factor (GM-CSF) (Zucali et al., 1986). Furthermore, following UVB irradiation of mice carrying a TNF promoter/reporter construct, most of the TNF-α produced was shown to be synthesised by fibroblasts, rather than keratinocytes and, since UVB irradiation does not penetrate beyond epidermis, this suggests a secondary response to keratinocyte-derived cytokines (de Kossodo et al., 1995). Thus, fibroblasts may facilitate a substantial amplification of secondary cytokine production in cutaneous immune responses, as well as playing a key role in maintenance of structural integrity of skin and in wound healing.

**Mast cells**

Mast cells are specialised secretory cells that derive from CD34+ve stem cells in the bone marrow and are distributed in connective tissues throughout the body. In the skin, mast cells are present in the greatest density in the papillary dermis, in sheaths of epidermal appendages and around blood vessels and nerves of the subpapillary plexus (Naukkarinen et al., 1996). Mast cells are characterised by their large cytoplasmic granules which were originally thought to be nutrient stores but in fact contain histamine, enzymes and other mediators which can be discharged as part of an inflammatory response. Mast cells bind immunoglobulin E (IgE) and play a key role in IgE-mediated allergic responses. In addition, it has been shown that mast cell degranulation and subsequent release of inflammatory mediators can be triggered by other stimuli, such as neuropeptides and complement components (Marshall and Bienenstock, 1994).
Dermal dendritic cells

The dermal dendritic cell is a stellate or sometimes spindle-shaped, highly phagocytic fixed connective tissue cell in the dermis of normal skin (Cerio et al., 1989). It is of uncertain histogenesis and may be bone marrow or fibroblast derived (Cerio, 1995). These cells are found in large numbers in the papillary dermis and upper reticular dermis, frequently in the proximity of vessels of the subpapillary plexus. Dermal dendritic cells are immunologically competent cells and probably function as effector cells in the afferent limb of an immune response (Lenz et al., 1993). The major difference between dermal dendritic cells and their epidermal counterparts, LC, is that the former express the blood clotting factor XIIIa, whereas LC do not. Furthermore, LC express higher levels of CD1a molecule than do dermal dendritic cells (Nestle et al., 1993). In inflammatory conditions such as psoriasis or eczema, the numbers of these cells increase and they are stimulated to express other markers of inflammation, including intercellular adhesion molecule-1 (ICAM-1). In addition, they are reduced or absent from malignant fibrotic tumours and fibroproliferative lesions, such as keloids, scars and scleroderma (Headington and Cerio, 1990; Cerio et al., 1989).

Specialised structures

These components are largely of epidermal origin and extend into the dermis. They include the pilosebaceous unit (hair follicle, sebaceous glands and arrector pili muscle), eccrine sweat glands (and, in some locations, apocrine sweat glands), and nails (Downing et al., 1993). The dermis also plays an important role in supporting the cutaneous vasculature, comprised of two main horizontal layers with vertical connections that terminate in a fine capillary network just beneath the epidermis.
Constriction and dilation of dermal arterioles is important in control of skin (and therefore body) temperature (Robinson *et al.*, 1988).

### 1.1.3 The hypodermis

The hypodermis is the deepest layer of the skin, composed primarily of fat. The key cells of the hypodermis are the adipocytes that provide energy, serve as a heat insulator for the body and act as a shock absorber to protect underlying tissue against mechanical trauma. Among mammals, only humans and marine mammals such as whales and dolphins have this subcutaneous layer of fat (Haake and Holbrooke, 1999).

### 1.1.4 The innate and adaptive protective functions of the skin

The skin is structured to prevent loss of essential body fluids, and to protect the body against the entry of toxic environmental chemicals. In the absence of a stratum corneum we would lose significant amounts of water to the environment, and rapidly become dehydrated. The stratum corneum, with its overlapping cells and intercellular lipid, makes diffusion of water into the environment very difficult (Roitt *et al.*, 1996).

The skin also plays a key role in the innate protection of the body against invasion by micro-organisms. The dryness and constant desquamation of the stratum corneum, the normal flora of the skin, the fatty acids of sebum and lactic acid of sweat all represent natural defence mechanisms against invasion by micro-organisms. Any form of trauma renders the skin permeable to infectious agents and their secreted products. One of the immediate protective responses of skin to barrier perturbation is release of lamellar bodies containing pre-formed lipid and hydrolytic enzymes, as well as cytokines, such
as TNF-α and IL-1 (Tsai et al., 1996; Nickoloff and Naidu, 1994). As a consequence of this cytokine release, endothelium can be activated allowing an influx of neutrophils and production of antibacterial defensins by keratinocytes is induced (Harder et al., 1997).

The defensins are recently identified natural antimicrobial molecules. They are divided into two classes, the α- and β- defensins based on the connectivity of their cysteine residues (Diamond and Bevins, 1998). Alpha-defensins are stored in neutrophils and have a broad spectrum of activity, killing Gram-negative and Gram-positive bacteria, fungi and enveloped viruses (Lehler and Ganz, 1996). Beta-defensins, on the other hand, are specifically produced by keratinocytes and other epithelial cells such as lung epithelia (Harder et al., 1997; Singh et al., 1998) and provide a protective role to organ surfaces that are continuously exposed to various microorganisms. Additionally, both α- and β-defensins are chemotactic for CD4 and CD8 positive T lymphocytes, as well as immature dendritic cells (Yang et al., 2001).

The innate immune response not only provides the first line of defense against microorganisms, but also provides the biological context that instructs the adaptive immune system to mount a response (Galluci and Matzinger, 2001). The adaptive immune response calls on the innate immune system to provide the professional phagocytes and granulocytes necessary to engulf small pathogens and contain larger parasites. Subsequently, antigen is picked up by dendritic cells in the tissue and carried into regional lymph nodes, where the dendritic cells activate naive T and B lymphocytes. Activated T and B lymphocytes then leave the lymph node and recirculate to the site of inflammation. The chemokine system has emerged as the crucial
regulator of the dendritic-cell and lymphocyte trafficking that is needed, first, to bring together antigen-loaded dendritic cells and naive T and B lymphocytes to generate an adaptive immune response and, second, to deliver this adaptive effector response to sites of inflammation and infection (Luster, 2002). Thus, effective host defense in skin depends on coordination between innate and acquired immunity.

1.2 THE EPIDERMAL LANGERHANS CELL

The principal task of the immune system is to protect the host by eliminating or neutralising foreign molecules, whilst simultaneously sparing or tolerating molecules that are intrinsic to the host. The epidermis is an integral part of the immune system and not only does it serve as a site of antigen entry and of antigen destruction, but it also contains a variety of cells (as described in the previous section) that participate in the initiation and regulation of immune responses. Although LC comprise only a minor population of epidermal cells (Tamaki et al., 1979), they can process and present antigens to T lymphocytes and thus are capable of initiating antigen-specific immune responses. LC are the principal antigen-presenting cells (APC) in the skin (Wolff and Stingl, 1983).
LC originate from cells in bone marrow and reside in the epidermis (Katz et al., 1979). In response to a variety of stimuli, LC migrate from epidermis via afferent lymphatics to draining lymph nodes where, as fully differentiated dendritic cells, they present processed antigen to T lymphocytes (Banchereau and Steinman, 1998; Steinman et al., 1995) (Fig. 1.2).

![Figure 1.2: Langerhans cell life cycle.](image)

**Figure 1.2: Langerhans cell life cycle.** a) LC derive from blood-borne cells which themselves originate from bone marrow precursors. LC represent a 2-5% of total epidermal cell population and form a semi-contiguous network in the epidermis. b) In response to a variety of stimuli, LC move away from epidermis via afferent lymphatic vessels to draining lymph nodes where they present antigen to T lymphocytes.

### 1.2.1 Structure and phenotypic markers of Langerhans cell

A number of structural features characterise the Langerhans cell. Firstly, they have a dendritic morphology when *in situ* within suprabasal epidermis, though this is less evident when epidermal cells are processed into a single cell suspension. Secondly, a unique hallmark of LC is the presence of characteristic intracytoplasmic “tennis racket”-
shaped organelles called Birbeck granules (Bucana et al., 1992) (Fig. 1.3). These cytoplasmic organelles are produced by fusion of cell membrane-derived structures with endosomes and lysosomes (Valladeau et al., 2000). Their function is unknown.

**Figure 1.3: Birbeck granules.** Birbeck granules are membrane-bound, rod shaped, 15-50nm long, 4nm wide, with central linear density and faint striations radiating from the linear density to the limiting membrane. They have characteristic "tennis-racket" shape (Taken from Bucana et al., 1992).

LC also possess a number of characteristic enzyme markers, including ATPase, ADPase and non-specific esterase (Romani et al., 1989). ATPase and ADPase appear to protect LC from the cytotoxic effects of exogenous ATP. Cytochemical markers include vimentin (an intermediate filament present in cells derived from mesenchymal tissue) and members of the S-100 family of proteins (De Panfilis et al., 1991).

A number of antigenic markers are expressed on the surface of LC. CD45 (also known as the common leukocyte antigen Ly-5) is a marker for cells of bone marrow origin (Romani and Schuler, 1992). Major histocompatibility complex (MHC) molecules are also present. MHC class I molecules include human leukocyte antigen (HLA)-A, -B, or -C in humans, and H-2K or H2-D in mice. MHC class II molecules include HLA-DP,
DQ, or -DR in humans and Ia or I-A and I-E in mice (Andersson et al., 1998). In normal, uninflamed, epidermis, LC are the only cells which express class II molecules. However, keratinocytes can be triggered to express class II molecules by the action of IFN-γ secreted by infiltrated lymphocytes in inflamed skin. CD1a and CD1b are amongst the most distinctive markers for LC (Ito et al., 1999). Recently, another marker, called Langerin, specifically expressed by LC was identified (Valladeau et al., 2000). Langerin is a type II Ca\(^{2+}\)-dependent lectin, and an endocytic receptor that functions as a potent inducer of Birbeck granule formation by superimposition and zippering of cell membranes.

LC can also express adhesion molecules, including those belonging to the cadherin, immunoglobulin and integrin families. ICAM-1 is an example of an adhesion molecule with close homology to immunoglobulins. Integrins of β1 (CD29) and β2 (CD18) subtypes have been shown on LC (Staquet et al., 1992). β1 integrins include very late antigen-1 (VLA-1) to VLA-6, whereas β2 integrins are a group of heterodimeric molecules, including lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and gp150, 95 (CD11c/CD18), which are involved in leukocyte adhesion and in antigen-specific activation of T lymphocytes (Hynes, 1992). In addition, activated LC can express a variety of accessory molecules, such as receptors for Fc fragment of IgG and IgE (FcγRII and FcεRI, respectively) and receptors for cytokines including IL-1 and IL-6, TNF-α, GM-CSF and IFN-γ (Larregina et al., 1996). Also, the co-stimulatory molecule B7 has been demonstrated to be expressed by LC (Symington et al., 1993).
1.2.2 Functional properties of Langerhans cell

Epidermal LC are the principal APC in the skin. Thus, they are primarily responsible for initiating immune responses, typified by that seen in allergic contact dermatitis. For exogenous proteins, antigen processing involves the binding of protein antigens to LC as a first step (Streilein and Grammer, 1989). LC then are able to ingest the antigen and, following internalisation, this becomes localised in endolysosomal compartments where unfolding of antigen tertiary structure and degradation into linear oligopeptides occurs (Bartosik, 1992). The final step of this process is antigen association with MHC class II molecules transport to the plasma membrane and expression of these MHC-peptide complexes on the LC surface (Kleijmeer et al., 1994).

However, for optimal T lymphocyte activation, additional APC attributes are required. These include the expression of adhesion molecules, whose engagement to appropriate ligands on T lymphocytes strengthens the LC-T lymphocyte interaction, and the presence of co-stimulatory molecules that transduce signals to the T lymphocytes independent of the T cell antigen receptor (TCR).

Thus, the phenotypic characteristics and functional properties of LC change as they travel to the draining lymph nodes. This process of differentiation is associated with, and is probably precipitated by, the receipt by LC of the signals necessary to induce their migration from the epidermis.

1.2.3 Langerhans cell maturation

The primary physiological role of dendritic cells is the presentation of antigen. In this respect, the functional activity of LC and their participation in the induction phase of contact hypersensitivity is intriguing as freshly isolated epidermal LC are comparatively
ineffective APC (Schuler and Steinman, 1985). However, the dendritic cells which arrive in lymph nodes, many of which derive from LC, are potent immunostimulatory cells (Cumberbatch et al., 2001). It is apparent that following skin sensitisation and during the process of movement to and entry into the lymph nodes, LC are subject to a functional maturation such that they acquire the antigen-presenting potential characteristic of dendritic cells found within lymphoid tissue. This functional maturation is reflected by phenotypic changes including an increase in the expression of membrane determinants required for effective interaction with and presentation of antigen to T lymphocytes. Thus, compared with epidermal LC, dendritic cells that arrive in draining lymph nodes display elevated expression of Ia and ICAM-1 (Cumberbatch et al., 1991; 1992). B7, leukocyte-associated antigen-3 (LFA-3) and CD40 accessory molecules are also up-regulated (Girolomoni et al., 1994).

There is a general consensus that LC within the skin are able to process antigen effectively and that this facility is lost during migration and the acquisition of antigen-presenting potential (Streilein and Grammer, 1989). Consistent with reduced antigen-processing activity is the fact that the maturation of LC is characterised by the loss of Birbeck granules and of endosomal antigens which reflect endocytotic, endosomal and lysosomal activity (Bucana et al., 1992; van Wilsem et al., 1994). Loss of Fc receptor expression has also been reported (Pierre et al., 1997). Another important event that characterises the process of LC maturation is the reduced expression of E-cadherin, which allows LC to dissociate from surrounding keratinocytes (Borkowski et al., 1994; Riedl et al., 2000), and the up-regulation of α6 and β1 integrin expression which comprise VLA-6. This confers laminin-binding activity on LC and facilitates interaction with the basement membrane (Price et al., 1997). Associated with this is
expression by LC of matrix metalloproteinase-9 (MMP-9), a collagenase that appears to be necessary for effective migration to lymph nodes, presumably by permitting passage across the basement membrane at the dermal-epidermal junction (Kobayashi et al., 1999).

Another series of changes during LC maturation involves the responsiveness of LC to chemokines and the regulation of the expression of chemokine receptors. For example, CCR7 receptor-ligand interactions promote the entry of T lymphocytes into lymph nodes and their localisation within the paracortex. This same receptor is up-regulated by dendritic cells during maturation, conferring the ability to respond to the relevant ligands produced by cells within the lymph node paracortex, including dendritic cells themselves. In contrast, expression of CCR1 and CCR5, two CC chemokine receptors, is reduced and in turn facilitates the movement of LC from the skin (Sozzani et al., 1998). Table 1.1 summarises the key differences between immature and mature LC.

Table 1.1: Comparison of immature and mature LC

<table>
<thead>
<tr>
<th>Markers/Properties</th>
<th>Immature LC</th>
<th>Mature LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC class I, II</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>LFA-3</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>B7-1, B7-2</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>FcR</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>CCR1, CCR5</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>α6 Integrin</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Antigen capture/process</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Antigen presentation</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Birbeck granules</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>
1.2.4 Initiation of Langerhans cell migration

Encounter with antigen in the skin is the prime stimulus to the mobilisation of LC and induction of their migration to draining lymph nodes. The molecular mechanisms that underlie this mobilisation have been the subject of intense research interest and it is now recognised that cytokine release in epidermis is responsible for the initiation and regulation of LC migration and maturation, IL-1β and TNF-α being of particular importance. These cytokines together with relevant chemokine receptor-ligand interactions, initiate the movement of LC from the epidermis and guide their directed migration to, and localisation within, peripheral lymph node tissue. In this section, the phenotypic changes induced during the activation of LC and the mechanisms through which their migration is regulated will be discussed.

Following epicutaneous application of antigen to skin, LC take up the antigen, process it and migrate towards the regional lymph nodes. During this process, LC convert from a resting into an activated functional state. This activation is initiated by keratinocytes that secrete inflammatory cytokines as a result of the presence of antigen, and is possibly also due to direct effects of antigens on LC themselves (Becker and Knop, 1993). LC activation is associated with induction of cytokine secretion, including IL-1β, IL-6, IL-12 and chemokines, enhanced expression of cell-surface molecules such as MHC class I and II, adhesion and costimulatory molecules, and finally altered antigen uptake, processing and presenting capacity (Romani and Schuler, 1992). Activation of LC and induction of migration towards the lymph node appears to depend on the capacity of antigens to induce IL-1β in LC, which is a selective and almost immediate effect of epicutaneous hapten application (Enk and Katz, 1992; Enk et al., 1993b; Zheng et al., 1995). In addition, other cytokines, such as TNF-α and GM-CSF, and
chemokines also contribute to LC activation and migration (Sozzani et al., 1995; Kaplan et al., 1992). Thus antigens themselves, by virtue of their capacity to induce a specific cytokine expression pattern, appear to be the primary stimuli that activate LC and induce the sensitisation process.

The process of LC migration is induced and regulated by epidermal cytokines which in turn provoke the phenotypic changes discussed above, necessary for the cells to leave the skin and travel to peripheral lymphoid tissue. The orchestrated interactions between LC, cytokines, chemokines and the surrounding tissue matrix provides an effective and well regulated mechanism for ensuring that encounter with antigen at skin surfaces is translated into an appropriate immune response.

1.2.5. Epidermal cytokines implicated in the regulation of Langerhans cell migration

Cytokines are small, secreted proteins or, more often, glycoproteins with pleiotropic functions that regulate and determine the nature of immune responses. They are involved in virtually every facet of immunity and inflammation, including antigen presentation, bone marrow differentiation, cellular recruitment and activation, adhesion molecule expression, and acute phase responses. Which cytokines are produced in response to an immune insult initially determines whether an immune response develops and subsequently has a major influence on whether that response is cytotoxic, humoral, cell-mediated or allergic. Because of their often potent activities, cellular cytokine release is generally controlled by an autocrine negative-feedback mechanism. They also function locally on contiguous cells in a paracrine function and may have distant effects on tissues and therefore act as endocrine agents (Abbas et al., 1997).
Considerable evidence suggests that epidermal cell-derived proinflammatory cytokines including TNF-α and IL-1 play an important role in promoting LC migration during the induction phase of contact hypersensitivity. In the next sections, the structure and the key functions of these and other cytokines related to LC migration and their role in the regulation of this process will be considered.

**TNF-α**

TNF represents two homologous 17kDa proteins primarily derived from mononuclear monocytes (TNF-α) and lymphocytes (TNF-β) (Beutler and Cerami, 1989; Vilcek and Lee, 1991). In addition to monocytes/macrophages, TNF-α may be produced by neutrophils, activated lymphocytes, natural killer cells, endothelial cells and smooth muscle cells (Peters et al., 1986; Takanashi et al., 1994). In the skin, TNF-α can be produced by resident leukocytes, dendritic cells, keratinocytes, fibroblasts and mast cells (Kock et al., 1990). Important biological activities of TNF-α include its ability to induce the proliferation of activated T and B lymphocytes and the expression of MHC class I and class II molecules (Sugarman et al., 1985; Pober et al., 1987). An important activity of TNF-α in the skin is the capacity to up-regulate cellular adhesion molecules such as ICAM-1 and E-selectin on the surface of vascular endothelial cells, thereby contributing to the accumulation of leukocytes at sites of inflammation (Detmar et al., 1992; Swerlick et al., 1992; Groves et al., 1995).

TNF-α appears to play an important role in the initiation of a protective as well as innate immune response against bacterial and viral intracellular pathogens (Beutler and Cerami, 1989; Flynn et al., 1995). This cytokine is one of the principal mediators of the sepsis response, which is triggered by bacterial-derived products such as
lipopolysaccharide (LPS) (Tracey et al., 1987). TNF-α can also stimulate secretion of other similar pro-inflammatory cytokines, such as IL-1 and IL-6, and therefore amplify its biological effects during local and systemic immune responses (Luheshi and Rothwell, 1996). In addition, TNF-α may induce the formation of new blood vessels and stimulate connective tissue deposition, and thus plays an active part in tissue remodelling responses after injury (Leibovich et al., 1987).

TNF-α is processed as a membrane-bound protein from which the soluble active factor is derived by cleavage of the extracellular domain by the convertase, TACE (Perez et al., 1990; Moss et al., 1997; Black et al., 1997). TNF-α exerts many of its effects by binding, as a trimer, to either a 55 kDa cell membrane receptor termed TNF-R1 or a 75 kDa cell membrane receptor termed TNF-R2. Both these receptors belong to the so-called TNF receptor superfamily. The superfamily includes FAS, CD40, CD27, and RANK. The defining trait of these receptors is an extracellular domain comprised of two to six repeats of cysteine rich motifs. Additionally, a number of structurally related decoy receptors exist that act to sequester TNF molecules, thereby rescuing cells from apoptosis (Naismith and Sprang, 1998; Kollias et al., 1999). These receptors are expressed on most types of cells. TNF-R1 signaling results in elevated activity of specific target genes such as NF-κB that control the transcriptional regulation of a number of pro-inflammatory molecules (Hohmann et al., 1990; Bazzoni and Beutler, 1996). TNF-R1 appears to be required for the successful host response to intracellular infectious pathogens (Flynn et al., 1995).

TNF-α in the skin induces chemokine production and up-regulation of cellular adhesion molecules on different cell types, and also alters the functional properties of
melanocytes as well as fibroblasts. The production of TNF-α by keratinocytes is markedly increased by UVB irradiation (Kock et al., 1990). Contact sensitisers and irritants also appear to up-regulate keratinocyte TNF-α expression (Lisby et al., 1995). Keratinocytes also express TNF-R1 (Tomic-Canic et al., 1998), which indicates that they have the potential to respond to secreted keratinocyte TNF-α in an autocrine fashion (Pillai et al., 1990). TNF-α also appears to be increased in LC in lesional psoriatic skin (Nickoloff et al., 1991).

TNF-α is also important in the development of contact dermatitis. Pre-treatment with anti-TNF-α antibody abolishes many inflammatory skin reactions, including ACD and ICD (Piguet et al., 1991). An important mechanism by which TNF-α influences the development of an inflammatory reaction is induction of the expression of cutaneous and endothelial molecules (Pober and Cotran, 1990; Groves et al., 1995). It has been demonstrated that TNF-α mRNA expression was increased in tributyltin-induced skin irritation in mice (Corsini et al., 1997). Furthermore, keratinocyte-derived TNF-α mRNA is rapidly up-regulated in a number of models of skin inflammation in mice (Enk and Katz, 1992; Holliday et al., 1997).

