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The Role of Chemokines in Oral Lichen Planus

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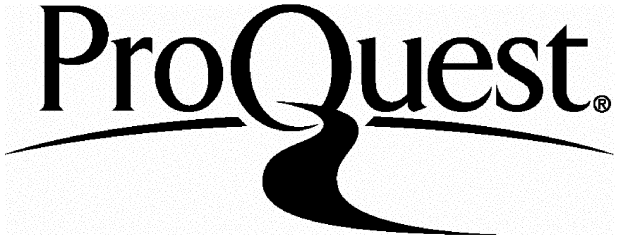
'The work presented in this thesis is my own, except where otherwise stated. No part of this work has been, or will be, submitted for any other degree, diploma or qualification.'



Abstract

Oral lichen planus (OLP) is an inflammatory disease characterised by a localised infiltration of T cells in a band-like structure below the oral epithelium. Chemokines are secreted proteins known for their ability to attract leucocyte populations through specific receptors on these cells. In this study, five chemokines that are known to attract memory T cell populations were investigated in order to establish their possible role in the pathogenesis of OLP. mRNA and protein of the studied chemokines (monokine induced by interferon-gamma (MIG), interferon-induced protein-10 (IP-10), interferon-induced T cell attractant chemokine (I-TAC), cutaneous T cell attractant chemokine (CTACK) and macrophage inflammatory protein-3alpha (MIP-3α)) was expressed by oral epithelial cells either constitutively or expressed following pro-inflammatory stimulation. IP-10 mRNA expression by oral epithelial cells was found to be enhanced by lipopolysaccharide stimulation, and IP-10 (and other relevant chemokines) were shown to have an anti-microbial effect against *Streptococcus sanguis*, perhaps suggesting that IP-10 is produced in OLP in response to, and/or to act against, an oral bacterial imbalance. It was confirmed that IP-10 and CTACK predominately attract memory T cells, however, CTACK produced from oral epithelial cells was more effective at chemoattraction after cytokine pre-treatment. mRNA transcripts of the CXC ELR- chemokines and CTACK were found to be upregulated in OLP lesional tissue in comparison to normal oral mucosal tissue. However, mRNA expression of MIP-3α or its receptor was not upregulated. The presence of CD40 and CD154 (whose ligation can enhance the production of some chemokines in epithelial cells) was demonstrated in oral lichen planus. In summary, chemokines are likely to play an important role in the migration of large numbers of T cells witnessed within OLP lesions. The exact initiating and precipitation factors accounting for the chronicity of OLP are not known but might include bacterial stimulation of chemokines.