Evidence for involvement of TNF-α in LC migration

TNF-α was the first cytokine to be identified as a stimulus for LC migration (Cumberbatch et al., 1997b; Stoitzner et al., 1999). Initial studies revealed that intradermal exposure of mice to recombinant TNF-α caused a rapid reduction, within 30 minutes, of LC from the epidermis local to the site of exposure. Moreover, this was accompanied by an increase after 2 hours in the number of dendritic cells (DC) in the draining lymph nodes (Cumberbatch and Kimber, 1992; Cumberbatch et al., 1994).
Further evidence suggests that TNF-α is not only able to induce LC migration on its own, but is also essential for the mobilisation of LC that is induced after epicutaneous application to mice of skin-sensitising allergens (Cumberbatch and Kimber, 1995). When mice were pre-treated with neutralising anti-TNF-α antibody prior to hapten application with the potent contact allergen, oxazolone, a marked inhibition of allergen-induced DC accumulation in draining lymph nodes was observed (Cumberbatch et al., 1997b).

A number of lines of evidence suggest that TNF-α delivers its signal directly to LC acting through membrane TNF-R2 receptors. Firstly, induction of LC migration by TNF-α is species-specific. When mice were intradermally exposed to human, rather than murine, recombinant TNF-α there was neither LC migration nor DC accumulation in the draining lymph nodes. These results suggest that there is a predominant role for the species-specific 75 kDa TNF-R2 receptor, rather than the species non-restricted 55 kDa TNF-R1 receptor (Kimber and Cumberbatch, 1992; Cumberbatch et al., 1994). Further evidence comes from studies where TNF-R1 gene knockout mice were used. These investigations revealed that TNF-α-dependent migration of LC to draining lymph nodes was normal in TNF-R1 gene knockout mice (Wang et al., 1996). However, hapten-induced LC migration was significantly suppressed in TNF-R2 deficient mice (Wang et al., 1997). Therefore, LC migration in response to skin sensitisation and skin irritation, including UVB irradiation (Moodycliffe et al., 1994) are dependent upon TNF-α acting directly on epidermal LC themselves via TNF-R2 receptors.

**IL-1**

Interleukin-1 is one of the most potent pro-inflammatory cytokines and affects nearly every cell type. The IL-1 family represents eleven peptides to date (Dunn et al., 2001),
of which IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) have been the most extensively studied (Dinarello, 1996). Monocytes/macrophages are the principal leukocytic source of IL-1 but in the skin, IL-1 is produced by keratinocytes, dermal dendritic cells, LC, melanocytes, fibroblasts and endothelial cells (Dinarello, 1996). IL-1ra is also produced by a number of epithelial cell types including keratinocytes in the skin and corneal epithelial cells (Bigler et al., 1992; Kennedy et al., 1995).

Despite their low degree of sequence similarity (~25% amino acid homology), IL-1α and IL-1β have similar biological activities, and these two proteins together with IL-1ra bind with comparable affinities to the two IL-1 receptors (IL-1R) (Dinarello, 1996). Both IL-1α and IL-1β are synthesised as 33 kDa precursor molecules. The IL-1α precursor peptide (pro-IL-1α) as well as the mature IL-1α processed peptide are biologically active (Lonnemann et al., 1989). Because IL-1α lacks a hydrophobic leader sequence required for normal secretion pathways, it may be released with cellular damage or it may bind to an internal receptor and act as an autocrine factor modulating cellular differentiation (Stevenson et al., 1992; Dinarello, 1996). The IL-1β precursor (pro-IL-1β) is also cell associated but biologically inactive until it is processed into the active form by a specific cysteine protease, IL-1β-converting enzyme (ICE/caspase-1) (Thornberry et al., 1992; Dinarello, 1997). Mature IL-1β is then released from the cell.

Two forms of IL-1R have been identified (Dinarello, 1996). Type I IL-1 receptors are found on T lymphocytes, fibroblasts, endothelial cells, hepatocytes, and numerous other cells. They have an extensive cytoplasmic domain and, after ligand binding, transduce the biologic effects attributed to IL-1 (Sims et al., 1993). In contrast, type II IL-1 receptors are predominately expressed on B lymphocytes, neutrophils and bone marrow
cells and have a truncated intracellular domain. The capture and sequestration of IL-1 by these inactive type II receptors may subserve an anti-inflammatory function and hence they are sometimes referred to as decoy receptors. Type II IL-1 receptors are the precursors for the soluble IL-1 binding factors, which, when shed, antagonise IL-1 activity (Sims et al., 1993; Re et al., 1994; Groves et al., 1995b). The capacity of IL-1ra to bind to the type I (pro-inflammatory) IL-1R rather than the type II, without transducing biological activity is the basis for its capacity to function as an anti-inflammatory cytokine antagonist (Eisenberg et al., 1990; Arend, 1993). A receptor accessory protein, IL-1R-AcP, is also required for signalling to occur following binding of IL-1α or IL-1β to IL-1RI (Greenfeder et al., 1995).

IL-1 induces the transcription of a number of genes involved in skin inflammation via activation of the transcription factor NF-κB. Membrane bound type-1 IL-1R can bind either IL-1α or IL-1β and, when complexed with the receptor accessory protein, will initiate the signal transduction cascade that ultimately results in stimulation of transcription of adhesion molecules, cytokines, and other pro-inflammatory genes (Huang et al., 1997; Cullinan et al., 1998). The precise signal transduction mechanisms resulting from ligation of IL-1 receptors is incompletely understood but has been the focus of intensive investigations in recent years. Briefly, current data suggests that the receptor/ligand complex recruits the adaptor molecule MyD88 that binds to the IL-1 receptor associated kinases (IRAKs) (Burns et al., 1998). The IRAKs then bind to another adaptor molecule, TNF receptor associated factor 6 (Cao et al., 1996), which associates with TAK1, the kinase that phosphorylates the NF-κB-inducing kinase (Malinin et al., 1997; Ninomiya-Tsuji et al., 1999). NF-κB-inducing kinase activates the IκB kinase complex, which can phosphorylate IκB, causing its rapid degradation.
 NF-κB is free to migrate into the nucleus, where it binds to specific sequences in the promoter region of multiple inflammatory genes.

In skin, IL-1 was one of the first cytokines identified as being produced by keratinocytes (Luger et al., 1981; Kupper et al., 1986). Release of this cytokine is induced by a variety of stimuli, such as contact sensitizers, irritant chemicals and UV irradiation (Enk and Katz, 1992). Human keratinocytes can release IL-1α in a biologically active form, whereas keratinocyte-derived pro-IL-1β is inactive under non-pathological conditions. Caspase-1 can be detected in low levels in normal keratinocytes and its expression is up-regulated by contact sensitisers and irritant chemicals which may thereby facilitate production of active IL-1β (Zepter et al., 1997). LC also constitutively produce IL-1 and its production is increased in a similar manner as with keratinocytes (Aiba et al., 1997; Lore et al., 1998). The role of IL-1 in contact dermatitis is a topic of ongoing investigation. It has been shown that IL-1β mRNA is up-regulated within 15 minutes of epicutaneous hapten application (Enk and Katz, 1992). The regulatory specificity of IL-1β was confirmed when monoclonal anti-murine IL-1β antibodies prevented primary sensitisation (Enk et al., 1993a). Investigators have demonstrated that local injection of IL-1ra in BALB/c mice significantly impaired the sensitisation and elicitation phases of contact hypersensitivity (Arend, 1993). IL-1ra injection did not suppress phenol-induced inflammation, a prototypic ICD reaction (Kondo et al., 1995). Mice deficient in IL-1β showed a defective ACD response to topically applied trinitrochlorobenzene (TNCB) (Shornick et al., 1996). These responses were reversible either by the application of very high doses of sensitising antigen or by intradermal injection of recombinant IL-1β immediately
before epicutaneous hapten application. Together, the data indicate that IL-1 may function as a potentiator of the immune response in ACD and is central to the contact dermatitis immunology.

One of the most important biologic activities of IL-1 is its function as a lymphocyte-activating factor. T helper cell activation requires the interaction of antigen-MHC complex with the T lymphocyte receptor and additional signals provided primarily by the interaction of B7 on APC with CD28 on the T lymphocyte. In addition, other signals contribute to optimal T lymphocyte activation and proliferation, and these may be provided by IL-1. IL-1 enhances the production of T lymphocyte-derived cytokines such as IL-2 as well as IL-2 receptors. In the absence of IL-1, either a diminished immune response or a state of tolerance develops. In addition to these effects on T lymphocytes, IL-1 can augment B lymphocyte proliferation and increases immunoglobulin synthesis. IL-1 acts synergistically with various CSFs to stimulate early bone marrow haematopoietic progenitor cell proliferation and the administration of IL-1 induces neutrophilia (Neta et al., 1990).

The production of IL-1 during an immune response produces a spectrum of changes associated with disease. IL-1 interacting with central nervous system is responsible for fever, lethargy, slow wave sleep and anorexia. An IL-1 hepatocyte interaction inhibits production of "house-keeping" proteins and stimulates the synthesis of acute phase response peptides (Maier et al., 1996). IL-1 activity in joints stimulates synovial cell proliferation, cartilage and bone resorption, and collagen deposition. Stimulation of endothelial cell adherence of leukocytes through the up-regulation of ICAM-1 is induced by IL-1 (Wegner et al., 1990). IL-1 also acts on blood vessels to induce
vasodilation, thus contributing to the hypotension of septic shock. Furthermore, IL-1 induces the synthesis of additional cytokines, including TNF, IL-6, GM-CSF and, as a positive-feedback mechanism, additional IL-1. Finally, like TNF, IL-1 is directly cytotoxic to cancerous and virus-infected cells (Pezzella et al., 1990).

The IL-1ra was the first described naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule (Arend, 1993). The first time that its IL-1-inhibitory activity was described was in the urine of patients with fever and in the supernatants of adherent monocytes cultured on IgG (Arend, 1993). Two forms of IL-1ra have been described. The first form, secreted IL-1ra (sIL-1ra), is a 177 amino acid protein that requires cleavage of a 25 amino acid leader sequence in order to be secreted as a variably glycosylated 152 amino acid protein (Eisenberg et al., 1990). The second form is an 18 kDa intracellular variant (icIL-ra) (Haskill et al., 1991) that arises as a result of alternative splicing of different first exons of the IL-1ra gene. As a result of this alternate splicing, icIL-ra lacks a functional leader sequence and therefore remains in the cytoplasm (Butcher et al., 1994). Two additional intracellular forms of IL-1ra (a 25 kDa and a 16 kDa proteins) have also been described (Muzio et al., 1995; Malyak et al., 1997).

Keratinocytes and other epithelial cells constitutively produce icIL-1ra, whereas sIL-1ra can be produced by most cells that can synthesise IL-1, with the exception of endothelial cells and hepatocytes (Andersson et al., 1992). Neutrophils have been shown to synthesise only sIL-1ra mRNA whereas fibroblasts can produce mRNA and protein of both IL-1ra isoforms following appropriate stimulation (Arend 1993). As a result of its potent antagonistic properties IL-1ra has been used experimentally as an
anti-inflammatory agent. In rabbits, IL-1ra prevents death from LPS-mediated septic shock (Ohlsson et al., 1990) and modulates immune complex-induced colitis (Ferretti et al., 1994). Furthermore, in animal models of asthma, IL-1ra suppresses the late asthmatic reaction and reduces airway inflammation (Okada et al., 1995).

**Evidence for involvement of IL-1 in LC migration**

While TNF-α is a mandatory signal for LC migration, it is not alone sufficient for the successful mobilisation and movement for these cells. There is a requirement also for IL-1β which in mouse epidermis is a constitutive and inducible product of LC themselves. As with TNF-α, intradermal administration of rIL-1β in mice causes LC migration and subsequent DC accumulation in draining lymph nodes. However, there is a difference in kinetics between the two cytokines as TNF-α will cause LC migration immediately after 30 min, whereas with IL-1β, a reduction in LC numbers is only observed 2 hours following injection (Cumberbatch and Kimber, 1992; Cumberbatch et al., 1997a). One explanation for this observation is that an important role of IL-1β is to induce TNF-α production by keratinocytes. In contrast, in TNF-α-induced LC migration, it is possible that there is already enough IL-1β, constitutively produced by epidermal cells, to deliver the other signal for the movement of LC away from the epidermis.

The obligatory requirement for IL-1β in the stimulation of allergen-induced migration is demonstrated by studies where neutralising anti-IL-1β antibody caused significant inhibition of this process when administered systemically to mice (Cumberbatch et al., 1997). Furthermore, treatment of mice with an anti-IL-1β antibody was able to inhibit TNF-α-induced migration. These data suggest that IL-1β is required for purposes
additional to the stimulation of TNF-α production and it is likely that this cytokine itself provides to LC a second independent stimulus for migration in an autocrine fashion. Further evidence for a central role of IL-1β in skin sensitisation in mice comes from studies indicating that a neutralising antibody to IL-1β is able to significantly suppress induction of contact hypersensitivity following intradermal injection prior to sensitisation (Enk et al., 1993b). Moreover, sensitisation is impaired in IL-1β gene knockout mice (Zheng et al., 1995; Shornick et al., 1996). In addition, it has been shown that LC express the type I receptor for IL-1β (IL-1RI) and that this receptor is necessary for LC migration (Cumberbatch et al., 1998; 1999).

Taking these data together, it can be concluded that mobilisation of LC is dependent upon receipt of two independent cytokine signals by these cells. The first signal comes from TNF-α, which is provided in a paracrine manner by keratinocytes and acts through TNF-R2 receptors. The second signal is from IL-1β, which is produced by LC and delivers an autocrine stimulus via IL-1RI receptors (Fig. 1.4).
Figure 1.4: The interactions between IL-1β, TNF-α and their receptors in LC migration. Following skin sensitisation (a), IL-1β is produced by LC providing an autocrine signal (b) and at the same time inducing TNF-α production by keratinocytes via IL-1RI (c,d). TNF-α then acts through TNF-R2 receptors on LC (e) and together with autocrine IL-1β triggers LC to migrate (f) from epidermis to draining lymph node.

IL-18

Interleukin-18 is a recent addition to the IL-1 family (Fantuzzi and Dinarello, 1999; Tsutsui et al., 2000). It was first purified from the sera or the extracts of liver tissues of mice that had been sequentially treated with P. acnes and LPS as interferon gamma inducing factor (Okamura et al., 1995a). IL-18 is synthesised as a 24 kDa inactive precursor that requires cleavage, like IL-1β, by caspase-1 in order to produce an 18 kDa mature IL-18 protein (Ghayur et al., 1997; Gu et al., 1997). Although IL-18 shows structural similarity to IL-1β, their functions are different with IL-18 sharing some biological properties with IL-12, including induction of IFN-γ production and
stimulation of NK cell activity, despite the fact that these two cytokines are structurally unrelated (Yamaguchi et al., 2001). Many cell types have been reported to produce IL-18, including macrophages, keratinocytes (Mee et al., 2000) and dendritic cells (Stoll et al., 1998; Nakanishi et al., 2001). IL-18 mRNA or protein is also seen in Kupffer cells (Seki et al., 2001), astrocytes and microglia (Prinz and Hanisch, 1999), intestinal and airway epithelial cells (Pizarro et al., 1999) and in osteoblasts (Udagawa et al., 1997). Interestingly, stimulation with FasL, can also release active IL-18 from cells, perhaps as a result of activation of caspases other than caspase-1 (Tsutsui et al., 1999).

Additionally, recently an exogenous pathway of IL-18 processing was identified in which a 29 kDa serine protease, proteinase-3, was shown to activate IL-18, albeit at a different cleavage site from that of caspase-1 (Fantuzzi and Dinarello, 1999).

The IL-18 receptor system and its signal transduction pathway are analogous to those of the IL-1R (Akira, 2000). Furthermore, toll-like receptors (TLRs) have been identified that also utilise a signalling pathway shared with IL-1R/IL-18R (Means et al., 2001). IL-18R was initially purified from a Hodgkin's disease cell line (Torigoe et al., 1997). It consists of a ligand-binding subunit, IL-1Rip, and a signal-transducing subunit, AcPL (Born et al., 1998). Recently, an IL-18 binding protein (IL-18BP) was found in the urine of healthy individuals (Novick et al., 1999). Due to the fact that IL-18BP does not have a transmembrane domain, it is secreted in a soluble form. Studies revealed that IL-18BP can inhibit binding of active IL-18 to its receptor and therefore suppress IL-18-induced IFN-γ production (Reznikov et al., 2000).

IL-18 has a wide spectrum of actions, although it was originally discovered as a factor that synergistically induces IFN-γ production from Th1 and NK cells in the presence of
IL-12. Both IL-12 and IL-18 are produced by macrophages and induce IFN-γ production from NK cells, T and B lymphocytes. IL-18 acts on Th1 cells to produce IFN-γ, IL-2 and GM-CSF. Furthermore, IL-18 contributes to elimination of virus-infected cells or tumour cells by inducing cytotoxic activity of CD8+ T lymphocytes and NK cells. A potential therapeutic usage of IL-12 and IL-18 for the treatment of allergic disorders arises from the fact that IFN-γ from IL-12 and IL-18 stimulated B lymphocytes inhibits IgE production and increases IgG production (Yoshimoto et al., 1997; Hofstra et al., 1998).

IL-18 may be involved in the pathogenesis of human skin disease. Keratinocytes express IL-18 as well as IL-1β but lack active caspase-1 under normal conditions (Stoll et al., 1997; Mizutani et al., 1991). They secrete biologically active IL-18 when stimulated by contact allergens in association with the activation of caspase-1 (Naik et al., 1999). IL-18 secreted locally may induce IFN-γ production from the dendritic epidermal T lymphocytes, particularly in collaboration with IL-12 produced by LC, resulting in an inflammatory skin reaction (Lamont and Adorini, 1996; Sugaya et al., 1999). Recently, IL-18 was additionally implicated in skin wound repair (Kampfer et al., 1999). Thus, IL-18 is emerging as a pleiotropic cytokine involved in a number of immune and inflammatory processes in skin.

Other cytokines involved in regulation of Langerhans cells function

A number of other cytokines potentially present in the epidermal microenvironment have also been implicated in the regulation of LC migration. These include IL-10, IL-12, TGFβ and GM-CSF. Data regarding these cytokines will be briefly reviewed below.
Interleukin-10 is a product of numerous cells, including Th1 and Th2 lymphocytes (Del Prete et al., 1993), cytotoxic T lymphocytes, B lymphocytes, mast cells and mononuclear phagocytic cells (Ghalib et al., 1993) and keratinocytes (Enk et al., 1995). IL-10 inhibits both production of IFN-γ and IL-2 and proliferation of Th1 lymphocytes (Fiorentino et al., 1991), IL-4 and IL-5 by Th2 cells (Del Prete et al., 1993), IL-1β, IL-6, IL-8, IL-12 and TNF-α by mononuclear phagocytes (Ralph et al., 1992; D'Andrea et al., 1993) and IFN-γ and TNF-α by NK cells (Hsu et al., 1992). In addition, IL-10 inhibits monocyte MHC class II, CD23, ICAM-1, and B7 expression. Inhibition of B7 expression may represent one of the most important biological activities of IL-10 through the resultant inhibition of the ability of the APC to provide accessory signals necessary for T lymphocyte activation (Ding et al., 1993; Schwartz, 1996).

The role of IL-10 in the suppression of cutaneous inflammation has not yet been clearly defined. IL-10 can be secreted by keratinocytes during the induction phase of contact sensitivity and may serve as a counter-regulatory molecule in contact hypersensitivity in mouse skin (Enk et al., 1993b; Enk and Katz, 1995). However, the inducibility of IL-10 synthesis by human keratinocytes is still a matter of debate. Several investigators have been unable to show any inducible IL-10 mRNA or protein expression in human keratinocytes after UVB irradiation, IL-1, TNF-α, IFN-γ or LPS treatment, while others have shown only mRNA expression after UVB irradiation without detectable IL-10 protein (Jackson et al., 1996; Teunissen et al., 1997). In contrast, other researchers have been reported that UVA and UVB irradiation can induce IL-10 mRNA and protein by human keratinocytes and suggest that the anti-inflammatory effects of IL-10 may account for the efficacy of phototherapy in inflammatory skin diseases (Grewe et al., 1995).
To attempt to resolve such discrepancies, the role of IL-10 in LC migration was examined. These studies revealed that when IL-10 knockout (-/-) mice were epicutaneously sensitised with hapten the number of LC leaving the epidermis and arriving in the draining lymph nodes were significantly greater compared to wild type mice. In addition, production of TNF-α, IL-1α and mRNA of IL-1β in the allergen-exposed epidermis was enhanced in IL-10 -/- mice and the enhanced dendritic cell accumulation could be inhibited with anti-TNF-α antibodies. Thus, these findings indicate an inhibitory effect of IL-10 in LC migration possibly via down regulation of TNF-α and IL-1 production (Wang et al., 1998; 1999). Furthermore, it was demonstrated that IL-10 inhibits and prevents the functional activity of LC and dendritic cells, in part due to regulation of the expression of certain costimulatory molecules and other membrane determinants (Steinbrink et al., 1997). Therefore these data indicate that IL-10, with respect to cutaneous immunity and inflammation in general, acts in terms of controlling these responses and ensuring that are not excessive to requirements in either magnitude or duration.

Interleukin-12 is a mononuclear phagocytic cell-derived peptide originally described as an NK stimulatory factor (Brunda, 1994; Grewe et al., 1998). The biologically active form is a heterodimer and secretion of heterodimeric IL-12 is always accompanied by production of p40 monomer and p40/p40 homodimer. The larger subunit (p40) is homologous to the soluble receptor for IL-6 and GM-CSF. Although a large number of cells appear to synthesise the small subunit (p35), p40 synthesis, which leads to the production of active IL-12, is mainly restricted to activated macrophages and dendritic cells (Stern et al., 1996; Trinchieri, 1998). The activity of IL-12 is mediated through the IL-12 receptor that consists of two subunits, IL-12Rβ1 and IL-12Rβ2 (Chizzonite et
IL-12 has been shown to be produced by LC and keratinocytes (Kang et al., 1996; Yawalkar et al., 1996). In addition, IL-12 has also been detected in spinal cord tissue, peripheral nerves and dermal nerve fibres (Turka et al., 1995).

In skin, human keratinocytes constitutively express IL-12 p35 mRNA, but the p40 mRNA that is needed for the production of the biologically active heterodimer is expressed only after exposure to contact allergen or UVB irradiation (Muller et al., 1994; Enk et al., 1996). Additionally, bioactive IL-12 is present in supernatants collected from allergen-stimulated epidermal cells (Muller et al., 1994). Since Th1 cells seem to be critically involved in delayed-type hypersensitivity and contact hypersensitivity, IL-12 may influence the fate of Th-mediated immune reactions in inflammatory dermatoses favouring Th1 responses (Aragane et al., 1994). LC maturing into dendritic cells have been shown to secrete functional IL-12 (Kang et al., 1996). Recent investigations evaluated whether the local presence of endogenous IL-12 p40 in skin is a cause or a consequence of cutaneous inflammation (Kopp et al., 2001). In these studies transgenic mice that constitutively express monomeric and homodimeric p40 in basal keratinocytes were used. These mice spontaneously developed an eczematous skin disease that was characterised by hyperkeratosis, focal epidermal spongiosis, and a mixed inflammatory infiltrate, indicating that IL-12 has a pro-inflammatory effect (Kopp et al., 2001).

Transforming growth factor-β (TGF-β) is another cytokine that is involved in skin inflammation. TGF-β represents a family of at least five peptides that regulate cell growth, having both stimulatory and inhibitory effects on different cells (Sporn and Roberts, 1993). Human keratinocytes produce TGF-β and express the type I, II and III
TGF-β receptors. TGF-β mRNA is constitutively expressed by keratinocytes and is upregulated after UVB irradiation. TGF-β induces transcription of genes for keratin 5 and keratin 14, the markers of basal cells. Transgenic mice that overexpress a dominant negative type II TGF-β receptor in the epidermis exhibit a significantly hyperkeratotic epidermis (Wang et al., 1997). Another effect of TGF-β is on LC. *In vitro* upregulation of class II antigen on the surface of murine LC by cytokines such as IL-1, TNF-α and GM-CSF is inhibited by the concomitant addition of TGF-β. Furthermore, TGF-β plays a key role in the synthesis of Birbeck granules to induce dendritic cells that phenotypically resemble LC (Geissmann et al., 1998). In addition, TGF-β -/- mice lack epidermal LC (Borkowski et al., 1996) and TGF-β causes a preference for LC production from dendritic cell precursors (Zhang et al., 1999).

Finally, GM-CSF is another pleiotropic cytokine involved in epidermal LC biology. In particular it seems to be important in the maintenance of viability of murine epidermal LC and augments their immunostimulatory activity (Burnham et al., 2000). In a murine model, the combination of anti-GM-CSF and anti-IL-3 monoclonal antibodies showed partial inhibition of the efferent or challenge phase of ACD (Piguet et al., 1991). In addition, IL-6 plasma levels were elevated after the challenge phase of ACD. Furthermore, immunohistochemical studies showed an increase in keratinocyte-bound IL-6 on serial biopsies from allergic and irritant patch test reaction sites (Oxholm et al., 1991).
1.2.6 Chemokines and the regulation of Langerhans cell migration

One of the most fundamental pathological defence mechanisms is the ability of damaged tissues to attract leukocytes in order to initiate inflammatory and immune responses, ultimately leading to structural repair. Cellular movement and positioning represent a key property of the immunocytes (Luster, 1998) and is crucial for their mobilisation and deployment to sites of pathogen challenge. Cell movement is also essential for the complex T lymphocyte, B lymphocyte and dendritic cell interactions that are needed for tolerance to self and effector responses to a variety of pathogens (Rossi and Zlotnik, 2000). One of the initial events in the inflammatory cascade is the release of primary cytokines such as IL-1 and TNF-α from damaged tissues. These primary messengers rapidly up-regulate the transcription of secondary cytokines in adjacent cells. The principal role of a subgroup of these secondary messengers, or chemokines, is chemotaxis, attracting circulating leukocytes to the sites of cellular damage. Recently, several studies have demonstrated that the traffic of dendritic cells (including LC) from the site of antigen capture to the draining lymphoid organs involves selective chemokines acting on maturing dendritic cells through chemokine receptors (Dieu et al. 1998; Sozzani et al., 1998; Sallusto et al., 1998). Thus, the structure of chemokines and their receptors along with their role in regulating LC maturation and migration will be reviewed.

Chemokine classification

More than forty chemokines have been identified to date and their activities have been defined (Rossi and Zlotnik, 2000). Chemokines are small 8-10 kDa proteins, 68-100 amino-acids in length, which share between 20 and 70% amino-acid sequence homology. The majority of family members are characterised by four positionally
conserved cysteine residues (Zlotnik and Yoshie, 2000) which link to form disulphide bonds, resulting in two connecting amino-acid loops within the folded structure of the protein. Chemokines possess a short amino terminal tail preceding the disulphide bonds and an α-helical structure positioned between the fourth cysteine residue and the carboxy-terminal (Rossi and Zlotnik, 2000).

There are four major chemokine families and their classification is based on the arrangement of the cysteine motifs. One family has two N-terminal cysteine (C) residues separated by one nonconserved amino acid residue. This family is called the CXC (X is any amino acid) or alpha chemokine family. The second family has two N-terminal, conserved cysteine residues in juxtaposition (the CC or beta chemokine family). The third family has a single cysteine residue in the conserved position (the C or gamma chemokine family). The fourth family is significantly different from the three previous families (Luster, 1998). Fractalkine, the only cloned member of this family to date, has a complex structure. It has an N-terminal, chemokine-like sequence followed by a mucin-like glycosylated stalk and a transmembrane region. The two N-terminal, conserved cysteine residues are separated by three nonconserved residues and has been called the CX3C family (Bazan et al., 1997; Luster, 1998). The majority of known chemokines, however, belong to the first two families, CXC and CC.

**Cellular sources and biologic activities of chemokines**

Chemokines are produced by a variety of cells and leukocytes and platelets are capable of secretion of most chemokines (Power et al., 1995). The exception is fractalkine, the CX3C chemokine produced by non-haematopoietic cells (Bazan et al., 1997). Monocytes are an important source of IL-8, growth factor-related oncogene (Gro), MIP-
1α and MIP-1β and monocyte chemotactic peptides (MCPs). T lymphocytes produce RANTES and small quantities of other CC chemokines. Eosinophils and basophils produce RANTES and MIP-1α (Kameyoshi et al., 1992; Li et al., 1996). Mast cells produce a number of chemokines, including MCP-1, MCP-3, MIP-1α, MIP-1β, RANTES and IL-8 (Selvan et al., 1994). Resident cells in the skin that have been shown to produce chemokines include keratinocytes, fibroblasts and endothelial cells (Gillitzer and Goebeler, 2001). Cells produce chemokines in response to a variety of factors, including viruses, bacteria, allergens or antigens, parasites, cytokines, oxidative stress, complement factors and mediators. IL-4 and IL-13 inhibit monocyctic production of chemokines (Marfaing-Koka et al., 1995).

As mentioned previously, the most important function of chemokines is chemotaxis. Other currently identified functions of chemokines include activation of inflammatory cells, antiviral immunity, immunoregulation, control of haematopoiesis, angiogenesis and other tissue cell growth and metabolism tasks. Most CXC chemokines have chemotactic activity for neutrophils. IL-8 is the best example of this activity and is considered the most potent chemotactic factor for polymorphonuclear leukocytes (Baggiolini et al., 1989; Walz et al., 1989). In addition to being chemotactic for neutrophils, IL-8 is also chemotactic for activated T lymphocytes, IL-2-activated NK cells, basophils and endothelial cells (likura et al., 2001; Inngjerdingen et al., 2001). The majority of CC chemokine family members are chemotactic for monocytes and T lymphocytes (Schall et al., 1993; Loetscher et al., 1994). Chemokines in this family include MCPs 1-4, RANTES, MIP-1α and -1β and eotaxin. Eotaxin has been reported to be a specific chemotactic factor for eosinophils, basophils and Th2 cells (Ponath et al., 1996), whereas MIP-1α is chemotactic for B lymphocytes (Alam et al., 1992).
Tables 1.2 and 1.3 summarise the main functions of selected CC and CXC chemokines (in brackets their new names are mentioned).

### Table 1.2: Selected CC chemokines and their main functions

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP group</td>
<td>Monocyte, eosinophil and T lymphocyte chemoattractant and haemopoietic stem cell inhibition</td>
</tr>
<tr>
<td>(CCL3, 4, 15, 19, 20)</td>
<td></td>
</tr>
<tr>
<td>MCP group</td>
<td>Basophil, eosinophil, monocyte and T lymphocyte chemoattractant</td>
</tr>
<tr>
<td>(CCL2, 7, 8, 13)</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>Basophil, eosinophil, monocyte and T lymphocyte chemoattractant</td>
</tr>
<tr>
<td>(CCL5)</td>
<td></td>
</tr>
<tr>
<td>Eotaxin (CCL11)</td>
<td>Eosinophil chemoattractant</td>
</tr>
<tr>
<td>TARC (CCL17)</td>
<td>Constitutive lymphoid tissue expression</td>
</tr>
</tbody>
</table>

### Table 1.3: Selected CXC chemokines and their main functions

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 (CXCL8)</td>
<td>Neutrophil chemoattractant and angiogenic</td>
</tr>
<tr>
<td>Inducible protein-10 (IP-10; CXCL10)</td>
<td>T lymphocyte chemoattractant, antitumour activity and angiostatic</td>
</tr>
<tr>
<td>MIG (CXCL9)</td>
<td>T lymphocyte chemoattractant, antitumour activity and angiostatic</td>
</tr>
<tr>
<td>Gro (CXCL1, 2, 3)</td>
<td>Neutrophil chemoattractant</td>
</tr>
<tr>
<td>Stromal cell-derived factor-1 (SDF-1) (CXCL12)</td>
<td>Monocyte and T lymphocyte chemoattractant, lymphopoiesis and myelopoiesis</td>
</tr>
</tbody>
</table>

**Chemokine receptors**

Reflecting the rapid expansion in our understanding of the chemokines, knowledge of their receptors has also grown considerably in recent years. The first chemokine receptor to be cloned was the IL-8 receptor in 1991 (Holmes et al., 1991) and more than 20 receptors have been identified to date. All chemokine receptors belong to the seven transmembrane-spanning, G protein-coupled-receptor superfamily. Most receptors bind more than one ligand, which may explain the overlapping functions of many chemokines. The exception includes IL-8 receptor type A, which only binds IL-8 with
high affinity (Holmes et al., 1991). Chemokine receptors are classified in a similar manner of that of chemokines. Thus, receptors binding to CXC chemokines are identified as CXCR, similarly, CCR for binding CC, CX3CR for binding fractalkine and CR for binding C chemokines. Tables 1.4 and 1.5 illustrate selected chemokine receptors and their ligands.

Table 1.4: CXC chemokine receptors and their ligands

<table>
<thead>
<tr>
<th>CXCR</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR-1</td>
<td>IL-8</td>
</tr>
<tr>
<td>CXCR-2</td>
<td>IL-8, Gro, NAP-2, ENA-78</td>
</tr>
<tr>
<td>CXCR-3</td>
<td>MIG, IP-10</td>
</tr>
<tr>
<td>CXCR-4</td>
<td>SDF-1</td>
</tr>
</tbody>
</table>

Table 1.5: CC chemokine receptors and their ligands

<table>
<thead>
<tr>
<th>CCR</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR-1</td>
<td>MIP-1α, RANTES, MCP-1, MCP-3</td>
</tr>
<tr>
<td>CCR-2</td>
<td>MCP-1, MCP-3, MCP-4</td>
</tr>
<tr>
<td>CCR-3</td>
<td>Eotaxin, RANTES, MCP-2, MCP-3, MCP-4</td>
</tr>
<tr>
<td>CCR-4</td>
<td>MIP-1α, RANTES, MCP-1</td>
</tr>
<tr>
<td>CCR-5</td>
<td>MIP-1α, MIP-1β, RANTES</td>
</tr>
</tbody>
</table>

In their primary role as mediators of leukocyte chemotaxis, the constitutive expression of chemokine receptors reflects the chemoattractant activity of their ligands. For example, CXCR1 is expressed predominantly on neutrophils and binds almost exclusively to IL-8, a potent neutrophil chemoattractant (Godaly et al., 2001). In contrast, CCR2, whose CC chemokine ligands include the MCP family, is widely expressed on haemopoietic cells, including basophils, monocytes, T lymphocytes, dendritic cells and NK cells (Andjelkovic et al., 2000). While certain receptors are constitutively expressed on leukocytes, others may be induced or down-regulated by inflammatory mediators, indicating mechanisms by which the inflammatory process may be controlled or directed. For example, CCR1 and CCR2, the receptors for MIP-
1α, MCP isoforms and RANTES, are only expressed on T lymphocytes following stimulation by IL-2 (Sallusto et al., 1999). Chemokines receptors are also differentially expressed on activated Th1 and Th2 T lymphocyte sub-types. Th1 cells predominantly express CXC receptors and Th2 cells CC receptors, hence permitting selective amplification of Th1 or Th2 responses by different chemokines. Thus, the expression of chemokine receptors seems crucial in the development and control of inflammation.

**Evidence for a role of chemokines in Langerhans cell migration**

Chemokines and their receptors are crucial elements for controlling directional migration of LC and dendritic cells. *In vitro* studies have demonstrated that a variety of chemokines induce migration of LC and dendritic cells (Xu et al., 1996; Sozzani et al., 1997). These chemokines include the CC chemokines, such as MIP-1α, MIP-1β, MCP-1 to MCP-4, RANTES, MDC and CXC chemokine SDF-1 (Sozzani et al., 1995; Godiska et al., 1997; Yamazaki et al., 1998). Dendritic cells express a variety of chemokine receptors, including CCR1, 2, 3, 6, 7 and CXCR1, 2, 4 (Zaitseva et al., 1997; Greaves et al., 1997).

It is therefore reasonable to consider that chemokines may play a role in attracting skin-derived dendritic cells to draining lymph nodes. Of particular importance is the CCR7 receptor, which as mentioned previously, is up-regulated in maturing dendritic cells and permits them to respond to CCR7 ligands (Sozzani et al., 1998). Recent studies using mutant mouse model have demonstrated that CCR7-ligand interactions are important for homing of LC to skin-draining lymph nodes (Gunn et al., 1999). Mice with a knock out of secondary lymphoid tissue cytokine (SLC), a CCR7 ligand, have a major defect in accumulation of antigen-bearing dendritic cells in draining lymph nodes following
hapten application (Gunn et al., 1999). However, the ability of LC to migrate from the epidermis was normal. Thus, SLC is required for effective movement of dendritic cells from lymphatic vessels to the paracortex of lymph nodes (Gunn et al., 1999). This assumption was strengthened by further studies where anti-SLC antibodies inhibited the accumulation of skin-derived dendritic cells in draining lymph nodes (Saeki et al., 1999). Furthermore, it was demonstrated that resting LC did not express mRNA for CCR7, but cytokine-activated cells did. TNF-α intradermal administration in mice induced expression of mRNA for CCR7 by LC (Saeki et al., 1999). These data provide evidence for complex interactions between cytokine and chemokine signals in order to stimulate LC migration. TNF-α production in the skin is induced in response to skin sensitisation, skin irritation or cutaneous infection. This cytokine in turn provides one stimulus for LC migration by inducing, among other changes in LC, an increase in the expression of CCR7 receptors.

Another chemokine involved in the regulation of LC migration is MIP-3α which acts through CCR6, a receptor mainly expressed on dendritic cells and lymphocytes. This chemokine appears to be the most powerful chemokine seen to date in inducing migration of LC precursors and freshly isolated LC but not any other dendritic cell population (Dieu-Nosjean et al., 2000). Like most other chemokines acting on LC, MIP-3α is inducible on inflammatory stimulation. Interestingly, mature dendritic cells lose their responsiveness to MIP-3α, presumably through receptor down-regulation or desensitisation. MIP-3α mRNA has been reported to be detected only within inflamed epithelial crypts of tonsils, a site of antigen entry known to be infiltrated by immature dendritic cells (Rossi et al., 1997).
Conversely, some chemokines are believed to signal in order to recruit dendritic cells to normal, non-inflamed tissue (Cyster, 1999). Therefore, for effective LC migration this kind of chemokine-ligand interaction should be decreased, and the corresponding chemokine receptor expression down-regulated. Such a scenario is possible with the observation that the induced maturation of cultured LC was associated with a rapid decrease in the expression of two CC chemokine receptors, CCR1 and CCR5. In these experiments the reduced expression of these receptors was affected by, amongst other activation stimuli, IL-1 and TNF-α (Sozzani et al., 1998).

Thus, the mobilisation of LC and their directed migration from epidermis to draining lymph nodes are processes of major importance in the initiation of cutaneous immune responses. These processes are regulated by the interactions between cytokines and chemokines and their associated receptors. Following local trauma in the epidermis, LC will respond and migrate to draining lymph nodes, where the initiation of immune responses will occur. Two independent cytokine signals are known to be required for the initiation of LC mobilisation, supplied by TNF-α and IL-1β. The same cytokines will stimulate alterations in adhesion molecules and increases of the expression of CCR7 receptor, together with downregulation of CCR1 and CCR5 receptors. These changes will liberate LC from the influence of local chemokines and allow them to dissociate from surrounding keratinocytes and migrate via afferent lymphatics to draining lymph nodes. This process of LC migration may be subject to counter-regulation by IL-10 (Fig. 1.5).
Figure 1.5: The interactions between cytokines and chemokines in LC migration. TNF-α and IL-1β are the two cytokine signals which are required for the initiation of LC mobilisation. These cytokines are induced or up-regulated following various forms of cutaneous trauma. Together TNF-α and IL-1β induce the changes in LC necessary for their emigration from the skin and directed movement to draining lymph nodes. Such changes include downregulated expression of chemokine receptors CCR1 and CCR5, upregulation of chemokine receptor CCR7 and altered expression of various adhesion molecules. The process of LC migration may be counter-regulated by IL-10, another epidermal cytokine, acting via by inhibiting TNF-α and IL-1β production. +: induction, -: inhibition (modified from Kimber et al., 2000).
1.3 LANGERHANS CELLS IN INFLAMMATORY SKIN DISEASE

The skin is the primary interface between the body and the environment. The spectrum of insults to which skin is susceptible includes disorders caused by chemical and microbial agents, thermal and electromagnetic radiation, and mechanical trauma. The most damaging consequence of the disruption of skin is invasion by pathogenic microorganisms, and the need for an effective means of protection against this challenge has been a fundamental force behind the evolution of the immune system. The translation of insults into cutaneous inflammation (innate immunity) and the potential recruit to memory T lymphocytes that will clonally expand in response to antigens encountered at the cutaneous interface with the environment (acquired immunity) are both required for successful cutaneous immune surveillance (Murphy et al., 2000).

Certain memory T lymphocytes appear to remember the anatomical site where they first encountered antigen. Specifically, there is an identifiable subgroup of memory T lymphocytes with the ability to circulate preferentially to the skin. These memory T lymphocytes, identified by a marker known as cutaneous lymphocyte-associated antigen (CLA), are generated in lymph nodes draining skin and are recruited back there during inflammation. Although their primary function is cutaneous immune surveillance, CLA-positive T lymphocytes have been implicated in the pathogenesis of many common skin diseases, including allergic contact dermatitis, psoriasis and atopic dermatitis (Robert and Kupper, 1999). Recent evidence suggest that the skin associated CC chemokine, CCL27 (Morales et al., 1999) and its receptor CCR10 (Homey et al., 2000) provide a skin specific signal for the recruitment of CLA+ve T lymphocytes to normal or inflamed skin (Homey et al., 2002).
Although each of these disorders can be viewed as an example of inappropriate cutaneous immune surveillance, their clinical manifestations and courses are determined by several factors: the functional phenotype and cytokine profile of the antigen-specific T lymphocyte, the type of antigen (e.g. pathogen, auto-antigen, or contact sensitising antigen), and the genetic background of the person.

1.3.1 Contact dermatitis

The two most frequent manifestations of skin toxicity are irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). Depending on the country, skin problems account for 20 to 70% of all occupational diseases, and between 20 and 90% of these are ICD, while most of the remaining contact dermatitis cases represent ACD (Wahlberg, 1996). Whereas ICD is a form of skin inflammation induced by primary contact with chemicals and is not thought to be driven by lymphocytes, ACD represents a lymphocyte-mediated delayed type hypersensitivity reaction that requires previous sensitisation by the same chemical(s). Contact dermatitis is a complex phenomenon that involves resident epidermal cells, fibroblasts of dermis and endothelial cells as well as invading leukocytes interacting with each other under the control of a network of cytokines and lipid mediators (Bos and Kapsenberg, 1993).

The primary lesions seen on physical examination are the same as for any other type of dermatitis. In the acute phase, erythema, edema, and vesiculation may be seen. Pruritus is usually very intense in this stage (Wahlberg, 1996). Over a period of hours to a few days, vesicles break open, leading to exudation, crusting, and increased scaling. In more chronic forms of dermatitis, the scaling is more pronounced and the skin is generally thickened in the affected areas. More than half of all cases of contact
dermatitis involve the hands (Lomholt et al., 2001). This is simply due to the fact that exposure to exogenous substances is more likely to occur here. ICD is most common over the fingertips, whereas ACD most frequently occurs on the dorsal side of the hands, where the skin is generally thinner and percutaneous absorption of allergens is facilitated (Wahlberg, 1996; Lomholt et al., 2001). The face is another very common site for contact dermatitis, and facial dermatitis is often related to the use of cosmetics that contain preservatives, fragrances, pigments and other potential sensitizers (Willis et al., 2001).

The first principal of management is to make an accurate diagnosis so that the causal agent can be removed from the patient’s environment. Patch testing is the most useful tool in the diagnosis of ACD (Wahlberg, 1998) and is the only way of objectively demonstrating that a given allergen is capable of inducing ACD in the patient. Patch testing attempts to artificially reproduce the circumstances that created the initial ACD. As such, it has certain limitations but in experienced hands, patch testing has a sensitivity and specificity of ~70% (Holness and Nethercott, 1997).