To Doug, for laughter and love

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Abbrevations

Ab -- antibody

Ag – antigen

APS – 3'-aminopropyltriethoxysilane

APC -- antigen presenting cell

bp -- base pairs

BSA -- bovine serum albumin

CD -- cluster of differentiation

CON or con -- control

CTACK -- cutaneous T cell attractant chemokine

CTL -- cytotoxic T lymphocyte

CTLA-4 -- cytotoxic T lymphocyte antigen-4

DAB -- 3', 3'-diaminobenzide

cDNA -- complementary deoxynucleic acid

dNTPs -- deoxynucleotides

DTH -- delayed-type hypersensitivity

E. coli -- Escherichia coli

ELISA -- enzyme-linked immunosorbent assay

FACS – florescence-activated cell sorter

FCS -- foetal calf serum

FSC – forward scatter

HLA – human leucocyte antigen

HPV-16 – human papilloma virus-16

ICAM -- intracellular adhesion molecule

idv – integrated density value

IFN-γ -- interferon-gamma

Ig -- immunoglobulin

IL-1β -- interleukin-1beta

IL-8 -- interleukin-8

IP-10 -- interferon-induced protein-10

I-TAC -- interferon-induced T cell chemoattractant

KGM -- keratinocyte growth medium

LC – Langerhans cell

LFA – lymphocyte function antigen

LP – lichen planus

LPS -- lipopolysaccharide

MEC -- mucosae epithelial derived chemokine

MHC -- major histocompatibility complex

MIG -- monokine induced by interferon-gamma

MIP-3α -- macrophage inflammatory protein-3alpha

M-MuLVRT -- moloney murine leukaemia virus reverse transcriptase

mRNA -- messenger ribonucleic acid

MyD88 -- myeloid differentiation antigen-88

NK cells -- natural killer cells

NO – nitric oxide

NOM – normal oral mucosal tissue

OLP -- oral lichen planus

PBMC -- peripheral blood mononuclear cells

PBL -- peripheral blood lymphocytes

PBS -- phosphate buffered saline

RANTES -- regulated upon activation T cell expressed and secreted

RT-PCR -- reverse-transcriptase polymerase chain reaction

SD – standard deviation

SDF-1α -- stromal-derived factor-1alpha

sIgA – secretory IgA

SSC – side scatter

S.sanguis -- Streptococci sanguis

TARC -- thymus and activation-regulated chemokine

TCR -- T cell receptor

TECK – thymus-expressed chemokine

TGF-α1 -- transforming growth factor alpha-1

Th -- T helper

TIRAP -- Toll-IL-1 receptor domain-containing adapter protein

TLR -- toll-like receptor

TNF-α -- tumour necrosis factor-alpha

TSB -- trypicase soya broth

VEGF -- vascular endothelium growth factor

YE -- yeast extract

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Chapter 1: Introduction

This study investigates immunological aspects of the oral epithelium in oral lichen planus. This first chapter details some of the immunological mechanisms that the host utilises to combat infection, the process of lymphocyte migration and immunological aspects of oral lichen planus.

1.1 An overview of aspects of immunity relevant to oral lichen planus

Many of the cells involved in immunological processes are cells derived from the blood-cell forming part of the bone-marrow and are known as haemopoetic cells. However, it has now been established that many non-haemopoetic cells, for example epithelial cells, are also involved in immunological reactions and thus play a probable role in some forms of immunologically-mediated disease of the oral mucosal membranes.

Cells involved in immunological processes are often distinguished by surface markers known as clusters of differentiation (CD). These antigens are recognised and normally differentiated by groups of monoclonal antibodies (Mason *et al,* 2001). The clusters of differentiation have been allocated into an arbitrary number system (eg. CD1, CD2 etc.) and thus the presence of certain CD markers on cells can assist in typing the cells involved in immunological reactions.

Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cytokine receptors can induce a number of activities in the cell, such as growth, differentiation, or death (Janeway & Travers, 1996). Although most cytokines have pleotrophic

effects, some are generally considered pro-inflammatory, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) (reviewed by Dinarello, 1997), whereas others are associated with anti-inflammatory effects, such as transforming growth factor-beta-1 (TGF-β1) (reviewed by Ling & Robinson, 2002).

1.2 Immune cell populations of relevance to oral lichen planus

Macrophages

Macrophages are phagocytic cells that can be distinguished from other leukocytes by the expression of CD14 on their cell surface. Phagocytes ingest antigen and are capable of destroying bacteria via phagocytosis and killing (reviewed by Djaldetti *et al,* 2002). Once antigen/s have been ingested, the cell utilises oxygen radicals, such as nitric oxide (NO) to destroy the bacteria. However, prolonged activation of phagocytes can cause excessive NO production which can cause tissue damage (reviewed by Ricevuti, 1997). As well as being aided by the action of complement, many phagocytes utilise receptors to bind to common bacterial repeated elements in order to help induce phagocytosis of these agents (reviewed by Peiser & Gordon, 2001). In addition, macrophages have a significant role in influencing the progression of immune responses during inflammation, by functioning as antigen presenting cells (APCs) and through production of cytokines.

Mast cells

Mast cells contain granules including mediators, such as histamine. These granules can be released during activation with IgE (Walsh *et al,* 2003). The presence of mast cells are often associated with Th2 reactions (see T-lymphocytes) and allergic

reactions, but can play a role in releasing pro-inflammatory cytokines, such as TNF-α (Boyce, 2003).