Relatively mild contact dermatitis of limited extent is treated topically. The mainstay of topical treatment consists of the application of topical corticosteroids of sufficient potency and within an appropriate vehicle. Systemic treatment is reserved for more severe cases or very extensive involvement (Levin and Maibach, 2001a). Systemic corticosteroids are the agents of choice and should be administered for as brief a time as possible because of their associated toxicity. Physical modalities of therapy are also occasionally useful. Ultra-violet light, UVB monotherapy, or UVA phototherapy in
combination with oral or bath psoralens (PUVA therapy) may be considered (Kalimo et al., 1989).

**Allergic contact dermatitis**

ACD is a cell-mediated or type IV immune reaction, which occurs after the exposure of susceptible individuals to sensitising chemicals and is dependent on the induction of a specific immune response (Xu et al., 2000). Contact hypersensitivity is characterised by an eczematous reaction at the point of contact with an allergen. It typically develops in two temporally distinct phases: i) the induction phase and ii) the elicitation phase (Xu et al., 2000). In susceptible individuals, exposure to allergen will induce the immune response necessary for sensitisation. Sensitisation takes 10-14 days in humans. Re-exposure to the same antigen will result in elicitation of the inflammatory reaction after a characteristic delay of usually 12 to 48 hours. The nature of the immune responses induced by chemical allergens is essentially no different from that which characterises protective immunity. ACD is a multifunctional disease, the onset of which depends on the nature of the chemical, concentration, type of exposure, age, sex and genetic susceptibility (Kimber and Gerberick, 1999).

Cutaneous antigens are generally of low molecular weight. It is clear that some allergens are very potent sensitisers and appear able to induce sensitisation in all normal people, whereas others (e.g. nickel salts) are weak antigens and appear only to sensitise susceptible individuals. The first step in the allergic process is the absorption of the hapten into the skin and covalent binding to a carrier protein. The antigen-carrier complex is then processed and presented associated with MHC class II molecules to histocompatible T lymphocytes by LC (Gerberick et al., 2001). As mentioned
previously, LC migrate from the epidermis through the dermis into regional lymph nodes via afferent lymphatic vessels. Then LC enter the paracortical area of regional lymph nodes and present the antigen to CD4+ve T lymphocytes. If the chemical is recognised by the immune system as non-self, a clonal expansion takes place and two populations of sensitised cells are formed, effector T lymphocytes, which travel to the skin surface in the peripheral blood, and long lived memory T lymphocytes, which proliferate to form new populations of sensitised cells on re-exposure to the antigen.

**Irritant contact dermatitis**

Skin irritation is defined as a non immunological local reversible inflammatory reaction, characterised by erythema and edema following a single or repeated exposure of chemical to the identical cutaneous site (Effendy et al., 2000). Acute ICD is characterised predominantly by inflammation, while chronic ICD is characterised by hyperproliferation and transient hyperkeratosis. When chemicals such as sodium dodecyl sulfate (SDS) or phenol is applied to the skin, an ICD reaction occurs. However, in contrast to haptens, irritants do not activate the immune cascade via the antigen presentation pathway. Primary skin irritation results in damaged keratinocytes, mononuclear cell infiltration and apposition of lymphocytes with LC (Sjogren and Anderson, 2001). Although the cellular mechanisms of ICD remain unknown, increasing evidence suggests that activated keratinocytes act as signal transducers in controlling the host homeostatic responses to exogenous stimuli.

Despite their mechanistic differences, ACD and ICD are often indistinguishable both clinically and histologically (Kaur et al., 2001). In ACD, the earliest cutaneous changes consist of extravasation of lymphocytes and other mononuclear cells in the uppermost
part of the dermis. Later changes include mononuclear cell migration into the epidermis, spongiosis, and vesiculation (Zhang and Tinkle, 2000). Approximately 24 h after hapten application, basophils appear, and by day 3, tissue mast cells increase in number. The presence of epidermal or dermal eosinophils is highly suggestive of ACD. In ICD, lymphocytes are relatively rare, and the bulk of the inflammatory infiltrate consists of neutrophils (Senaldi and Piguet, 1997).

Cytokine profiles in contact dermatitis

ICD and ACD are two similar appearing diseases and, as mentioned above, differentiating between them can be difficult clinically. Several studies have suggested that distinct cytokine profiles may be useful tools for differentiation of ICD and ACD (Muller et al., 1996). Early evidence for a distinct regulation of LC activation by irritants and allergens was provided by a study which investigated phenotypic and functional changes of in-vivo activated LC (Aiba and Katz, 1990). In another study, the early events that occur following allergen treatment of the skin of non-sensitised mice were investigated in an attempt to identify cytokine production that may be critical in the induction of ACD (Enk and Katz, 1992). This study demonstrated by semi-quantitative RT-PCR that only contact allergens up-regulated keratinocyte-derived IL-1α, IP-10, MIP-2 and LC-derived IL-1β and I-A mRNAs. Both irritants and allergens induced the expression of TNF-α, GM-CSF and IFN-γ. These studies underscore the complex array of signals that characterise the induction phase of ACD and suggest that certain cytokines might serve as differentiating markers for ACD and ICD. Furthermore, p40 chain mRNA of IL-12, a cytokine important for the development of Th1 responses, was shown to be induced only following allergen challenge (Trinchieri, 1993). Irritants failed to induce p40 chain mRNA and therefore release of bioactive IL-
12 from epidermal cells. Taken together these studies suggest that ICD and ACD, at least in mice, can be differentiated by their respective cytokine patterns.

1.4 AIMS

The evidence discussed so far indicates that the stimulation of LC migration from skin and their subsequent accumulation as antigen-presenting cells in draining lymph nodes are dependent upon the availability of signals provided by epidermal cytokines, particularly IL-1β and TNF-α.

IL-1β requires cleavage by the cysteine protease caspase-1 in order to release its biologically active form, a 17 kDa molecule. In view of the role of caspase-1 in regulating the processing and release of IL-1β, we hypothesised that this protease may represent a useful target for manipulation. To this end, in the first part of the project a number of approaches were used to define the role of capsase-1 in experimental LC migration and induction of contact hypersensitivity.

After demonstrating that functional caspase-1 is required for LC migration and optimal contact sensitisation, the next question of this thesis involves the role of IL-18, a cytokine structurally similar to IL-1β, in regulation of LC migration and the induction of cutaneous immune responses. There are several reasons why it is relevant to question the potential influence of IL-18 on LC mobilisation. Firstly, IL-1β and IL-18 are not only structurally similar, but also they both require enzymatic cleavage by caspase-1 in order to release their active forms. Secondly, both dendritic cells
(including LC) and keratinocytes have been shown to express IL-18. Thirdly, it has been reported that IL-18 release by keratinocytes can be enhanced by chemical allergens and elevated expression of this cytokine has also been observed in contact hypersensitivity. Furthermore, the relationship between IL-1β, TNF-α and IL-18 in LC migration and contact hypersensitivity remained to be determined.

Lastly, the presence of several inhibitory IL-1 molecules within the epidermis suggest that tightly-co-ordinated regulation of this cytokine system is essential for the cutaneous microenvironment. Although counter-regulation of IL-1 and its inhibitors has long been suspected, formal evidence for this is lacking. Thus, in the final chapter, the suspected homeostatic mechanisms were assessed using a murine keratinocyte line and mice over-expressing either IL-1α or secreted IL-1ra (sIL-1ra) in basal keratinocytes both in vitro and in vivo in the epidermis.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Cytokines, antibodies and other reagents

Recombinant murine IL-1β (specific activity 1-2 x 10^8 U/mg), IL-18 (specific activity 1-2 x 10^8 U/mg) and TNFα (specific activity 2 x 10^8 U/mg by L929 cytotoxicity assay) were purchased from R & D Systems (Oxon, UK), PeproTech EC Ltd. (London, UK) and Genzyme Diagnostics (West Malling, Kent, UK) respectively. Cytokines were diluted in sterile PBS containing 0.1% BSA as carrier and were administered locally by intradermal injection into ear pinnae (30 μl). Biotin-conjugated rat anti-mouse I-A^d/I-E^d monoclonal antibody (clone 2G9) and streptavidin-FITC conjugates were purchased from Pharmingen (San Diego, CA). Caspase-1 inhibitor II (Ac-YVAD-cmk) and caspase-3 inhibitor III (Ac-DEVD-cmk, a YVAD analogue which does not inhibit caspase-1) were from Calbiochem (Beeston, Nottingham, UK). PAM212 keratinocytes were a gift from Dr Stuart Yuspa (NIH, Bethesda, USA). Recombinant mIL-1α, mIL-1ra and goat anti-mouse IL-1ra antibody were purchased from R&D Systems. Anti-CD45 antibody was purchased from Calbiochem. Recombinant human sIL-1RI was a gift from Dr John Sims (Immunex Corp., Seattle, USA). The monoclonal antibodies against murine IL-1RI (35F5) and murine IL-1RII (4E2) were kind gifts from Dr Richard Chizzonite (Hoffman-La Roche, Nutley, USA). All other reagents, including contact sensitisers 2, 4-dinitrofluorobenzene (DNFB) and oxazolone (OX), and skin irritant sodium lauryl sulphate (SLS) were purchased from Sigma Chemicals Ltd. (Poole, Dorset, UK) unless otherwise indicated.
2.1.2 Animals

Transgenic mice used in the present studies overexpressing IL-1α (IL-1.2) have been described previously (Groves et al, 1995b). To generate mice that overexpress IL-1ra in basal epidermis, cDNA encoding the secreted form of murine IL-1ra (a gift from Dr Alexander Whitehead, University of Pennsylvania School of Medicine, Philadelphia, USA; (Zahedi et al, 1991)) was cloned by blunt-end ligation into the BamH1 site of the K14-hGH vector as previously described (Groves et al, 1995b). Transgenic mice were generated in the FVB strain by pronuclear microinjection of the K14-IL-1ra-hGH construct and two founder lines were identified by Southern blot analysis, designated RA1 and RA10. Both lines passed the transgene in a Mendelian fashion and the skin of these mice was both grossly and histologically normal. All transgenic animals had previously been generated by Professor Groves and they were a kind gift from Professor Kupper, Harvard Skin Disease Research Center, Boston, USA.

IL-18 knockout (IL-18 -/-) and wild type (WT) mice have previously been described in detail (Wei et al., 1999) and they were a kind gift from Professor Liew, Department of Immunology and Bacteriology, University of Glasgow. Caspase-1 knockout (caspase-1 -/-) and WT control mice were the kind gift of Dr. Wong, BASF Corporation, Worcester, MA and have also been described in detail (Li et al., 1995; Li et al., 1997). For caspase inhibitor experiments, female Balb/c mice were purchased from Harlan. All animals were housed in a conventional animal facility with a 12-hour light/dark cycle. Mice were used between 8 & 12 weeks of age and in individual experiments were age-matched to within 2 weeks. All experiments were carried out under provisions of the Animals (Scientific Procedures) Act, 1986.
2.2 GENERAL METHODS

2.2.1 Genotyping

All animals used in this project were analysed for presence or absence of incorporated transgene. Selected mice were scruffed and ear marked for identification, prior to dissection of 0.2 cm of tail tip. Tail tips were transferred to individually labeled 0.5 ml eppendorf tubes and stored at -20°C. Upon removal from storage, genomic DNA was prepared and incorporated transgene detected by specific polymerase chain reaction (PCR).

2.2.2 Genomic DNA preparation

Tissue samples were incubated in 20 μl of crude DNA extraction buffer (50mM Tris-HCl, pH 8.0; 20mM NaCl; 1mM EDTA; 1% SDS) containing 0.5 mg/ml proteinase K (Roche) at 55°C for 1 hour. 180 μl of ddH₂O was added and solutions were heated at 99°C for 5 minutes, prior to cooling on ice. Crude genomic DNA samples were stored at -20°C.

2.2.3 PCR amplification

PCR amplification reactions were prepared as mastermixes and consisted of the following reagents per reaction: 35.5 μl of ddH₂O, 5 μl of 10x PCR buffer (500mM KCl, 200mM Tris-HCl pH 8.4; Gibco), 2 μl of MgCl₂ (50mM; Gibco), 4 μl of dNTP mix (dATP, dCTP, dGTP and dTTP at 2.5mM each; Promega) and 1 μl of each primer (diluted to 100 pmole/ul in ddH₂O). 0.5 μl of taq DNA polymerase (5 U/μl; Gibco) and 1 μl of DNA template (150 pg of plasmid DNA, 500 ng of genomic DNA) were then
added to each reaction prior to thermocycling using specific PCR conditions as outlined below (Table 2.1).

Table 2.1: PCR conditions

<table>
<thead>
<tr>
<th>Target Sequence</th>
<th>Primer Sequence</th>
<th>Program</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human keratin 14 promoter (K14)</td>
<td>K14-for: 5' CACGATAACCTGACTAGCTGGGTG 3'</td>
<td>94°C 5' (x1) 94°C 30&quot; 60°C 30&quot; (x35) 72°C 30&quot;</td>
<td>514</td>
</tr>
<tr>
<td></td>
<td>K14-rev: 5' CATCACCCACAGGCTAGCGCCAAC 3'</td>
<td>72°C 5' (x1)</td>
<td></td>
</tr>
</tbody>
</table>

Primer sequences are shown as forward (sense) primer above reverse (antisense) primer. Size=PCR product size (bp); *=minutes; **=seconds; (xN)=number of cycles.

2.2.4 Agarose-gel electrophoresis

A 1% (w/v) agarose (molecular biology grade, Roche) solution in 1x TBE was heated for 4 minutes in a 500W microwave oven. 2.5 µl of a 10mg/ml ethidium bromide solution was added to 200 ml of agarose solution which was left to cool at room temperature for 30 minutes. A gel tray was prepared by sealing both ends with autoclave tape and positioning a comb approximately 1cm from the end of the tray. The agarose solution was then poured into the prepared tray and left to set at room temperature for 30 minutes. Loading samples were prepared by addition of 15% (v/v) of appropriate loading dye (either bromophenol blue 40% (w/v) sucrose solution in 1x Tris borate ethylenediaminetetra-acetic acid (TBE) with 0.16% (w/v) bromophenol blue crystals) for products <1200 base pairs (bp), or xylene cyanol (as bromophenol blue, but
with 0.16% (w/v) xylene cyanol crystals) for products < 1200 bp. 100 bp DNA ladder (Roche) (0.25 μm/μl, comprising of fragments between 100 and 1500 bp increments and an additional band of 2642 bp) was also prepared by mixing the stock DNA ladder solution with bromophenol blue in a 2:1 ratio. The set gel was transferred to an electrophoresis tank (DNA Mini or Midi Sub Electrophoresis Tnaks, Biorad) which was filled with 1x TBE to a level approximately 5 mm above the gel surface. 20 μl of each sample and 5 μl of DNA ladder was then loaded into the appropriate wells and the gel run at 95V for 90 minutes. DNA was visualized under ultra-violet (UV) transillumination and the results photographed (Polaroid 677 print film).

2.2.5 Animal anaesthesia

Preparation of WT, knockout and transgenic animals for non-surgical procedures was performed by placing individual animals in a dedicated anaesthetic chamber supplied with inhalational anaesthetic (Halothane Ph. Eur.; Merial Animal Health Ltd) via a series 5 T.C. Vaporiser (IMS). Animals were closely observed until the desired level of anaesthesia was attained.

2.2.6 Non-reversible anaesthesia

Sacrifice of animals to facilitate tissue harvest was performed by placing individual animals in a dedicated anaesthetic chamber supplied with inhalational anaesthetic (Halothane Ph. Eur.; Merial Animal Health Ltd) via a series 5 T.C. Vaporiser (IMS). Animals were left in the chamber for 5-10 minutes and then their neck was dislocated to ensure death.
2.2.7 Statistical analysis for all experiments performed in this thesis

The statistical significance of differences of means of experimental groups was calculated using a two-tailed student’s t-test. Mean differences were considered to be significantly different when p<0.05. All data are presented as mean +/- SEM and error bars are indicated on figures where SEM > 5% of the mean.

2.3 IMMUNOLOGICAL METHODS

2.3.1 Immunohistochemical staining of LC

LC numbers and morphology were evaluated en-face in epidermal sheet preparations using a published protocol (Cumberbatch et al., 1994). Briefly, ears were harvested and split into dorsal and ventral halves using fine forceps. Dorsal ear halves were then incubated in 0.02M ethylenediamine tetra-acetic acid (EDTA) at 37°C for 90 minutes to allow separation of epidermis and dermis. Epidermal sheets were carefully peeled away from dermis, washed twice in phosphate-buffered saline (PBS), fixed in acetone at -20°C for 20 minutes and washed again. Epidermal sheets were then incubated with rat anti-mouse I-A^d/I-E^d antibody diluted to 5μg/ml in PBS with 0.1% bovine serum albumin (BSA) for 45 minutes at room temperature, washed and subsequently incubated for a further 45 minutes with FITC-labelled streptavidin diluted 1:100 in PBS with 0.1% BSA. Following further washing, epidermal sheets were mounted whole in glycerol, slides were coded and LC were counted in 10 high power fields in the central portion of the ear using an eye-piece with a calibrated grid (0.25mm x 0.25mm at x 40 magnification) and a fluorescence microscope (Axiophot, Zeiss). Codes on slides were
covered with mask tape prior enumeration ensuring that LC numbers were counted without known the nature of each slide. Results are expressed as mean +/- SEM numbers of LC/mm² epidermis. At least four ears were used per experimental group.

2.3.2 Flow cytometry analyses of bone marrow derived DC

Approximately $10^5$ DC were incubated in round-bottomed 96-well plates for 30-45 min on ice with monoclonal antibodies directed against I-A^d/I-E^d (clone 2G9, rat IgG2ak; Pharmingen), CD11c (clone HL3; Pharmingen), DEC205 (clone GL9; Pharmingen) or isotype control (clone G235-2356, hamster IgG; Pharmingen), each diluted to 5 μg/ml in RPMI-FCS. Cells were used washed twice in cold Roswell Park Memorial Institute medium (RPMI)-Fetal calf serum (FCS) by centrifugation for 5 minutes (300g) at 2-8°C and the supernatant discarded. DC were then incubated for a further 30-45 minutes on ice with FITC-conjugated F(ab')₂ goat anti-rat IgG diluted 1:100 in RPMI-FCS. Finally, the cells were washed and resuspended in 0.4ml cold BSA (0.5%)/PBS/sodium azide (0.05%) and retained on ice prior to analysis using a FACSCalibre™ Flow Cytometer (Becton Dickenson).

2.3.3 Enzyme Linked Immunoabsorbent Assays (ELISAs)

The reagents for these experiments were provided from Dr Steve Poole, Division of Endocrinology, NIBSC, UK. For the measurement of mIL-1ra protein, 96 well plates (‘Maxisorp’, NUNC, Roskilde, Denmark) were coated with a polyclonal sheep anti-rat IL-1ra antibody at 2 μg/ml in bicarbonate coating buffer (0.1M NaHCO₃, 0.1M NaCl, pH 8.2) overnight at 4°C. Non-specific binding sites were blocked with 1% bovine serum albumin (Sigma) for 1 hour at room temperature. Samples or standards prepared
from rmIL-1ra (R&D Systems) were added at 100 µl/well and incubated for 2 hours at room temperature. Following washing (wash buffer; 0.5M NaCl, 2.5 mM NaH2PO4, 7.5 mM Na2HPO4, 0.1% v/v Tween 20), 100 µl of biotinylated polyclonal sheep anti-rat IL-1ra antibody (1:1000 dilution in wash buffer) was added to each well and incubated for 1 hour at room temperature, followed by a further wash. Detection was achieved with 100 µl avidin-HRP (1:5000 dilution in wash buffer; Dako) for 30 minutes, followed by addition of 100 µl of substrate solution (1 OPD tablet (Sigma) in 10 ml substrate buffer (34.7 mM citric acid, 66.7 mM Na2HPO4) + 2 µl H2O2) for 20 minutes and termination with 150 µl 1M H2SO4 prior to measurement of absorbance at 490 nm. The IL-1ra ELISA was sensitive to 50 pg/ml. IL-1ra concentrations obtained from the ELISA were normalised to total protein content in each sample for comparison.

Murine IL-1α was assessed utilising a similar protocol, using a polyclonal sheep anti-mouse IL-1α antibody at 0.5 µg/ml for coating and a biotinylated polyclonal sheep anti-mouse IL-1α antibody at 1:1000 dilution in wash buffer as a secondary antibody. Standards were prepared from rmIL-1α (R&D Systems) and the IL-1α ELISA was sensitive to 30 pg/ml. IL-1α and IL-1β at concentrations up to 1 µg/ml were not detected in the ELISA for mouse IL-1ra, and IL-1β and IL-1ra at concentrations up to 1 µg/ml were not detected in the ELISA for mouse IL-1α.
2.4 *IN VIVO* TECHNIQUES

2.4.1 Contact hypersensitivity protocol

Groups of at least three mice were sensitised by application of 150 μl 0.5% DNFB in acetone/olive oil (AOO; 4/1) to abdominal skin. Five days later, 10 μl of 0.25% DNFB was applied to dorsal and ventral surfaces of the right ear and AOO alone was applied to the left ear. Twenty-four, 48 & 72 hours later, challenge-induced ear swelling (relative to the vehicle-treated ear) was measured using a modified (spring loader was taken off) micrometer (Mitutoya Inc., Japan). In parallel experiments, OX was used in the same volumes and diluent at concentrations of 1% (sensitisation) and 0.5% (elicitation). In experiments to assess the effect of caspase-1 inhibitors on CHS, mice were pretreated with 300 μl of 400 μM Ac-YVAD-cmk, Ac-DEVD-cmk, or vehicle (DMSO) 1 hour before application of sensitisers and 0, 2, 4, 6 and 8 hours after sensitisation. Care was taken to ensure that sensitiser was only applied to the pretreated area of skin. The final concentration and the time of application of inhibitors had to be optimised. For optimization experiment group of mice (n=5) were pre-treated with 100, 200, 300, 400, 500 and 600 μM of Ac-YVAD-cmk prior to sensitisation with 0.5% DNFB and ears were challenged with 0.25% DNFB 5 days later as described above. Ear swelling was measured at 24 hours and the effect of Ac-YVAD-cmk was compared with untreated mice used as controls. As illustrated in Figure 2.1 there was minimal inhibition of ear swelling with 100, 200 or 300 μM of Ac-YVAD-cmk. Significant inhibition of ear swelling response occurred with 400, 500 or 600 μM of the inhibitor (with no difference between them). Thus, it was decided that a concentration of 400 μM was optimal for these series of experiments.
Figure 2.1: Optimisation of Ac-YVAD-cmk concentration in CHS experiments. Groups of mice (n=5) were pre-treated with different concentration of Ac-YVAD-cmk prior to sensitisation with 0.5% DNFB. 5 days later ears were challenged with 0.25% DNFB and ear swelling was measured after 24 hours. Pre-treatment with 100, 200 or 300 mM of Ac-YVAD-cmk resulted in minimal suppression of the ear swelling response. In contrast, application of 400, 500 or 600 mM of Ac-YVAD-cmk suppressed ear swelling > 55% compared to untreated mice. Data shown are mean +/- SEM of three experiments.