Dendritic cells

Dendritic cells are antigen presenting cells with a unique ability to induce primary immune responses. They have a prime role in regulating immune responses by their specialised ability to acquire, process and present antigen to T cells. Dendritic cells appear to be the only class of antigen-presenting cells that have the capacity to stimulate the expansion of naïve T cells and thereby initiate primary immune responses (Randolph, 2001). Immature dendritic cells do circulate in the blood, but are more abundant within epithelial and connective tissues, where they are ideally positioned to acquire antigens that typically initially establish infection in the periphery. After the correct stimulatory signals, dendritic cells will migrate to the lymph nodes to interact and activate T-cells locally. The factors that activate maturation of dendritic cells are numerous and include proinflammatory cytokines, bacterial products or stimulation with specialised ligands or necrotic cells, as well as members of the heat shock protein family (Randolph, 2001).

Langerhans cells

Langerhans cells (LC) are dendritic cells located exclusively in the epidermis of skin and oral mucosal membranes. It is believed that their main role is the transport of antigens to lymphoid tissue, where they then differentiate to present antigen to T cells. Compared with other dendritic cells, LC are relatively inefficient in antigen uptake, processing and presentation and this may serve to avoid hyper-responsiveness to harmless protein antigen likely to be frequently encountered in skin and oral mucosal

membranes (Momaass *et al,* 1999). However, LC are vital for the induction of immune responses to antigens encountered via the skin and oral mucosal membranes, and are particularly important in primary immune responses by their ability to activate naïve T cells, whereas non-professional APC can induce effector function in previously activated cells (Lappin *et al,* 1996). As such, LCs play a key role in contact hypersensitivity reactions (Gorbachev & Fairchild, 2001). LC can normally be distinguished from other cells in the skin due to the presence of CD1a or T6 (Murphy *et al,* 1982) on their cell surface or Birbeck granules (part of the endosomal compartment) in their cytoplasm (McDermott *et al,* 2002).

Lymphocytes

Adaptive immune responses are mediated by lymphocytes. The adaptive immune system is the response of specific lymphocytes towards antigen, which includes the development of immunological memory. These responses are generated by the clonal selection of lymphocytes that bear a specific receptor to antigen. The process of initiating the adaptive response takes place in the secondary lymphoid organs. Lymphocytes bear cell-surface receptors for antigen with many different specificities, due to unique gene rearrangements in these cells (Janeway & Travers, 1996).

T-lymphocytes

These are a subset of lymphocytes that are derived from interactions within the thymus. These cells have a heterodimeric receptor, known as the T cell receptor (TCR), consisting either of alpha-beta ($\alpha\beta$) or gamma-delta ($\gamma\delta$) chains, which is associated with the CD3 complex.

Alpha-beta T-cells

Alpha-beta ($\alpha\beta$) T-cells fall into two major classes that differ in the class of MHC molecule that they recognise. These two main classes differ in respect to effector function and are distinguished by cell-surface proteins known as CD4 and CD8. CD4 binds to an invariant portion of MHC class-II whereas CD8 binds to an invariant portion of the MHC Class I molecule. During antigen presentation, CD4 and CD8 associate on the cell surface with the T-cell receptor (Reviewed by Gao *et al,* 2002).

CD4 cells are known for the ability to assist the immune system; whereas Th2 CD4+ cells assist B-cell activation, Th1 CD4+ cells assists cell-mediated immunity. The effector functions of Th CD4+ cells are mostly dependant upon cytokine production; Th1 cells are primarily associated with IFN-γ production, Th2 cells predominately produce IL-4 (Reviewed by Murphy & Reiner, 2002). A subset of CD4+ T cells that express CD25 represent cells that are thought to be involved in regulation of immune responses and the suppression of inflammation (Reviewed by Shevach, 2002).

On the other hand, CD8+ cells are known for their ability to recognise foreign antigen associated with MHC class-1 and cytotoxically destroy cells bearing this molecule with a 'foreign' antigen. On activation, cytotoxic T-cells release granules from their cytoplasm which include perforin and granzyme. Granzymes are serine esterases which can induce apoptosis in target cells, by inducing DNA fragmentation. Alternatively, cytotoxic T cells can induce apoptosis in their target cells through binding with TNF receptors which induce an intracellular pathway, leading to cellular death (Reviewed by Barry & Bleakley, 2002).