2.4.2 Intradermal injections of IL-18 or IL-1β cytokine experiments prior to sensitisation with contact allergens

In these experiments, the dorsal surface of the right ear was pre-treated by intradermal injection of 30 μl of IL-18 (50ng), IL-1β (50ng) or diluent alone 30 minutes prior to sensitisation with allergen (30μl 1% OX or 0.5% DNFB). 5 days later, the left ear of the mice was challenged with 30μl 0.5% OX or 0.25% DNFB respectively and ear swelling (relative to pre-challenge) was measured as described above.

2.4.3 Irritant dermatitis

Groups of at least three mice received 25 μl of the skin irritant SLS (10%) dissolved in dimethylformamide (DMF) on the dorsal and ventral surfaces of the right ear and DMF alone to the left ear. At intervals thereafter (2, 6 and 24 hours), ear swelling (relative to the DMF-treated ear) was measured using a modified spring-loaded micrometer (Mitutoya Inc., Japan).
2.4.4 LC migration in response to DNFB or OX

Dorsal mouse ear skin (four ears per experimental group) was painted with DNFB (0.5% in AOO) or OX (1% in AOO) and harvested 4 hours later. Epidermal sheets were then prepared, immunostained and LC were counted as described above. In some experiments ear skin was pre-treated with topical application of 10μl caspase inhibitors Ac-YVAD-cmk or Ac-DEVD-cmk (200μM in DMSO) 1 hour prior to application of DNFB.

2.4.5 LC migration in response to IL-1β and TNF-α

Cytokines were either supplied as, or reconstituted in, sterile solutions of PBS containing 0.1% (BSA) as carrier protein. Mice (n=3 per group) received 30 μl (50ng) intradermal injections of cytokine into both ears. Controls included mice that had received either an equivalent volume of diluent with carrier protein alone or that were untreated. Ears were harvested either 30 min after TNF-α injection or 4 hours after IL-1β injection and processed for immunohistochemical evaluation of LC numbers. Previous studies had shown these intervals to be optimal for evaluation of cytokine-induced LC migration in mice (Cumberbatch et al., 1997).

2.4.6 Isolation and enumeration of lymph node dendritic cells

Draining (auricular) lymph nodes were excised at various periods following treatment. Nodes were pooled for each experimental group, and single-cell suspensions of lymph node cells (LNC) prepared by mechanical disaggregation through 200-mesh stainless steel gauze. LNC were washed with, and suspended in, RPMI-1640 growth medium (Gibco) supplemented with 25 mM HEPES, 400 ug/ml streptomycin, 400 ug/ml
ampicillin and 10% heat-inactivated FCS (RPMI-FCS). Viable cells counts were performed by exclusion of 0.5% trypan blue and the cell concentration adjusted to 5x10^6 cells/ml in RPMI-FCS. DC-enriched populations were prepared using a protocol that has been published by Macatonia et al., 1986. Briefly, 2 ml of Metrizamide (14.5% in RPMI-FCS; Sigma) was layered gently under 8 ml of the cell suspension, and tubes centrifuged for 15 minutes (600g) at room temperature. Cells accumulating at the interface were collected, washed once, and resuspended in RPMI-FCS. The frequency of DC in such low buoyant density fractions was assessed routinely by direct morphological examination using phase contrast microscopy. Results were expressed as DC/node. (This technique was performed by Marie Cumberbatch, Syngenta, Central Toxicology Laboratory, UK).

2.4.7 Bone marrow preparation

Femurs and tibiae of female, 8-12 weeks old caspase-1 -/- and WT mice were removed and cleaned from the surrounding muscle tissue by rubbing with Kleenex tissues. Thereafter intact bones were left in 70% ethanol for 2-5 minutes for disinfection and washed with PBS. Then both ends were cut with scissors and the marrow flushed with PBS using a Syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. After one wash in PBS, about 1-1.5 x 10^7 leukocytes were obtained per femur or tibia.
2.5 IN VITRO TECHNIQUES

2.5.1 In vitro LC migration

Ears were harvested from mice and split into dorsal and ventral halves as described above. The dorsal half only (which does not have cartilage attached) was placed in organ culture, a model that other researchers have also been used (Price et al., 1997). Briefly, dorsal ear halves were floated individually on 2 ml of RPMI/10% fetal calf serum with or without caspase-1 inhibitor or control peptide in 16mm diameter wells of 24-well cluster trays (Costar Corp., Cambridge, MA) and kept at 37°C in a 5% CO₂ incubator. At 24 and 48 hours, explants were removed and epidermal sheets were prepared and analysed for the presence of LC as described in immunological methods section.

2.5.2 Bone marrow cell culture with GM-CSF

The principal method for generating bone marrow DC with GM-CSF was adapted from previous publication (Inaba et al., 1998). Modifications were as follows. Instead of 24-well plates, bacteriological petri dishes with 100 mm diameter were used (Falcon, No 1029/Becton Dickinson). Cell culture medium (R10) was RPMI-1640 (Gibco) supplemented with penicillin (100 U/ml; Sigma), streptomycin (100 µg/ml; Sigma), L-glutamin (2 mM; Sigma), 2-mercaptoethanol (50 µM; Sigma), and 10% heat-inactivated and filtered FCS. At day 0 bone marrow leukocytes were seeded at 2x10⁶ per 100 mm dish in 10 ml R10 medium containing 200 U/ml (=20 ng/ml) rmGM-CSF (5x10⁶ U/mg; Peprotech). At day 3 another 10 ml R10 medium containing 200 U/ml rmGM-CSF were added to the plates. At days 6 and 8 half of the culture supernatant was collected,
centrifuged and the cell pellet re-suspended in 10 ml fresh R10 containing 200 U/ml rmGM-CSF, and given back into the original plate. At day 10 non-adherent cells (bone marrow DC population) were collected and used for Flow cytometry.

2.5.3 Culture of PAM 212 cells

PAM212 murine keratinocytes were maintained in RPMI 1640 medium supplemented with 10% new-born calf serum, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and HEPES buffer (10 mM), (all reagents from Gibco). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. Confluent layers of PAM212 cells in 48 well plates were incubated in triplicate with increasing concentrations of IL-1α, IL-1ra or sIL-1RI in a total volume of 0.5 ml per well in the presence or absence of anti-IL-1 receptor antibodies at a final concentration of 10 µg/ml for 24 hours at 37 C. Subsequently, supernatants were collected, cleared by centrifugation and stored at -20°C prior to ELISA analysis. For cell viability experiments, supernatant lactate dehydrogenase (LDH) levels were assessed using a kit (Sigma), according to the manufacturer’s instructions.

2.5.4 LDH assay

LDH release from PAM 212 keratinocytes was determined using the LDH Assay Kit (Sigma) according to the manufacturers instructions. Briefly, conditioned medium from PAM 212 keratinocyte culture was retained, cleared by centrifugation at 1000g and stored on ice. Cell monolayers were refreshed with RPMI serum free medium containing 1% (v/v) LDH lysis solution and incubated at 37°C. After 45 minutes, the PAM 212 lysate (100% control) was harvested, cleared by centrifugation at 1000g and
stored on ice. The LDH assay mixture was prepared in a 1.5 ml eppendorf tube as a master mix and consisted of the following reagents per reaction: 40 µl of LDH Substrate Solution, 40 µl of LDH Enzyme and 40 µl of LDH Dye Solution. 120 µl of LDH assay mixture was transfected to wells of a 96-well tissue culture plate (Costar) containing 60 µl of PAM 212 conditioned medium or 100% control (each sample was analysed in triplicate wells). Reactions were mixed thoroughly and incubated in the dark at room temperature for 30 minutes. Reactions were terminated by the addition of 18 µl of 1N HCl (4.3 ml of concentrated hydrochloric acid mixed with 45.7 ml of ddH2O) per well and solutions mixed thoroughly, prior to spectrophotometrical analysis at 490 nm (SpectraCountTM plate reader; Packard). LDH levels in PAM 212 keratinocyte conditioned medium was determined as a percentage of total LDH (100% control).

2.5.5 Preparation of epidermal homogenates

Ears were removed and split into dorsal and ventral halves prior to incubation at 37 °C for 60 minutes in the presence of dispase I (2.5 U/ml in PBS; Roche). Epidermis was separated from dermis and washed thoroughly in PBS. Epidermal sheets were then homogenised in extraction buffer (50 mM Tris base, 150 mM NaCl) utilising a motorised mixer for 90 seconds. Homogenates were cleared by centrifugation and stored at -20°C. Total protein content was assessed using a modified Bradford assay, according to the manufacturer’s instructions (Sigma). Briefly, a 10 mg/ml BSA solution in ddH2O was prepared and diluted to obtain standards of 2, 1, 0.5, 0.25 and 0.125 mg/ml. 6 µl of sample or standard was mixed with 474 µl of ddH2O in clean 1.5 ml eppendorf tubes, and 120 µl of Bradford Reagent added. Reactions were mixed thoroughly and 120 µl removed to 96-well plates (Costar), prior to spectrophotometry at 595 nm (SpectraCount plate reader, Packard). Protein levels (mg/ml) were determined
by reference to the BSA standard curve, and samples diluted to equal concentration,
prior to storage at -80°C.

2.5.6 Organ culture of epidermal sheets

Epidermal sheets prepared by dispase separation as described above were placed in
duplicate into wells of a 24-well plate with 1 ml of RPMI 1640 medium (Gibco) and
incubated for 24 hours at 37°C. Supernatants were subsequently aspirated, clarified by
centrifugation and stored at -20°C. For neutralisation studies, anti IL-1RI (35F5) or
anti-IL-1ra antibody were added to the culture medium at a final concentration of 10
µg/ml at the onset of incubation. An isotype-matched, irrelevant control antibody was
used at the same concentration in all experiments.
CHAPTER 3

CASPASE-1 IS A CRITICAL REGULATOR OF LANGERHANS CELL MIGRATION AND THE INDUCTION OF CONTACT HYPERSENSITIVITY
3.1 INTRODUCTION

A considerable body of data now indicates that, upon antigen stimulation, LC capture antigens and migrate into regional lymph nodes where they activate antigen specific T cells (Steinman et al., 1995; Banchereau and Steinman, 1998). The migration of LC from the epidermis into draining lymph nodes following encounter with antigen is a complex series of events that are regulated by an assortment of chemokines, cytokines and adhesion molecules, many of which may be induced by encounter with antigen (Wang et al., 1999a; Cumberbatch et al., 2001).

Considerable evidence suggests that epidermal cell derived proinflammatory cytokines, including IL-1β and TNF-α, play important roles in promoting LC migration during the induction phase of CHS (Cumberbatch et al., 1997a; Stoitzner et al., 1999). Under normal conditions, resting epidermal cells synthesise low levels of cytokines. However, stimuli including contact allergens induce epidermal cells to produce significant amounts of proinflammatory cytokines, such as IL-1β and TNF-α. Both cytokines are available in the epidermal microenvironment, where TNF-α may be produced by both keratinocytes (Kock et al., 1990) and LC (Larrick et al., 1989; Schreiber et al., 1992), although in murine skin IL-1β is primarily a product of LC (Larrick et al., 1989; Mohamadzadeh et al., 1997). It is clear also that keratinocytes and LC express surface receptors for both cytokines and thus can respond appropriately to them (Groves et al., 1994; Wang et al., 1997; Cumberbatch et al., 1999). As reviewed in the introduction the evidence that these two cytokines provide the key signals for LC mobilisation comes from studies demonstrating that intradermal injection of either cytokine in murine skin leads to LC migration from epidermis with subsequent accumulation of dendritic cells.
in draining lymph nodes (Cumberbatch and Kimber, 1992; Cumberbatch et al., 1997b). Furthermore, both anti-IL-1β and anti-TNF-α neutralising antibodies inhibited hapten-induced LC migration from murine skin in vivo (Cumberbatch et al., 1997b).

While there is no doubt that in normal conditions the initiation of LC migration in response to contact sensitisation is dependent upon the induced or up-regulated expression by epidermal cells of these cytokines, this does not exclude the involvement of other factors (Cumberbatch et al., 2001). For instance, it has been proposed that IL-10 may serve to limit or control LC migration secondary to regulation of TNF-α synthesis (Wang et al., 1999a; 1999b). In addition, it has been shown that modified responses of LC to certain chemokines, such as MIP-1α, -1β, -3β, via regulated expression of CC chemokine receptors, may have important role in the mobilisation and homing of these cells (Sallusto et al., 1998; Gunn et al., 1999; Saeki et al., 1999).

Although TNF-α is synthesised in its active form, IL-1β requires processing by caspase-1, an intracellular cysteine protease that cleaves pro-IL-1β at position Asp_{116}-Ala_{117} in order to release the mature 17 kDa form of IL-1β (Thornberry et al., 1992). Caspase-1 is a cysteine protease that is produced by LC amongst other cells. It is synthesised as a 45 kDa proenzyme and requires two internal cleavages before becoming the enzymatically active heterodimer composed of a 10- and 20 kDa chain (Figure 3.1). The active site is located on the 20 kDa chain (Dinarello, 1996).
At the onset of the present study, there was no data available for the role of caspase-1 in LC migration and in the induction of cutaneous immune responses. However, caspase-1 inhibitor, Ac-YVAD-cmk, has been used in animal models in a several studies (Rouquet et al., 1996; Ona et al., 1999), but not in any way related to skin. In these studies was reported that, in vivo, pre-treatment of mice with Ac-YVAD-cmk protected them from the lethal effect of anti-Fas antibody and from liver failure induced by injection of TNF-α. In addition, Ac-YVAD-cmk administration was also highly effective in rescuing mice that have been pretreated with anti-Fas antibody from rapid death, despite extensive hepatic apoptosis (Rouquet et al., 1996). More recently, it was demonstrated that intra-cerebroventricular administration of caspase-1 inhibitor, Ac-YVAD-cmk, delayed disease progression and mortality in the mouse model of Huntington's disease (Ona et al., 1999). In view of the role of this enzyme in regulating the processing and the release of IL-1β, which is known to be central in the LC
migration response and its clear activity in other disease models in vivo, we wished to investigate the influence of caspase-1 in the initiation of antigen-specific cutaneous inflammation, hypothesizing that it might represent an attractive potential therapeutic target in inflammatory skin disease.
3.2 RESULTS

3.2.1 Contact hypersensitivity is suppressed in caspase-1 -/- mice

The first question was to determine whether caspase-1 -/- mice could be sensitised normally to epicutaneously applied hapten. Thus, caspase-1 -/- and wild type mice (WT) were sensitised epicutaneously on abdominal skin with DNFB and challenged on ear skin 5 days later. Although WT mice responded with a vigorous ear swelling response as expected, significant attenuation of this response was observed in caspase-1 -/- animals (Figure 3.2a). When another allergen, oxazolone, was used a similar degree of suppression was observed, indicating that this phenomenon is not specific to DNFB (Figure 3.2b). Overall ear swelling as anticipated with oxazolone was less compared with DNFB-induced response.

![Figure 3.2: Contact hypersensitivity response in WT and caspase-1 -/- mice. Mice were sensitised with DNFB (a) or oxazolone (b) and challenged 5 days later on one ear as described. Marked ear swelling occurred in wild type mice (□) but there was significant suppression of this response in caspase-1 -/- animals (○) *: p<0.05; **: p<0.01. Unsensitised controls (wild type (●), caspase-1 -/- (Δ)) demonstrated negligible ear swelling. n=3 mice/group. Results are representative of three independent experiments.](image-url)
3.2.2 Hapten-induced LC migration is impaired in caspase-1 -/- mice

We hypothesised that the impaired CHS in caspase-1 -/- mice might relate to a defect in LC migration, and therefore experiments were performed in which epidermal LC numbers were determined following topical exposure of mice to DNFB. This allergen has previously been shown to induce rapid migration of LC from murine epidermis (Bergstresser et al., 1980). Although LC were present in WT and caspase-1 -/- epidermis with similar morphology (Figure 3.3 a,c), LC numbers in epidermal sheets from untreated caspase-1 -/- mice were significantly lower than in WT mice (573 +/-37 LC/mm² in caspase-1 -/- mice compared to 670 +/-22 LC/mm² in WT mice, p<0.05, n=4 ears/group).

Figure 3.3: MHC class II +ve LC morphology in epidermal sheet preparations derived from WT and caspase-1 -/- mice. a, b: WT; c, d: caspase-1 -/- a, c: untreated skin; b, d: epidermal sheets prepared 4 hours after challenge with 0.5% DNFB. Steady state levels of LC density were lower in caspase-1 -/- animals (c) compared to wild-type (a). DNFB induced increased intensity of MHC class II staining in LC from both WT (b) and caspase-1 -/- mice (d).
In WT mice, application of DNFB lead to a rapid decline in LC numbers to ~72% resting levels (Figure 3.4a), a reduction comparable to that we routinely observe in BALB/c mice following hapten challenge. In contrast, DNFB treatment failed to provoke significant LC migration in caspase-1 -/- mice (Figure 3.4b).

Figure 3.4: LC numbers in epidermal sheets prepared following topical application of 0.5% DNFB in AOO or AOO alone. a) WT mice, b) caspase-1 -/- mice. DNFB resulted in a marked fall in LC density in WT mice, whereas LC numbers did not significantly change in caspase-1 -/- mice. *: p<0.05 compared to vehicle, n=4 ears/group. Data shown are from three independent experiments.

Interestingly, although LC numbers did not change in caspase-1 -/- mice following application of DNFB, their morphology did alter in a manner typical of allergen-
induced activation, with shortening of dendritic processes and an increase in intensity of MHC class II expression, changes also seen in the WT mice (Figure 3.3b,d). Similar data were obtained using the alternate sensitiser oxazolone (Figure 3.5a,b).

These experiments showed that there was a defect in LC migration in caspase-1 -/- mice and this was also associated with an impaired CHS reaction in response to contact allergens, such as DNFB and oxazolone. This was the first indication that caspase-1 has an important role in the process of LC migration.

In order to investigate if this defect in LC migration might resolved at a period later than 4 hours after the exposure of mice to allergens, the kinetics of LC migration following topical application of allergen between WT and caspase-1 -/- mice were then assessed at intervals after DNFB application. In WT mice, DNFB caused a steady decline in the
frequency of LC in the epidermis 4, 8 and 24 hrs after application of the allergen. In contrast, this decrease in LC numbers was not observed in caspase-1 -/- mice indicating that this defect in LC mobilisation is associated with caspase-1 and the cells do not appear to be able to receive an alternative signal to overcome this problem even 24 hrs after DNFB application (Figure 3.6).

Figure 3.6: LC migration kinetics following topical application of DNFB. DNFB resulted in a significant decrease in the numbers of LC in the epidermis of WT mice (a) 4, 8 and 24 hrs after its application compared to naive and AOO-treated mice. This decrease in LC numbers was absent in caspase-1 -/- mice (b) where LC numbers were not significantly different between naïve, DNFB- and AOO- treated groups. *: p<0.05; **: p<0.01; ns: not significant. Data shown are of three independent experiments (n=4 ears/group).
3.2.3 Bone marrow-derived DC numbers are lower in caspase-1 -/- mice

In the experiments described above it was repeatedly found that resting LC numbers in the epidermis of caspase-1 -/- were significantly less compared to LC numbers from the epidermis of WT mice. One explanation for this could be that the numbers of bone marrow-derived DC are generated less efficiently in the absence of caspase-1. To examine this, bone marrow-derived DC were generated in vitro from WT and caspase-1 -/- mice and their numbers were compared by flow cytometry using specific markers for DC. As illustrated in figure 3.7 flow cytometric analysis revealed that CD11c, DEC205 and MHC class II positive cells (DC-specific markers) numbers were significantly lower in caspase-1 -/- mice, indicating that bone marrow-derived DC were less efficiently generated in caspase-1 -/- mice compared to WT.

Figure 3.7: Bone marrow-derived DC numbers are lower in caspase-1 -/- mice. Bone marrow-derived cells were obtained from caspase-1 -/- and WT mice. Cells were cultured for 10 days in the presence of GM-CSF. On day 10, cells were collected and analysed by flow cytometry. Cells positive for CD11c, DEC205 or MHC class II markers were then counted. In each case, DC +ve cell numbers obtained from caspase-1 -/- mice cultures were significantly lower (*: p<0.05) compared to WT mice derived cell cultures. Data are the mean of three independent experiments +/- SEM.
3.2.4 Response of LC to cytokine stimulation in caspase-1 -/- mice

Because LC migration in response to hapten is thought to be largely dependent upon availability of IL-1β and TNF-α, the response of LC in WT and caspase-1 -/- mice to intradermal injection of these cytokines was next examined. In WT mice, both TNF-α and IL-1β lead to a rapid decrease in LC numbers (Figure 3.8a) but, although caspase-1-/- LC responded normally to IL-1β, they failed to migrate after injection of TNF-α (Figure 3.8b). Clearly therefore, given the correct signal, caspase-1 -/- LC are capable of normal migration, but are unable to do this after hapten stimulation alone.

Figure 3.8: LC numbers in epidermal sheets prepared following intradermal cytokine injection. a) WT mice, b) caspase-1 -/- mice. IL-1β and TNF-α induced a significant fall in epidermal LC numbers in WT mice and injection of carrier protein alone (BSA) was without effect. In caspase-1 -/- only IL-1β caused significant LC migration, whilst TNF-α injection failed to induce significant LC movement. *: p<0.05, **: p<0.01 compared to BSA injected skin. Data shown are of three independent experiments.
To summarise, the data obtained thus far indicated that caspase-1 -/- mice developed significantly suppressed CHS reaction following exposure to allergens, DNFB or OX. This was associated with impaired LC migration in caspase-1 -/- following topical application of the same allergens even 24 hours after application. Although caspase-1 -/- LC did not move in response to allergens, they were activated in a comparable manner with normal LC. However, when exogenous IL-1β was administered to caspase-1 -/- mice, LC migrated normally away from the epidermis, indicating that caspase-1 -/- LC were capable of normal migration if they receive the correct signal. Interestingly, intradermal injection of TNF-α failed to induce significant migration in caspase-1 -/- mice suggesting that TNF-α induced migration is dependant on IL-1β availability. Therefore, our data are in agreement with previous studies suggesting that IL-1β is a mandatory signal for LC migration and that caspase-1 has a key role in this process being the enzyme that cleaves IL-1β from its inactive to the active form. To further investigate the role of caspase-1 in LC migration and CHS, the next step was to use caspase-1 inhibitors both \textit{in vitro} and \textit{in vivo}.

3.2.5 Caspase-1 inhibitor, Ac-YVAD-cmk, prevents LC migration \textit{in vitro}

To determine whether pharmacological caspase-1 inhibition might inhibit LC migration the effect of Ac-YVAD-cmk, a cell permeable irreversible caspase-1 inhibitor (Rouquet \textit{et al.}, 1996; Thornberry \textit{et al.}, 1994), on LC migration in organ culture was assessed. Previous data have indicated that \textit{in vitro} organ culture of skin results in migration of LC from epidermis into dermis, where they accumulate in “cords” in lymphatic vessels prior to passing into the culture medium (Price \textit{et al.}, 1997; Stoitzner \textit{et al.}, 1999). Thus, dorsal ear skin from BALB/c mice was cultured for up to 48 hours and LC numbers were evaluated in the presence of Ac-YVAD-cmk or a control peptide, Ac-
DEVD-cmk, which has little effect on caspase-1 function (Thornberry et al., 1994). In skin cultured in medium alone there was a steady decline in epidermal LC numbers over 48 hours to 501 +/-18 LC/mm², compared to 902 +/-58 LC/mm² in BALB/c skin prior to organ culture (Figure 3.9). However, skin cultured in the presence of 100μM Ac-YVAD-cmk showed a significant (p<0.05) reduction in this decline, whereas control peptide had no inhibitory effect, indicating that inhibition of caspase-1 impairs LC migration\textit{ in vitro}.

![Figure 3.9: Inhibition of LC migration by caspase inhibitors \textit{in vitro}. BALB/c ear skin was prepared as described and incubated in organ culture for 48 hours in the presence or absence of Ac-YVAD-cmk (a caspase-1 inhibitor) or Ac-DEVD-cmk (control). At times indicated, ear halves were removed from organ culture and MHC class II +ve LC were enumerated in epidermal sheet preparations. In the absence of inhibitor there was a rapid decline in LC remaining in epidermis (□) and this was significantly inhibited by Ac-YVAD-cmk (○) though not by Ac-DEVD-cmk (○). Data shown are of three independent experiments. n=3 ear halves/group. *: p<0.05; **: p<0.01.](image)

Similar experiments were performed using skin derived from caspase-1 -/- mice. In these animals (Figure 3.10b), a much slower decline in LC numbers occurred,
paralleling the effect seen in WT mice in the presence of caspase-1 inhibitor, and addition of caspase-1 inhibitor was without effect. Controls using skin from WT control mice (Figure 3.10a) showed similar migration to that seen in BALB/c skin with inhibition by the caspase-1 inhibitor (Figure 3.9).

a) **Wild-type**

b) **Caspase-1 -/-**

Figure 3.10: LC migration in organ culture in caspase-1 -/- mice. WT (a) or caspase-1 -/- (b) ear skin was incubated in organ culture in medium alone (□) or in the presence of 100μM Ac-YVAD-cmk (○) or Ac-DEVD-cmk (ο). At 24 or 48 hours, ear halves were removed from organ culture and MHC class II +ve LC were enumerated in epidermal sheet preparations. In WT skin LC numbers decreased over 48 hours, and this decrease was inhibited by Ac-YVAD-cmk but not by Ac-DEVD-cmk. In skin derived from caspase-1 -/- mice only very low level LC migration under organ culture conditions was observed and caspase inhibitors were without effect. Data shown are of three independent experiments. n=3 ear halves/group. *: p<0.05.
3.2.6 Caspase-1 inhibition prevents LC migration and suppresses CHS \textit{in vivo}

Having demonstrated that Ac-YVAD-cmk inhibits LC migration \textit{in vitro} the next step was to determine whether a similar effect could be achieved \textit{in vivo}. Ear skin of BALB/c mice (n=3) was painted with 200 µM Ac-YVAD-cmk in DMSO 1 hour prior to application of 0.5% DNFB in AOO. The final concentration of caspase inhibitors and the time of application prior to allergen treatment was optimised as explained in detail in Chapter 2. Controls included application of DMSO alone or application of the control peptide Ac-DEVD-cmk. As expected, DNFB alone lead to a mean 24% decrease in epidermal LC numbers after 4 hours (Figure 3.11). DMSO alone had no effect on this decrease but pre-treatment of skin with Ac-YVAD-cmk inhibited 67% of the induced fall (p<0.05). Control peptide Ac-DEVD-cmk was without effect.

![Figure 3.11: Inhibition of DNFB-induced LC migration \textit{in vivo} by topical application of caspase inhibitors. BALB/c ear skin was pre-treated 1 hour prior to application of 0.5% DNFB or vehicle (AOO) with 200µM caspase inhibitors in DMSO, or DMSO alone. 4 hours later ears were harvested and MHC class II +ve LC were enumerated in epidermal sheet preparations. DNFB induced a marked fall in LC density that was significantly inhibited by Ac-YVAD-cmk pre-treatment. Ac-DEVD-cmk and vehicle alone were without effect. *: p<0.05; ns: not significant. Data are representative of three independent experiments.](image-url)
In order to investigate the effect of caspase-1 inhibitor with a different allergen, in parallel experiments oxazolone was used instead of DNFB to trigger LC migration. Figure 3.12 demonstrates that similar data were obtained with oxazolone, indicating that pharmacological caspase-1 inhibition is able to block LC migration in response to a variety of antigens, not only DNFB.

![Figure 3.12: Inhibition of Oxazolone-induced LC migration in vivo by topical application of caspase inhibitors. BALB/c ear skin was pre-treated 1 hour prior to application of 1% Oxazolone or vehicle (AOO) with 200μM caspase inhibitors in DMSO, or DMSO alone. 4 hours later ears were harvested and MHC class II +ve LC were enumerated in epidermal sheet preparations. Oxazolone induced a marked fall in LC density that was significantly inhibited by Ac-YVAD-cmk pre-treatment. Ac-DEVD-cmk and vehicle alone were without effect. *: p<0.05; ns: not significant. Data are representative of three independent experiments.](image)

It becomes clear from the data described above that as well as with caspase-1 -/- mice, pharmacological inhibition of caspase-1 resulted in the suppression of normal LC migration in response to contact allergens. Thus, we speculated that if Ac-YVAD-cmk can suppress LC migration, this compound may be able to inhibit the allergic contact dermatitis reaction that occurs by contact allergens in vivo. Therefore, having determined the inhibition of allergen-induced LC migration in vivo by topical application of
caspase-1 inhibitor, the effect of this inhibitor in the CHS reaction was then explored. The first step was to optimise the final concentration of Ac-YVAD-cmk and the time of treatment in conjugation with allergen application (described in detail in Chapter 2). After optimising the conditions for the experiment, mice treated with Ac-YVAD-cmk 400 µM prior to and immediately after sensitisation exhibited marked suppression of the ear swelling response to DNFB (Figure 3.13), suggesting that the migration inhibition demonstrated in figures 3.11 and 3.12 results in functional inhibition of the CHS response. Ac-DEVD-cmk pre-treatment was without effect and control mice showed negligible ear swelling.

Figure 3.13: Suppression of CHS by topical application of caspase inhibitors. Unshaved BALB/c abdominal skin was treated with caspase-1 inhibitor Ac-YVAD-cmk (□), control peptide Ac-DEVD-cmk (○) or DMSO vehicle (○) as described. Mice were then sensitised in the treated area with 0.5% DNFB and challenged 5 days later with 0.25% DNFB on one ear. Unsensitised mice (Δ) used as controls. Ac-YVAD-cmk pre-treatment resulted in significant suppression of the resulting ear swelling response (*: p<0.05) at 24, 48 and 72 hours, whereas ear swelling in mice treated with Ac-DEVD-cmk control peptide did not differ from vehicle treated animals. Data shown are of three independent experiments.
3.3 DISCUSSION

The induced migration of epidermal LC from skin, and their arrival as immunostimulatory DC in regional lymph nodes is a crucial step in the development of an antigen specific immune response and most notably in contact sensitisation. Previous studies have demonstrated that the mobilisation of LC and their migration through afferent lymphatics and their localisation within the paracortical areas of draining lymph nodes are processes stimulated and regulated by cytokines and chemokines. Of particular importance for the initiation of migration are the epidermal cytokines IL-1β and TNF-α and in this study these observations were extended to demonstrate that caspase-1 is central to this event.

Initial characterisation of caspase-1 -/- mice revealed that resting numbers of epidermal LC were significantly lower in these animals compared with wild-type controls. This may be explained by the fact that there is a decreased recruitment of LC or increased exit of LC in the absence of caspase-1 and also by a generalised deficiency of LC numbers, when this enzyme is not available. The dynamics of LC recruitment to epidermis remain unclear, although mechanisms must exist to ensure that LC precursors home to epidermis in correct numbers and at appropriate times (Robert et al., 1999). The data obtained suggest that caspase-1, or caspase-1 dependent cytokines, are required for optimal LC homing to skin. Another possible explanation could be that caspase-1 -/- LC circulate constitutively out of epidermis more rapidly in the absence of caspase-1 and a lower steady-state number is thereby achieved. However, this is argued against by our finding that, in almost all circumstances examined by us, LC are less able to migrate in the absence of caspase-1 than they might normally do. Our preliminary
data on *in vitro* generation of LC precursors suggest that caspase-1 -/- mice do have an inability to generate normal numbers of bone marrow-derived DC compared to WT mice. Therefore, fewer progenitor cells may give rise to a lower number of LC in the epidermis of caspase-1 -/- mice.

Our results demonstrate that antigen-induced LC migration is impaired in caspase-1 -/- mice. Similarly, TNF-α a cytokine which in WT mice leads to rapid mobilisation of LC injected intradermally to ear skin of caspase-1 -/- mice failed to induce migration. However, LC migrated normally in response to intradermal injection of IL-1β in caspase-1 -/- mice. Taken together, these data demonstrate that caspase-1 is required for normal migration of LC and that caspase-1 -/- LC are able to migrate if the appropriate stimuli are available. These data are in agreement with the hypothesis that there is a requirement for two independent cytokine signals supplied by TNF-α and IL-1β for efficient LC migration (Cumberbatch *et al.*, 1997b). The implication is that in caspase-1 -/- mice TNF-α alone is unable to initiate LC movement because the absence of caspase-1 results in an inability of epidermal cells to inducibly or constitutively secrete IL-1β. In contrast, exogenous IL-1β is able to stimulate migration because in addition to supplying directly one signal to LC, it is also able to induce production, probably by keratinocytes (Kock *et al.*, 1990), of TNF-α. Thus these findings confirm and extend those of Enk *et al.*, who demonstrated that blockade of IL-1β by antibody injection inhibits both LC migration and the CHS response (Enk *et al.*, 1993). That TNF-α also plays a critical role has been demonstrated both by antibody inhibition studies and in mice deficient in its p75 receptor (Wang *et al.*, 1997; Dekaris *et al.*, 1999).
The phenotypic studies demonstrate that although topical application of DNFB to caspase-1 -/- mice failed to initiate LC migration, activation was induced, as indicated by increased expression of MHC class II. Therefore, these findings indicate there can be dissociation of activation and migration and suggest that different signals are responsible for each. Caspase-1 is present in both LC (Ariizumi et al., 1995) and keratinocytes (Zepter et al., 1997), though whether it is required in one or both cell types for LC migration to occur requires further investigation. Previous data demonstrating that IL-1β is primarily a product of LC in murine skin (Ariizumi et al., 1995), and that caspase-1 is involved in IL-1β processing in these cells suggest that it is likely to be of primary importance in LC, though our current data cannot exclude a role for caspase-1 in keratinocytes. Indeed, recent evidence suggests that caspase-1 in keratinocytes may be active in certain states and result in the release of biologically active IL-1β (Zepter et al., 1997).

Recently a number of caspase-1 inhibitors have been identified. The present investigations demonstrate that Ac-YVAD-cmk, a prototypic caspase-1 inhibitor, is able to suppress LC migration both in vivo and in vitro. Short-term organ culture largely recapitulates normal LC migration in vitro (Price et al., 1997; Stoitzner et al., 1999), with accumulation of LC in “cords” within dermal lymphatic vessels and subsequent exit into the culture medium. This process is likely to result from release of IL-1 and/or TNF from injured keratinocytes, and our finding with skin derived from caspase-1 -/- mice and with caspase-1 inhibitors in BALB/c skin indicates that is clearly caspase-1 dependent. Moreover, topical application of Ac-YVAD-cmk in vivo resulted in a marked inhibition of LC migration in response to topical DNFB and effectively inhibited CHS. Clearly therefore, Ac-YVAD-cmk is able to penetrate epidermis and
interact with caspase-1 inside viable epithelial cells and our lab is currently exploring the possibility of utilising such compounds as cutaneous immune modulators \textit{in vivo}.

Taken together the data presented here indicate that caspase-1 is central in LC migration and its activity can be controlled by specific inhibitors both \textit{in vivo} and \textit{in vitro}. Furthermore, these observations indicate that there may be considerable potential for development of Ac-YVAD-cmk or other specific caspase-1 inhibitors as therapeutic agents for the treatment of immunologically mediated skin diseases such as allergic contact dermatitis, atopic eczema and psoriasis.
CHAPTER 4

INTERLEUKIN-18 PROVIDES AN ESSENTIAL PROXIMAL SIGNAL IN LANGERHANS CELLS MIGRATION AND CONTACT HYPERSENSITIVITY
4.1 INTRODUCTION

In Chapter 3 it was demonstrated that the cysteine protease caspase-1 plays an important regulatory role governing murine LC migration and CHS. This enzyme is responsible for processing IL-1β from its inactive precursor form into mature cytokine capable of receptor binding and initiation of its signaling pathway. During the course of this work, IL-18, a cytokine structurally similar to IL-1β, was identified as a caspase-1 processed cytokine (Fantuzzi and Dinarello, 1999).

IL-18 was identified as a factor promoting IFN-γ production and it was originally called IFN-γ-inducing factor (Okamura et al., 1995). However, since then it has been found to play a role in a variety of immune and inflammatory responses (Dinarello et al., 1998; Akira, 2000; McInnes et al., 2001). The hypothesis that IL-18 might be involved in LC migration and contact hypersensitivity can be justified by several reasons. The most important is that IL-18 is structurally similar to IL-1β and, although they utilise different receptors, these cytokines share similar signal transduction elements (Bazan et al., 1996; Dinarello et al., 1998; Akira, 2000). Furthermore, it has recently been shown that many epidermal cells, including dendritic cells, LC and keratinocytes express IL-18 (Stoll et al., 1997; Naik et al., 1999; Mee et al., 2000). Finally, recent studies have been shown that chemical allergens can stimulate keratinocytes to secrete IL-18 (Naik et al., 1999) and that there is increased expression of this cytokine in the elicitation phase of CHS (Xu et al., 1998).

Taken together the conclusions made from these studies strongly suggested that, like IL-1β and TNF-α, IL-18 was a candidate cytokine involved in the regulation of LC
migration from epidermis to lymph node. However, functional evidence for this was lacking and the following series of experiments were therefore undertaken.

As identified elsewhere in this thesis, a considerable body of evidence has been accumulated characterising of the nature of cytokine signals required for chemical allergen-induced LC migration. However, it must be recognised that other stimuli, such as skin irritants, can also cause migration of LC from epidermis (Brand et al., 1992; Cumberbatch et al., 1993). Interestingly, the skin irritant sodium lauryl sulfate (SLS) induces dendritic cell accumulation in lymph nodes in a TNF-α-dependent manner (Cumberbatch et al., 1993; 1995) and expression of IL-1β, a key cytokine in allergen-induced LC migration, was not up-regulated by LC in response to SLS treatment (Enk and Katz, 1992). However, these are the only available evidence of signals regulating LC migration in response to a skin irritant rather than a skin sensitiser. From these data one can therefore hypothesise that the cytokine requirements for stimulation of LC migration by skin irritants differ from those that are necessary for migration induced by skin sensitisers.

Thus, the aim of this part of my work was to establish whether IL-18 plays a role in the cutaneous inflammatory responses typified by CHS and to compare this with non-antigen specific inflammation induced by skin irritants.
4.2 RESULTS

4.2.1 IL-18 stimulates epidermal LC migration and draining lymph node DC accumulation

In order to investigate a possible role for IL-18 in LC migration the effect of intradermal injection of this cytokine in BALB/c mice was assessed. It has been reported previously that intradermal administration to mice of homologous recombinant IL-1β stimulates a time-dependent reduction in epidermal MHC class II+ LC reaching approximately 68% of control levels found in BSA-treated mice within 4 hrs of exposure. This reduction in epidermal LC numbers was accompanied by a significant increase in draining lymph node DC numbers measured 17 hrs later (Cumberbatch et al., 1997). Given the similarities between IL-18 and IL-1β, the ability of these two cytokines to stimulate LC migration and the subsequent accumulation of DC in lymph nodes draining the site of exposure, each measured 4 hrs and 17 hrs after cytokine administration respectively, was compared (Figure 4.1a). As with previous experiments, injection of carrier protein alone (0.1% BSA/PBS) was without effect on control epidermal LC numbers measured 4 hrs following exposure. In contrast, IL-18 or IL-1β administration caused a marked decrease in epidermal LC numbers. Thus, the data illustrated in figure 4.1a revealed that IL-18 induced 26% reduction in LC numbers, whereas IL-1β caused 21% reduction in the same experiments. Importantly, under the same conditions of exposure, IL-18 induced a corresponding increase in the number of DC arriving in regional lymph nodes. IL-18 and IL-1β were found to cause similar increase (3 fold) in lymph node DC numbers when compared with groups of mice that had been exposed to carrier protein alone (Figure 4.1b). (This experiment was performed by Dr Cumberbatch, Central Toxicology Laboratory, Syngenta).
4.2.2 IL-18 is required for an intact contact hypersensitivity response in murine skin

Having demonstrated a role of IL-18 in LC migration, the apparent association of this cytokine with CHS response was examined. To explore the role of IL-18 in murine CHS, IL-18 -/- and WT mice were sensitised on abdominal skin with 1% OX and challenged on one ear 5 days later with 0.5% OX. Ear swelling was measured over the following 72 hours. Although a vigorous swelling response occurred in WT mice, a significant reduction of ear swelling was evident in IL-18 -/- animals (Figure 4.2a). A similar reduction of CHS was seen when DNFB, an alternate contact sensitiser, was used (Figure 4.2b). These data strongly suggest that IL-18 is an obligate part of the normal contact hypersensitivity response in murine skin, and that this requirement is not dependent on the antigen involved.
4.2.3 IL-18 is not required for irritant contact dermatitis in murine skin

Having shown a role for IL-18 in allergen mediated murine skin inflammation the next step was to determine whether cutaneous inflammatory responses were generally suppressed in the absence of IL-18. To explore this speculation the ear swelling induced by the skin irritant SLS was then examined. 10% SLS in dimethyl formamide was painted onto ear skin of IL-18 -/- and WT mice and ear thickness was measured over the following 72 hours. Ear swelling occurred in both groups, maximal at 24 hours, and no significant difference was evident between IL-18 -/- and WT mice (Figure 4.3). Consistent with this is that the irritant response to contact allergens observed in unsensitised IL-18 -/- mice shown in Figure 4.2 did not differ from that observed in unsensitised WT mice. These data suggest that although IL-18 is required for allergen-induced cutaneous immune responses, it does not have an obvious role in irritant responses.
Figure 4.3: Ear swelling in response to skin irritant SDS is normal in IL-18 -/- mice. 25 μl of 10% SDS in DMF applied on both sides of the right ears of WT and IL-18 -/- mice. Ear swelling was measured at 0, 24, 48 and 72 hrs after application of the irritant and there was no significant difference between WT (○) and IL-18 (□) -/- mice. n=3 mice/group. The results are representative of three independent experiments.

4.2.4 IL-18 is required for normal epidermal LC migration and the afferent phase of CHS

Because of previous data suggesting that IL-18 is capable of initiating LC migration from skin the hypothesis was made that LC migration might be deficient in IL-18 -/- mice, and that this might account for the decreased CHS response shown in figure 4.2. Therefore LC were enumerated in epidermal sheet preparations derived from IL-18 -/- and WT mice before and 4 hours after epicutaneous application of either 1% OX or 0.5% DNFB in AGO (Figure 4.4). In WT mice both allergens induced a significant fall (OX 25%, DNFB 29%, both p<0.05 compared to AGO) in epidermal LC numbers suggestive of LC migration away from epidermis. Resting LC numbers and morphology in IL-18 -/- mice were equivalent to those observed in WT animals, and no significant change in epidermal LC numbers was seen following allergen application. Interestingly however an increase in MHC class II staining intensity of IL-18 -/- LC after stimulation was detected, suggestive of LC activation. Confirmation that LC in
IL-18 -/- mice can be mobilised and are not simply fixed in epidermis was obtained by examining epidermal LC numbers following application of the skin irritant SLS (Figure 4.4). Both WT and IL-18 -/- mice displayed an equivalent drop (WT 36%, IL-18 -/- 38%, both p<0.01 compared to AOO) in remaining epidermal LC 4 hours following skin painting with SLS indicating that IL-18 -/- LC can, given an appropriate signal, be induced to migrate. The equivalent reduction in IL-18 -/- and WT LC from the epidermis following topical application of SLS is consistent with the observation that ear swelling response following SLS treatment was without significant different between IL-18 -/- and WT mice (Figure 4.3).