CD45RA is a marker for naïve cells involved in primary immune reactions, whereas CD45RO is considered a marker for memory cells, which are associated with a secondary immune reaction (Reviewed by Swain, 2003). Depending upon antigen dose and co-stimulation, memory cells require less time to reach commitment compared to naïve cells during antigen presentation (Lanzavecchia & Sallusto, 2001).

Gamma-delta T-cells

A high proportion of gamma-delta ($\gamma\delta$) receptor T-cells reside in the epithelial surfaces such as skin and the vagina. Most of these gamma-delta cells are CD8+, but express the CD8 molecule as a homodimer of two alpha chains instead of the conventional alpha-beta chains. Regardless of site investigated, $\gamma\delta$ T-cells represented up to 2% of the T-cell population in human healthy oral mucosa (Pepin *et al,* 1993).

Although the exact functions of $\gamma\delta$ T-cells are not clear, they are thought to be involved in immune defence, regulation and tissue homeostasis. There is evidence to suggest that these cells are important mediators of mucosal tolerance and therefore, regulate autoimmunity. Unlike $\alpha\beta$ T-cells, $\gamma\delta$ TCRs are able to recognise intact peptides and non-peptides and do not require presentation through MHC molecule/s (Reviewed in Hanninen & Harrison, 2000 and Allison & Garboczi, 2002), thus differing in specificity to $\alpha\beta$ T-cells. $\gamma\delta$ cells may also have a role in the skin homeostasis, in recognising stressed mucosal epithelial cells. Certain $\gamma\delta$ T cell populations are implicated in recognising the 60kDa heat shock protein (hsp60) and in $\gamma\delta$ T-cell deficient mice, infections cause an exaggerated inflammatory response with accompanied tissue necrosis. Thus these cells may be crucially important in prevention of chronic diseases, through production of immunoregulatory cytokines

(Reviewed in Carding & Egan, 2000). These cells can also act in a cytotoxic manner, similar to CD8+ $\alpha\beta$ cells.

Natural killer (NK) cells

In humans, NK cells efficiently lyse abnormal cells that either lack the expression or express inadequate amounts of HLA class I molecules. Down-regulation of HLA class I expression is a frequent event during tumour transformation or subversion of the immune system during viral infection, therefore NK cells represent a first line of defence of the immune system against transformed and virally infected cells (Biassoni *et al,* 2000). Natural killer cells can discriminate between the normal and HLA class I deficient cells due to the expression of specific inhibitory receptors that when activated inhibit NK-mediated cytotoxicity (Biassoni *et al,* 2000).

B-lymphocytes

B-lymphocytes bear specific receptors to antigen. When in contact with specific antigen B cells are capable of generating specific antibodies to that antigen (Janeways & Travers, 1996). Different classes of antibodies can be induced in B cells after specific cytokine stimulation and the reaction is synergised by ligation of CD40 on B cells (Zhang, 2003).

1.3 Antigen Presentation

To recognise antigen, T cells (require to establish) contact with antigen-presenting cells by forming an immunological synapse, whereby cell receptors and costimulatory molecules are congregated in a central area surrounded by a ring of adhesion

molecules. The amount of signal that T cells receive is dependent upon three factors: 1) the level of peptide-MHC complexes that initiate signal transduction, 2) the level of costimulatory molecules that amplify the signalling process, and 3) the stability of the synapse that determines for how long the signalling process is sustained (Lanzavecchia & Sallusto, 2001).

The major histocompatibility complex (MHC)

The major histocompability complex (MHC)-Class I (or human leucocyte antigen (HLA-A, B, C)) is a cell surface molecule, which can present peptide antigens to T cells and is expressed upon all cells of the body, except red blood cells (Hofmann *et al,* 2001). MHC-Class II (or HLA-D) is expressed almost exclusively on professional antigen presenting cells, such as dendritic cells, macrophages and B-cells (Guermonprez *et al,* 2002). MHC Class I molecules present peptides almost exclusively from intracellular sources, to CD8+ T cells (Reviewed by Yang, 2003), and MHC class II molecules generally present internalised and processed antigen to CD4+ T cells (Robinson & Delvig, 2002).