**Figure 4.4:** LC numbers in epidermal sheets prepared following topical application of skin allergens DNFB (0.5% in AOO) or OX (1% in AOO) and skin irritant SLS (10% in DMF). a, WT mice; b, IL-18 -/- mice. Application of DNFB or OX resulted in a significant decline in LC numbers in WT mice, whereas this decline was absent in IL-18 -/- mice. In contrast, SLS application induced a marked fall in LC density in both WT and IL-18 -/- mice. The results are expressed as the mean number of cells/mm² (+/- SEM). n = 4 ears/group. *: p<0.05, **: p<0.01. The results are representative of three independent experiments.
If the role played by IL-18 in CHS was largely in regulation of LC migration it was reasoned that injection of IL-18 into skin at the time of sensitisation might restore the CHS ear swelling response. Thus, IL-18 -/- mice were sensitised on the skin of one ear with 1% OX 30 minutes after intradermal injection of 50ng rmIL-18, rmIL-1β or diluent alone. Five days later, mice were challenged with 0.5% OX on the contra-lateral ear pinna and swelling of the challenged ear was monitored over the following 72 hours. As with abdominal sensitisation (Figure 4.2), IL-18 -/- mice showed a suppressed ear swelling response in this model but this could be completely rescued by pre-injection of IL-18 or IL-1β (Figure 4.5). Vehicle alone was without effect, and IL-18 or IL-1β pre-injections did not enhance the swelling observed in WT mice. These data confirm that IL-18 has an obligate role in the murine CHS response and strongly suggest that it is required in the sensitisation, phase of CHS.

Figure 4.5: Intradermal administration of exogenous IL-18 prior to sensitisation with OX (1% sensitisation; 0.5% elicitation) restores CHS response in IL-18 -/- mice. WT and IL-18 -/- mice ear skin was pretreated locally by intradermal administration of 30 μl of IL-18, IL-1β (50ng) or same volume of BSA, prior to sensitisation with OX and 5 days later ears were challenged. IL-18 -/- mice that did not receive cytokine treatment prior to sensitisation showed suppressed ear swelling, but this attenuated response was completely restored in IL-18 -/- mice pretreated with IL-18 or IL-1β. BSA alone had no effect. IL-18 or IL-1β pretreatment did not induce more swelling in WT mice. n = 5 mice/group. **: p<0.01, ns: not significant. The results are representative of three independent experiments.
4.2.5 IL-18 acts proximally to IL-1β and TNF-α in initiation of LC migration and murine CHS.

Having shown that IL-18 is required for LC migration, the possible relationship between IL-18 and the other cytokines (notably IL-1β and TNF-α) previously implicated in regulation of LC migration was determined. Thus, 50ng IL-1β, IL-18 or TNF-α were injected into IL-18 -/- or WT ear skin as previously described and remaining epidermal LC at 4 hours (IL-1β and IL-18) or 30 minutes (TNF-α) were enumerated by immunostaining of epidermal sheet preparations. All three cytokines induced an equivalent reduction of epidermal LC numbers in IL-18 -/- and WT mice (Figure 4.6), indicating that the migratory response to these cytokines is not dependent upon secondary release of IL-18. Thus in response to allergen, IL-18 most likely acts proximally to IL-1β and TNF-α.

Figure 4.6: Intradermal injection of TNFα, IL-1β or IL-18 induces equivalent LC migration in IL-18 -/- mice. WT and IL-18 -/- mice received 30 μl intradermal injections into their ears of equivalent amounts (50ng) of either TNF-α, IL-1β or IL-18 suspended in 0.1% BSA or of 0.1% BSA alone. Control mice were untreated. Epidermal sheets were prepared 4 hrs (IL-1β or IL-18) or 30 min (TNF-α) after treatment and LC numbers were determined. Administration of all three cytokines resulted in similar decline in LC numbers in both WT and IL-18 -/- mice. The results are expressed as the mean number of cells/mm² (+/- SEM; n=4 ears/group; **: p<0.01). The data shown are of three independent experiments.
It was demonstrated in Chapter 3 that the CHS response is absent in caspase-1-/- mice that lack functional IL-1β and IL-18, the only cytokines known to be processed by this enzyme. To investigate the relationship of IL-18-induced responses to IL-1β, LC migration in caspase-1-/- mice following intradermal administration of IL-18, IL-1β or carrier protein (BSA) alone was examined. Exposure of WT mice to IL-18 or IL-1β caused a similar decline in LC numbers of 26.4% and 30.2%, respectively. BSA had no effect in LC migration (Figure 4.7a). In caspase-1-/- mice treated concurrently with these cytokines, although IL-1β induced a comparable reduction in LC numbers (24.7%) to those obtained for WT mice, IL-18 failed to initiate LC migration in these mice (Figure 4.7b). As these animals are deficient in both IL-18 and IL-1β these data further support the concept that IL-18 acts upstream of IL-1β.

Figure 4.7: Epidermal LC migration stimulated by IL-18 is absent in caspase-1-/- mice. Groups of mice (n=4 ears/group) received 30 µl intradermal injections into both ear pinnae of 50 ng of IL-18 or IL-1β each suspended in BSA, or of BSA alone. Control mice were untreated. IL-1β and IL-18 caused significant reduction (**: p<0.01 compared to BSA-treated mice) in LC numbers in WT mice (a). In caspase-1-/- mice, IL-1β injection resulted in comparable reduction (*: p<0.05; **: p<0.01 compared to BSA-treated mice) of that observed in WT mice, whereas IL-18 injection failed to provoke LC migration (b). Data shown are of three independent experiments.
Finally, to further explore the inter-dependency of these two cytokines in the initiation of CHS the ability of each to rescue the defective CHS response in caspase-1 -/- mice was examined. In the absence of cytokine pre-treatment caspase-1 -/- mice displayed a suppressed CHS response, confirming the data obtained in Chapter 3. Intradermal injection of 50ng IL-18 immediately prior to sensitisation was without effect whereas injection of 50ng IL-1β fully restored the CHS in these mice (Figure 4.8). Similar experiments performed in IL-18 -/- mice demonstrated that both cytokines were equally capable of restoring the defective CHS response (Figure 4.5).

**Figure 4.8: Intradermal injection of exogenous IL-1β prior to sensitisation with OX rescues CHS response in caspase-1 -/- mice, whilst administration of IL-18 is without effect.** WT and caspase-1 -/- mice received intradermal injection of equivalent amounts (50ng) of murine IL-1β or IL-18 in 0.1% BSA or 0.1% BSA alone prior to sensitisation with 1% OX and 5 days later ears were challenged with 0.5% OX. Ear swelling was measured 24 hrs after challenge. Caspase-1 -/- mice without cytokine pretreatment showed suppressed CHS response. This response was completely rescued with IL-1β pretreatment. However, administration of IL-18 had no effect. *: p<0.05; ns: not significant. n = 5 mice/group. The data are representative of three independent experiments.
4.3 DISCUSSION

The experiments described in this Chapter indicate that IL-18 may play a key proximal role in the induction of allergic contact hypersensitivity in murine skin. It is required for normal LC migration in response to allergen and appears to act upstream of IL-1β and TNF-α, two cytokines that have previously been shown to govern LC migration from epidermis to lymph node.

IL-18 bears strong structural relationships to IL-1β (Dinarello et al., 1998). Both molecules require proteolytic cleavage by caspase-1 to generate the mature functional cytokine from an inactive precursor form (Fantuzzi and Dinarello, 1999). No other cytokines are known to be cleaved by caspase-1 and in Chapter 3 it was demonstrated that caspase-1 plays a key regulatory role in LC migration, presumably by modulation of processing and release of IL-1β and IL-18. Within the epidermal microenvironment both keratinocytes (Mee et al., 2000) and LC (Ariizumi et al., 1995; Stoll et al., 1998) express IL-18 and caspase-1 and thus either cell may be the source of this signal. Recent data indicates that IL-18 may also be released in a non-caspase-1 dependent fashion following the ligation of fas ligand (Tsutsui et al., 1999). Keratinocytes have been shown to express both fas and fas-ligand in a number of inflammatory states (Gutierrez-Steil et al., 1998; Viard et al., 1998) and thus this mechanism may also be of importance in the generation of mature IL-18 in the context of human inflammatory skin disease.

A considerable body of experimental evidence indicates that both IL-1β and TNF-α are required for the induction of LC migration in response to allergen (Cumberbatch et al.,
The current model is that following allergen contact with epidermis, LC release IL-1\(\beta\) that then stimulates production of TNF-\(\alpha\) by neighbouring keratinocytes that express the signal transducing type-1 IL-1 receptor. LC, which express both IL-1 (Cumberbatch et al., 1998) and TNF (Wang et al., 1997) receptors, are stimulated by the presence of both cytokines to enter the migration pathway, with loss of E-cadherin expression, induction of MMP-9 and other markers associated with maturation and antigen presentation (Cumberbatch et al., 2000). The data presented herein, taken together with antibody inhibition studies published previously (Cumberbatch et al., 2001), clearly indicate that IL-18 acts upstream of both TNF-\(\alpha\) and IL-1\(\beta\). These observations do not define the source of IL-18 release, though either keratinocytes or Langerhans cells may be responsible. That IL-18 should be a proximal mediator in LC migration is in accord with previous data indicating that IL-18 can induce synthesis and release of a number of secondary cytokines, including TNF-\(\alpha\), IL-6, IL-8 and IL-1\(\beta\). In some cells this is a direct effect (Netea et al., 2001), in others it requires the presence of TNF-\(\alpha\) (Puren et al., 1998). Although formal proof is awaited it is therefore highly likely that IL-18 could induce production of both downstream cytokines involved in regulation of LC migration within epidermis.

The finding that IL-18 has no apparent role in irritant contact dermatitis is of interest for a number of reasons. Firstly, it indicates that the role for IL-18 in antigen-specific inflammation is not simply part of a general requirement for IL-18 in skin inflammation. Secondly, it implies that IL-18, which is stored preformed in considerable quantities in murine and human keratinocytes (Mee et al., 2000) is not immediately bioactive. Were IL-18 directly pro-inflammatory in skin we would have predicted that at least part of the ICD ear swelling response, which occurs due to release
of preformed proinflammatory cytokines such as IL-1α from damaged keratinocytes (Kupper and Groves, 1995), would have been absent in IL-18 -/- mice. This was not the case. An alternate explanation is that otherwise bioactive IL-18 does not have a direct pro-inflammatory effect in skin, though this is unlikely as IL-18 is capable of inducing expression of endothelial adhesion molecules (Morel et al., 2001) as well as promoting synthesis and release of secondary cytokines including TNF-α that are known to be pro-inflammatory when released in the cutaneous microenvironment (Netea et al., 2001).

Finally, and perhaps most importantly, it demonstrates that the presence of IL-18 is not obligatory for LC migration in all circumstances. Although the mechanisms and biological role of LC migration in irritant dermatitis are unclear, there is increasing evidence that preformed IL-1α release is important in initiating this response (manuscript in preparation). It therefore seems likely that, given such a signal, LC are able to avoid the requirement for IL-18 in migration in some situations.

A considerable body of evidence is now accumulating that indicates that IL-18 plays a key role in epithelial defence against microorganisms. Several skin-specific viruses (e.g. human papilloma virus (Lee et al., 2001; Cho et al., 2001) and pox viruses (Xiang and Moss, 1999; Smith et al., 2001) have evolved means of suppressing IL-18 in the skin by production of inhibitory binding proteins analogous to the endogenously produced IL-18 binding protein. Moreover, mice deficient in IL-18 are prone to experimental infection by a number of organisms including Leishmania and Staphylococcus aureus (Wei et al., 1999), both of which are major skin pathogens. A central role for IL-18 in the initiation of LC migration following antigen penetration of epidermis fits in well with this role and underscores the central part played by IL-18 in epithelial defence.
CHAPTER 5

HOMEOSTATIC MECHANISMS OF THE IL-1 SYSTEM

OPERATE BOTH IN VITRO AND IN VIVO IN THE EPIDERMIS
5.1 INTRODUCTION

It was demonstrated in the previous Chapters that TNF-α, IL-1β and IL-18 are the key players in the process of LC migration and the subsequent accumulation of dendritic cells in draining lymph nodes. These studies also revealed that caspase-1 has a regulatory role in this process. In addition, recent studies (Cumberbatch et al., manuscript in preparation) revealed that IL-1α, a cytokine associated with skin injury and inflammation, is also able to stimulate the activation and migration of LC from the epidermis (Figure 5.1). Taken together these data clearly indicate that IL-1 molecules have a central role in the initiation of cutaneous responses.

![Graph showing LC migration and DC accumulation](image)

**Figure 5.1: IL-1α induces LC migration and DC accumulation in draining lymph nodes.** Groups of mice (n=10) received 30 μl intradermal injections into both ear pinnae of equivalent amounts (50 ng) of either IL-1α or IL-1β suspended in 0.1% BSA or of 0.1% BSA alone. Control mice were untreated. After 17 hrs, (a) ears were removed from 4 ears/group and MHC +ve LC determined. IL-1α and IL-1β induced equivalent LC migration. (b) Draining auricular lymph nodes were excised and DC numbers were determined. IL-1α and IL-1β caused similar increase in DC numbers. (These experiments were performed by Dr Cumberbatch, Central Toxicology Laboratory, Syngenta).

In order to further investigate the role of the IL-1 system in LC migration and CHS we considered using keratin-14 promoter transgenic mice that selectively overexpress IL-1α
or IL-1ra in basal epidermis. These mice had previously been generated by Professor Groves and were available in the laboratory. During the characterization of these animals fascinating data were revealed suggesting a co-ordinated regulatory mechanism between IL-1 molecules and these data are presented in this Chapter.

Interleukin-1 is a pleiotropic cytokine and primary mediator of cutaneous inflammation (Kupper, 1990; Dinarello, 1996). All biological effects of IL-1 are mediated by two agonistic IL-1 receptor ligands, IL-1α and IL-1β and can be specifically antagonised by a third ligand, IL-1 receptor antagonist (IL-1ra), all of which lie in a 430 kb region of chromosome 2 in man (Nicklin et al., 1994). Both IL-1α and IL-1β are synthesised as 31 kDa molecules lacking signal peptides, and whilst IL-1α remains primarily intracellular in its precursor form, IL-1β is predominantly secreted, following cleavage to a 17 kDa molecule by the cysteine protease caspase-1 at the cell surface (Singer et al., 1995). Unlike IL-1β, the 31 kDa form of IL-1α has full biological activity.

IL-1ra exists in three alternatively spliced forms; one containing a signal peptide and predominantly released from monocytes, whereas the other two lack a leader sequence and are found within keratinocytes and other epithelial cells (Bigler et al., 1992; Muzio et al., 1995). IL-1α, IL-1β and IL-1ra exert their effects through interaction with two specific cell surface receptors. The type I IL-1 receptor (IL-1RI) is an 80 kDa transmembrane molecule with an extensive cytoplasmic domain, through which all IL-1 mediated responses are transduced (Sims et al., 1989). IL-1RI deficient mice fail to respond to IL-1 and display reduced inflammatory responses (Labow et al., 1997). The type II IL-1 receptor (IL-1RII) is a smaller molecule (68 kDa) with a truncated cytoplasmic domain, which functions as a non-signalling decoy target for IL-1 receptor
ligands (McMahan et al., 1991; Colotta et al., 1993). Both IL-1 receptors can be cleaved from the cell surface, although soluble type II IL-1 receptor is the predominant shed form in vivo (Arend et al., 1994).

Epidermis is physiologically unique in being the only organ in the body to store prodigious quantities of preformed IL-1α (Dinarello, 1996) and keratinocytes have been shown to synthesise both IL-1α and β molecules in vitro (Kupper et al., 1986), in addition to both IL-1 receptors (Groves et al., 1994). Release of keratinocyte-derived IL-1α is sufficient to trigger cutaneous inflammation in human (Camp et al., 1990; Groves et al., 1992) and murine (Groves et al., 1995b) skin through the induction of secondary cytokines such as IL-8 and upregulation of endothelial adhesion molecules. Presumably, in order to control the potentially harmful effects of excessive IL-1α release in the epidermis, significant quantities of both splice variants of intracellular IL-1ra (icIL-1ra) are detectable in keratinocytes (Bigler et al., 1992; Muzio et al., 1995; Gabay et al., 1997). In addition to quenching excessive IL-1α activity, icIL-1ra may contribute to the inhibition of IL-1 responses by destabilising mRNA of some IL-1 inducible genes such as gro-α (Watson et al., 1995). Moreover, activation of keratinocytes by mitogens or interferon-γ results in marked up-regulation of IL-1RII in vitro and release from the cell surface, adding a further level of control to IL-1 within the epidermis (Groves et al., 1995a).

Perturbation of the tightly regulated cutaneous IL-1 network can result in profound inflammatory changes in vivo. For example, transgenic mice that overexpress IL-1α under the control of a keratin-14 (K14) promoter display a phenotype characterised by spontaneous focal inflammatory skin lesions (Groves et al., 1995b). Further, crossing
such animals with mice that overexpress type-1 IL-1 receptor on keratinocytes results in a significantly more severe phenotype than that seen in the IL-1α transgenic mice alone (Groves et al., 1996). By contrast, mice that overexpress IL-1RII in basal keratinocytes display a marked reduction in localised inflammatory responses (Rauschmayr et al., 1997). Additionally, alterations in the balance of IL-1 molecules has been shown in human epidermis from individuals with inflammatory skin diseases such as psoriasis, where IL-1α is reduced (Cooper et al., 1990) and IL-1ra is increased (Hammerberg et al., 1992) and in atopic dermatitis (Terui et al., 1998).

The numerous anti-IL-1 molecules present in epidermis (two species of icIL-1ra, cell surface and shed type-2 IL-1 receptor) suggest that tightly-co-ordinated regulation of IL-1 activity is essential in the cutaneous microenvironment (Kupper and Groves, 1995). Although counter-regulation of the IL-1 system has long been suspected, formal evidence for this is lacking. In the present study, a murine keratinocyte line and mice over-expressing either IL-1α or secreted IL-1ra (sIL-1ra) in basal keratinocytes were utilised to assess whether homeostatic mechanisms operate both in vitro and in vivo in the epidermis.
5.2 RESULTS

5.2.1 Counter regulation of IL-1α and IL-1ra release from epidermis of transgenic mice that overexpress IL-1 family molecules in vivo

Epidermal sheets were harvested from ears of mice derived from lines overexpressing either IL-1α (line IL-1.2) or IL-1ra (lines RA1 and RA10) and IL-1RI (line 1R10), in addition to wild-type animals (FVB). Homogenates were prepared and cytokine content was analysed by ELISA. Epidermis from line IL-1.2 displayed levels of IL-1α approximately four-fold higher than wildtype (FVB) littermates (21.2±2.3 ng/mg total protein vs 5.5±0.4 ng/mg, respectively) (Table 5.1). IL-1α levels were unchanged from wildtype levels in both IL-1ra lines and IL-1RI line. Similarly, sIL-1ra concentrations were three fold higher than FVB mice in one line over-expressing sIL-1ra (RA1) and increased over four-fold in line RA10 (67.1±3.3 ng/mg total protein vs 194.8±3.6 ng/mg and 284.7±15.0 ng/mg, respectively), whilst no differences were observed in the IL-1.2 line and 1R10 line, as expected. The approximate ten-fold excess of IL-1ra over IL-1α in epidermal homogenates from wildtype mice is in agreement with previous studies analysing this ratio in keratinocytes (Kutsch et al., 1993). These data indicate appropriate expression of the relevant transgene and suggest that transgenic over-expression of IL-1α or IL-1ra in keratinocytes does not affect the large intracellular stores of the opposing molecule in vivo.
Table 5.1: Analysis of epidermal IL-1α and IL-1ra content in IL-1α and IL-1ra transgenic mice

<table>
<thead>
<tr>
<th>Line</th>
<th>IL-1α a</th>
<th>icIL-1ra b</th>
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<tr>
<td></td>
<td>(ng/mg total protein)</td>
<td>(ng/mg total protein)</td>
</tr>
<tr>
<td>FVB</td>
<td>5.5 ± 0.4</td>
<td>67.1 ± 3.3</td>
</tr>
<tr>
<td>IL-1.2</td>
<td>21.2 ± 2.3</td>
<td>62.7 ± 3.6</td>
</tr>
<tr>
<td>RA1</td>
<td>4.1 ± 0.5</td>
<td>194.8 ± 3.6</td>
</tr>
<tr>
<td>RA1 0</td>
<td>4.5 ± 0.5</td>
<td>284.7 ± 15.0</td>
</tr>
<tr>
<td>1R1 0</td>
<td>4.8 ± 0.6</td>
<td>55.6 ± 5.2</td>
</tr>
</tbody>
</table>

a - Epidermal homogenates were prepared from the ears of mice derived from IL-1 transgenic lines, as described and analyzed by ELISA for IL-1α protein. Data represent mean ± SEM (n=3).

b - The epidermal homogenates described in (a) were subjected to mIL-1ra ELISA. Data represent mean ± SEM (n=3).

The next step was to determine whether transgenic overexpression of IL-1α or IL-1ra had any effect on release, as opposed to storage, of the opposing molecule. Thus, epidermal sheets were prepared from transgenic and wild-type mouse ears and placed in organ culture for 24 hours, prior to determination of supernatant IL-1α, IL-1ra or IL-1β. As expected, a large increase in IL-1α release was observed from epidermis derived from IL-1.2 mice compared with wildtype littermates (16 fold; 2,733±177 pg/ml vs 164±29 pg/ml). Moreover, significant increases in epidermal IL-1α release were also observed in supernatants from epidermal sheets prepared from both RA1 (3-fold; 530±120 pg/ml; p <0.01) and RA10 (4-fold; 678±35 pg/ml; p <0.01) lines (Figure 5.2a). In contrast, minimal release of IL-1β was observed in epidermal sheets prepared from RA1 and RA10 lines. Interestingly, increased levels of IL-1β were also released from IL-1.2 derived epidermis (Figure 5.2c).
Figure 5.2: Epidermal sheets from transgenic mice that overexpress IL-1α or IL-1ra in basal keratinocytes release increased quantities of the opposing molecule. (a) Epidermal sheets prepared from 3 different lines of transgenic mice were subjected to 24 hour organ culture and supernatants subsequently assayed by ELISA for mIL-1α, as described. The same supernatants were also used for IL-1ra ELISA (b) and IL-1β ELISA (c). Data represent mean ± SEM (n=3). *: p<0.01; **: p<0.001.

In epidermal sheets derived from IL-1ra transgenic mice, supernatant sIL-1ra levels were markedly increased in both RA1 and RA10 lines (RA1: 9,131±396pg/ml; RA10: 23,060±450 pg/ml; FVB: 61±19 pg/ml). More surprisingly however, an eight-fold increase in sIL-1ra release from IL-1.2 epidermal sheets was also observed (5,006±140 pg/ml; p <0.001, Figure 5.2b). Addition of neutralising antibody against IL-1ra to the organ culture medium at the onset of incubation significantly reduced IL-1α release from RA10 epidermal sheets (570±42 pg/ml to 224±9 pg/ml, p <0.001), suggesting that IL-1ra was acting extracellularly (Figure 5.3a). Addition of isotype-matched control antibody was without effect. Addition of a neutralising antibody against IL-1RI to the
organ culture decreased supernatant sIL-1ra levels from 5,827±89 pg/ml in IL-1.2 sheets to 810±66 pg/ml (p <0.001, Figure 5.3b), again suggesting that the IL-1 was acting extracellularly, whereas isotype control antibody was without effect.

Figure 5.3: Counter-regulation of IL-1 receptor ligand production in mice transgenic for IL-1α or IL-1ra is inhibited by neutralising antibodies. (a) Epidermal sheets from RA10 (K14-IL-1ra transgenic) mice were co-incubated with either a neutralising antibody against mIL-1ra or an irrelevant control antibody and supernatants were subsequently assayed for IL-1α by ELISA. Data represent mean ± SEM (n=3). (b) Epidermal sheets from lL-1.2 (K14-IL-1α transgenic) mice were co-incubated with either a neutralising antibody against mIL-1RI or an irrelevant control antibody and supernatants subsequently were assayed for IL-1ra by ELISA. Data represent mean ± SEM (n=3). **:p<0.001.

5.2.2 Counter regulation of IL-1α and IL-1ra release by murine keratinocytes in vitro

Having shown that there is a counter regulation of IL-1α and IL-1ra in vivo we next examined if this regulatory mechanism also exists in vitro. The murine keratinocyte cell line PAM212 was initially exposed to increasing concentrations of recombinant mIL-1α for 24 hours, prior to analysis of supernatants for IL-1ra immunoreactivity. In the absence of IL-1α, PAM212 cells released negligible quantities of IL-1ra, but this
rose in a dose dependent manner to 1,924±97 pg/ml following exposure to 10 ng/ml IL-1α (Figure 5.4).

![Graph](image)

Figure 5.4: Keratinocytes respond to increasing concentrations of IL-1α by induction of IL-1ra release in a dose dependent manner. PAM212 cells were exposed to recombinant muIL-1α for 24 hours, prior to collection of supernatant and subsequent ELISA for muIL-1ra. Data shown represent the mean and SEM from 3 experiments.

We then investigated the effect of IL-1ra in PAM212 cells. Conditions were similar to previous experiment. Following exposure to increasing concentrations (0.03 – 3 ng/ml) of recombinant IL-1ra, release of IL-1α from PAM212 cells increased from 529±69 pg/ml to 1,043±57 pg/ml (Figure 5.5). Interestingly, it was revealed that PAM212 cells constitutively release IL-1α even in the absence of IL-1ra. The amount of IL-1α released from PAM212 cells was increased in a dose dependent manner and it was maximal after exposure of cells to 1ng/ml of IL-1ra.
Figure 5.5: Keratinocytes respond to increasing concentrations of IL-1ra by up-regulating IL-1α release in a dose dependent manner. PAM212 cells were exposed to recombinant muIL-1ra for 24 hours, prior to collection of supernatant and subsequent ELISA for muIL-1α. Data represent mean ± SEM (n=3).

The constitutive release of IL-1α by PAM212 cells is in agreement with previous studies (Miyazaki et al., 2000) and the increased release of IL-1α and IL-1ra did not occur as a consequence of cell death as no increases in supernatant lactate dehydrogenase (LDH) activity were observed in parallel experiments (Figure 5.6). Moreover, separate experiments revealed no interference between IL-1α and IL-1ra in their respective ELISA assays.

Figure 5.6: LDH assay. The percentage of cell viability was determined by LDH release from PAM 212 keratinocytes after stimulation with various concentrations of either IL-1α or IL-1ra. In both cases, the cell death was minimal. Data represent mean ± SEM (n=3).
5.2.3 Responses to changes in extracellular IL-1α and IL-1ra concentrations in keratinocytes are mediated through IL-1RI

Having shown counter-regulation of IL-1 receptor ligands in keratinocytes, the mechanism of control was investigated utilising IL-1R antibodies. PAM212 cells were incubated with 3 ng/ml of either IL-1α or IL-1ra in the presence or absence of monoclonal neutralising antibodies against either mIL-1RI or mIL-1RII for 24 hours prior to ELISA analysis of clarified supernatants. In both cases, the increases observed with the IL-1 receptor ligands were completely abrogated in the presence of anti IL-1RI antibody (790±8 pg/ml to 535±19 pg/ml IL-1α and 1,001±46 pg/ml to 361±41 pg/ml IL-1ra), whereas co-incubation with anti IL-1RII antibody was without effect (Figure 5.7).

![Figure 5.7: Counter-regulation of IL-1α and IL-1ra is mediated through the type I IL-1 receptor in keratinocytes in vitro.](image)

(a) PAM212 cells were incubated with or without IL-1ra (3 ng/ml) in the presence or absence of antibodies against either mIL-1RI or mIL-1RII (10 μg/ml) for 24 hours at 37°C prior to ELISA analysis for IL-1α. Data represent mean ± SEM (n=3). (b) PAM212 cells were incubated under the same conditions as (a) in the presence of IL-1α (3 ng/ml) and assayed for IL-1ra. Data represent mean ± SEM (n=3).
5.2.4 Exposure of keratinocytes to sIL-1R results in a dose-dependent increase in IL-1α release *in vitro*

Finally, PAM212 keratinocytes were incubated overnight at 37°C in the presence of increasing concentrations of recombinant human sIL-1RI, a potent pharmacological IL-1 inhibitor, and supernatants subsequently analysed by ELISA for IL-1α. Supernatant IL-1α concentrations increased in a dose-dependent manner from 230±33 pg/ml to 500±47 pg/ml in the presence of 100 ng/ml sIL-1RI (Figure 5.8). IL-1α/IL-1R complexes were shown not to interfere with the detection of IL-1α in the ELISA.

![Figure 5.8: Keratinocytes increase release of IL-1α following exposure to soluble type-1 IL-1R. PAM212 cells were incubated overnight at 37°C in the presence or absence of increasing concentrations of recombinant sIL-1RI and supernatants subsequently analysed by ELISA for IL-1α. Data represent mean ± SEM (n=3).](image-url)
5.3 DISCUSSION

In this chapter the counter-regulation of IL-1α and IL-1ra in murine keratinocytes was described, evidenced by increases in IL-1α and IL-1ra release following addition of IL-1ra or IL-1α, respectively, to PAM212 cells in vitro and increased release of IL-1α and IL-1ra in an ex vivo organ culture model using epidermal sheets derived from mice that overexpress epidermal sIL-1ra and IL-1α, respectively. The compensatory changes were observed in released rather than stored proteins. The regulatory mechanism was shown to be mediated through the type I IL-1 receptor using neutralising monoclonal antibodies and the findings were extended by demonstrating an up-regulation in IL-1α release in keratinocytes following addition of sIL-1RI.

The complex autoregulatory loops involving released forms of IL-1α and β, sIL-1ra and IL-1 receptors have been extensively studied in leukocytes. For example, binding of IL-1α to IL-1RI results in induction of bio-active IL-1α (Dinarello et al., 1987; Manson et al., 1989) and sIL-1ra (Jenkins and Arend, 1993), whereas sIL-1ra inhibits both the activity and synthesis of IL-1α and β in monocytes (Granowitz et al., 1992). Incubation of peritoneal macrophages with sIL-1RI has been reported to induce a significant release of IL-1α in a concentration-dependent manner, in keeping with the data presented herein, and the same study reported a rapid and substantial systemic release of IL-1α following intra-peritoneal injection of sIL-1RI in mice (Netea et al., 1999).

In cutaneous cells, icIL-1ra has been shown to be elevated in lysates from human dermal fibroblasts overexpressing proIL-1α, although in the converse experiment, fibroblasts transduced to over-express icIL-1ra failed to show any increases in IL-1α.
production (Higgins et al., 1999). Primary murine keratinocytes have been shown to up-regulate icIL-1ra mRNA production following IL-1α exposure in a time and dose-dependent manner (La et al., 1999) and these findings have been extended recently to the characterisation of two important transcription factor binding sites on the icIL-1ra promoter responsible for the IL-1α-induced increase (La and Fischer, 2001). The data presented here therefore extend this observation by the demonstration of a dose-dependent induction of IL-1ra protein release by IL-1α and our observation that IL-1α release is stimulated following IL-1ra exposure in keratinocytes is also novel.

It is important to note that the effects observed in murine epidermis relate only to extracellular IL-1 molecules. This has several unexpected implications, most importantly, that a low level of pericellular IL-1 activity is likely to be present around normal keratinocytes. The lack of regulation of opposing IL-1 agonists and antagonists in keratinocyte homogenates noted in our study strongly suggests that the important site for constitutive IL-1α activity is extracellular, rather than intracellular. Indeed, the release of IL-1α in significant quantities is surprising in itself, since this molecule lacks a signal peptide and the mechanism of its release has been the subject of much speculation. However, application of mechanical stress to human keratinocytes has been shown to permit the release of large quantities of IL-1α and IL-1ra in the absence of cell death (Lee et al., 1997) and since IL-1 derived biological activity requires extracellular ligand to bind IL-1RI, there can be little doubt that release occurs.

The observed regulation of keratinocyte IL-1α release by IL-1ra would not be predicted from the known functions of these molecules and underscores the importance of IL-1 homeostasis in the normal cutaneous environment. For a putative IL-1 homeostatic
mechanism to function, it is likely that keratinocytes signal an excess or deficit of extracellular IL-1 molecules through the type-1 IL-1 receptor, and our data indicate that this is indeed the case. The hypothesis made that low level ligation of type-1 IL-1 receptor occurs in normal keratinocytes both in vivo and in vitro. Should there be perturbation of this low level receptor occupancy, either by the presence of excess receptor agonists or antagonists, keratinocytes respond by release of the opposing molecule (Figure 5.9).

Figure 5.9: Schematic illustration of the homeostatic mechanism involved in maintaining extracellular IL-1 activity in murine keratinocytes (☐: IL-1α, □: IL-1ra).

The physiologic role of constitutive cutaneous release of IL-1α is uncertain. One hypothesis is that keratinocyte-derived IL-1 is required for the production of other molecules involved in tissue homeostasis such as KGF and GM-CSF from dermal fibroblasts (Szabowski et al., 2001). Alternatively, it may simply act to maintain a
“tick-over” of the cutaneous IL-1 system, ensuring that keratinocytes are primed for IL-1 release in case of injury or other noxious stimulus.

If such a homeostatic role exists, IL-1α and IL-1ra are unlikely to be the sole players in the pathway since neither IL-1α nor IL-1ra knockout mice show any gross cutaneous abnormalities (Horai et al., 1998; Nicklin et al., 2000). Six new members of the IL-1 superfamily have been recently cloned (Dunn et al., 2001) and, whilst none has been reported to exhibit high affinity binding to cell surface IL-1RI, one molecule provisionally named IL-1H1/IL-1α is strongly induced in murine and human keratinocytes following treatment with IL-1α and TNF-α and activates NFκB following binding to a novel IL-1 receptor (Kumar et al., 2000; Debets et al., 2001). Whether these new molecules influence the delicate balance of IL-1 in the epidermis awaits further investigation.

The data presented here represent the first description of mutual counter-regulation of IL-1 receptor ligands in keratinocytes and underscore the necessity for tight control of this cytokine system in the extracellular microenvironment around epidermal keratinocytes. The findings in this Chapter indicating that IL-1ra can induce a significant release of IL-1α in keratinocytes may explain the cutaneous inflammatory response that follows therapeutic administration of IL-1ra (Bresnihan, 1999) and illustrates the importance of fully understanding the biology of the IL-1 system in the cutaneous microenvironment.
CHAPTER 6

CONCLUDING DISCUSSION
The skin is continuously exposed to a broad array of environmental hazards such as pathogens or injury. In order to cope with these threats, human skin contains a tightly regulated network of pro-inflammatory cytokines which become active during infection or after trauma. Major pro-inflammatory cytokines that are known to be involved in such threats, include members of the IL-1 family (IL-1α, IL-1β and IL-18) and TNF-α. This group of cytokines plays a critical role in generating host responses against noxious agents, such as viral, fungal and bacterial pathogens as well as toxic chemicals, allergens and ultraviolet light. These cytokines can mediate either innate or acquired immune responses by activating the endothelium, inducing secondary cytokines and activating immunocytes.

Within the epidermis, LC, dendritic cells and keratinocytes are the key players in cutaneous defense. The mobilization of LC and their directed migration from the epidermis to draining lymph nodes are processes of pivotal importance in the generation of cutaneous immune responses. Thus, LC are likely to be of key pathogenic importance in a number of human skin diseases, including allergic contact dermatitis, atopic eczema, and psoriasis. Over the past several years, considerable evidence has accumulated which suggests that epidermal cytokines play a crucial role in LC migration. At the onset of this project, several observations had led to the hypothesis that this migratory response is under control of at least two cytokines derived from epidermal cells, IL-1β and TNF-α.

This project aimed to investigate and elucidate further the roles played by IL-1 family molecules and the IL-1β processing enzyme, caspase-1, in the induction and regulation of LC migration and the initiation of cutaneous immune responses. Three hypotheses
were formulated in the general introduction of this thesis: (1) since caspase-1 regulates the processing and release of IL-1β, it might represent a useful target for LC manipulation; (2) because IL-18 has many structural similarities with IL-1β, including processing and release of mature protein by caspase-1, it may have a role in LC migration; and (3) as LC migration is a tightly controlled process, regulation of the activity of the cytokines involved is essential in the cutaneous microenvironment and therefore, a homeostatic mechanism controlling the IL-1 system was suspected and sought.

This project provided an invaluable opportunity for training and gaining experience in a wide range of laboratory techniques including enumeration, isolation, purification and characterization of epidermal LC and dendritic cells within regional lymph nodes. Furthermore, phenotypic characterization of these cells provided training in immunohistochemistry and analytical flow cytometry. In addition, valuable experience gained in experimental procedures in mice, including the induction and elicitation of contact hypersensitivity and the intradermal or intraperitoneal administration of cytokines at different sites. Finally, during this project a broad training was received in experimental techniques involving genetically modified mice, including in vivo immunological techniques, genotypic and animal colony management.

Novel data have been demonstrated, implicating caspase-1 as a key regulator of LC migration and the subsequent induction of CHS. Most interestingly, the caspase-1 inhibitor Ac-YVAD-cmk, when applied topically had a marked inhibitory effect on LC migration in response to allergen application to skin and significantly suppressed CHS. Thus, these findings suggest that there might be a potential for development of YVAD
and other specific caspase-1 inhibitors as therapeutic agents for the treatment of immunologically mediated skin diseases such as ACD.

The next stage of this project focused on the role of IL-18 in the regulation of LC migration and CHS. The data presented herein, in combination with antibody inhibition studies published recently (Cumberbatch et al., 2001), demonstrated that IL-18, when administered intradermally to mice, was able to stimulate both the migration away from the epidermis of a proportion of LC local to the site of exposure and the accumulation of DC in draining lymph nodes. Furthermore, LC migration was absent in IL-18 -/- mice and CHS was significantly suppressed in response to topical sensitisation with contact allergens in these animals. However, IL-18 was shown to have no apparent role in irritant contact dermatitis, since LC migration in response to the irritant agent SLS was normal in IL-18 -/- mice. The study also indicated that IL-18 acts upstream of both TNF-α and IL-1β in the migratory process (Figure 6.1).

**Figure 6.1: IL-18 acts upstream of IL-1β and TNF-α in LC migration.** Interaction of the allergen with LC provides a signal for IL-18 activation (1) and subsequent up-regulation of IL-1β (2) which in turns acts on adjacent keratinocytes to provoke the production of TNF-α (3) and then these events stimulate LC migration (4).
In the final section of this project we demonstrated the existence of a homeostatic feedback mechanism in the epidermal IL-1 system, in which not only increased levels of agonist (IL-1α) lead to increases in antagonist production (IL-1ra), but also that increases in antagonist release lead to up-regulation of agonist, underscoring the complexity of cytokine networks in epidermis. Interestingly, this counter-regulation was observed only in extracellular IL-1 molecules and was shown to be mediated through IL-1RI.

A number of fundamental questions concerning the findings arising from this work remain unclear and will be worth pursuing:

1. Which cells produce and which cells respond to IL-1β, TNF-α and IL-18 following contact of antigen with the skin?

It was reviewed in the introduction that these three cytokines have been shown to be released from keratinocytes, LC and fibroblasts (the predominant cell types in the cutaneous microenvironment) under various conditions and there is clear evidence that these cytokines are key signals in LC migration and CHS. However, from our current data it is unclear which cell types provide, and which ones respond to these major mediators during initiation of LC migration. This question is difficult to examine using conventional methodologies such as immunohistochemistry and Western blotting as these techniques provide only limited functional information. A better approach will be to perform reconstitution experiments using cytokine gene knockout mice, exploiting the fact that LC derive from precursor cells in bone marrow. Thus, if an irradiated IL-1β knockout mouse is reconstituted with WT bone marrow it will be possible to assess the effect of keratinocyte IL-1β deficiency in the context of IL-1β competent LC. The
reverse experiment can equally easily be done, in which WT mice are reconstituted with marrow taken from an IL-1β deficient mouse, thereby generating chimeric mice in which LC lack IL-1β in the context of an epidermis fully able to produce this molecule. Similar experiments can be conducted using IL-1β, IL-18, TNF-α and relevant receptor knockout animals, in order to precisely identify which cells are secreting and responding to each molecule. Preliminary experiments done in our lab, using TNF knockout animals have confirmed the feasibility of this approach and these experiments will be critical in extending our understanding of LC cytokine signaling.

2. What are the interrelationships between IL-1/TNF/IL-18 in LC migration?

Previously, it was considered that topical sensitisation causes the up-regulation of expression of IL-1β by LC (Enk and Katz, 1992) and that this cytokine, as well as delivering one signal for migration, acts in an autocrine fashion through IL-1RI receptors, thereby stimulating the production of TNF-α by keratinocytes. The latter cytokine provides a second signal to adjacent LC acting via TNF-R2 receptors (Kimber et al., 1998; 2000). It is clear from data presented in Chapter 4 that this sequence of events now has to be modified and extended in order to accommodate the requirement for IL-18. The findings observed herein suggest that the availability of bioactive IL-18 is an early event during skin sensitisation and may act as the initial trigger for IL-1β production, which in turn stimulates the production of TNF-α. Another hypothesis is that IL-18 might serve as the direct stimulus for the induction or upregulation of both IL-1β and TNF-α. The precise contribution of IL-18 to IL-1β and/or TNF-α production will be dependent upon whether LC and/or keratinocytes prove to express the functional IL-18R complex. The interrelationships between these cytokines require further investigation which should provide valuable information on the sequence of events in
the early stages of LC migration. It also remains unclear whether the various cytokines
duce distinct phenotypic changes in LC they are required all to be present for migration
to occur. As with the first question, the use of individual cytokine gene knock out mice
should be an invaluable tool.

3. What is the functional role of the retained LC in the epidermis and what are the
functional differences between mobilised and non mobile LC?
The studies presented in this thesis together with previously published data
(Cumberbatch et al., 1997) revealed that only a proportion of LC migrate away from the
epidermis to regional lymph nodes following encounter with an antigen. Thus, a key
question that needs to be resolved is why not all LC respond to a given stimulus and
what the role of the retained LC is. A possible explanation is that LC left behind after
antigen stimulation are there to ‘guard’ the epidermis and make sure that there is still
protection from other immunological insults since epidermis is vulnerable whilst the
responding LC have gone. However, it could be argued that since memory T cells may
already be in transit (having been primed by the responder LC), it should not make any
difference if the non-responders move later on. Possibly a more powerful argument
which addresses both questions is that there is some form of antigen specificity built
into LC such that only certain subsets will respond to particular types of stimulus. This
hypothesis is supported by the recent discovery that Toll-like receptors (TLRs) act as
antigen-specific cell-surface markers on various immunocytes and recently, it was
demonstrated that LC express TLRs (Akira et al., 2001). To examine these questions
experimentally, it will be important to examine how sub-populations (perhaps defined
by TLR expression) of LC behave following treatment with different stimuli.
Alternatively, one could examine the numbers of LC remaining in epidermis following
sequential distinct stimuli (DNFB and oxazolone for example) to determine if different LC migrate in response to each stimulus.

4. Do the recently identified novel IL-1 family gene members have a role in LC migration?

Recently five new IL-1 gene family members have been described (Dunn et al., 2001; Debets et al., 2001). At least two of these (IL-1F5 and IL-1F9) are expressed in human skin and are upregulated in psoriatic lesions (Debets et al., 2001). The function of these two IL-1 isoforms is currently unknown but, considering their expression in diseased skin and the important role of other IL-1 isoforms in skin they may well have important roles in cutaneous biology and be involved in regulation of LC migration and function. Thus, investigation of these novel IL-1 molecules and their function in skin could yield new perspectives on the regulation of inflammatory processes in skin.

In the last decade, major advances have been made in the understanding of the regulation of LC migration and in defining the key role played by epidermal cell derived cytokines, including IL-1β and TNF-α, in the initiation and maintenance of cutaneous inflammation (Cumberbatch et al., 2000). The present studies have confirmed that IL-1β and TNF-α are key cytokine signals in the regulation of LC migration and CHS. Furthermore this thesis has provided evidence of the key roles of IL-18, as well as caspase-1, in the process of LC migration. The data presented here provide important information on the role of the IL-1 system in the initiation and regulation of cutaneous immune responses and provide further evidence of the complexities of the cutaneous immune system.


72. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2