Class II MHC molecule expression on Langerhans cells

The presence of MHC-Class II on the surface of LCs is indicative of cellular activation, as acquisition of MHC-II molecules tends to coincide with migratory properties. For example, the application of haptens to the skin leads to changes that include an increase of the major histocompatibility complex II expression and migration towards lymph nodes to activate T cells (Rougier *et al,* 1998). Therefore, the proportion of cells that express CD1a in comparison to those also expressing HLA-D antigens can be considered a reflection of dendritic cell maturation (Reviewed

by Bancereau & Steinman, 1997). IFN-γ is particularly effective in inducing non-HLA-DR positive LCs to express these MHC molecules (Berman *et al,* 1985).

Costimulatory molecules

Co-stimulatory molecules are molecules that associate during antigen presentation between the APC and T cell and enhance antigen presentation. Although there are many different co-stimulatory molecules during antigen presentation, some of the most important are thought to be the B7 molecules. The B7 family members; B7.1 and B7.2 (or CD86/ CD80 respectively) are cell surface molecules almost exclusively located on 'professional' antigen-presenting cells, such as LC. Both molecules are involved in the co-stimulation of T-cell responses during MHC-TCR antigen presentation (reviewed in McAdam *et al,* 1998). Either molecule can bind to the ligands, CD28 or cytotoxic T-lymphocyte associated molecule-4 (CTLA-4), located upon resting and activated T cells. Whilst CD28 binding, in conjunction with a TCR-MHC signal, upregulates T cell stimulation and clonal expansion (Vella *et al,* 1997) (through stimulation of multiple cytokines), binding of CTLA-4 is thought in most instances to down-regulate this process (Kuhns *et al,* 2000).

In the presence of co-stimulation, naïve T cells can respond to approximately 100-fold lower doses of antigen and respond more rapidly than without co-stimulatory signals. It is also been postulated that antigen delivered without the presence of co-stimulatory signals can induce tolerance in T-cells specific for that antigen (Lanzavecchia & Sallusto, 2001).

Antigen presenting cell - T lymphocyte adhesive interactions

Antigen presentation involves a number of interactions between the T-cell and the antigen-presenting cell. Some of the important interactions involved include lymphocyte function antigen(LFA)-1 (a member of the integrin family) with ligands ICAM-1, -2, -3 and CD2 with LFA-3, which bring the T-cell and APC respectively into contact, in order that they can present antigen through MHC-TCR and co-stimulatory molecule interactions (Reviewed by Elangbam *et al,* 1997).

1.4 Aspects of the delayed-type hypersensitivity reactions

The delayed type hypersensitivity reaction (DTH) (or Type IV hypersensitivity) is a well-characterised T-cell mediated inflammatory reaction. The reaction is antigen-specific and results in erythema and induration at the site of antigen injection in immunised animals or humans. The nature of the antigen can be varied. The histology generally consists of an influx of immune cells at the site of injection, including macrophages or basophils within 24-72 hours. T cells (either CD4+ or CD8+ depending on the antigen) are required to initiate the reaction (Reviewed by Black, 2001) and they migrate to the lesion, where they act specifically upon cells presenting specific antigen.

The DTH reaction that occurs in the skin after contact with irritant or allergen is known as contact hypersensitivity. There are three critical events that must occur in generating a reaction; sensitisation, trafficking and elicitation. There is primary sensitisation by an inflammatory agent resulting in antigen-presenting cell migration to the local lymph nodes and presentation of antigen to T-cells. Memory T cell clones generated in the lymph node/s then respond to a secondary exposure to that particular

antigen (Reviewed by Sallusto & Lanzvecchia, 2001). Although erythema and induration are not normally associated with OLP lesions, this may due to the possible involvement of the secondary reaction when the OLP reaction is clinically relevant.

1.5 Lymphocyte homing

In a process of immune surveillance, lymphocytes continuously re-circulate through the blood into the organised lymphoid tissue, and then return to the blood. Importantly, the movement of lymphocytes or leukocytes into peripheral tissues is an essential part of immune reactions, both during inflammation and tissue damage.

The migration of lymphocytes into lymphoid and peripheral tissue requires the expression of a number of chemokine and adhesion molecules in the involved tissue and expression of specific receptors upon the target cells. This process occurs in sequential steps, firstly cells make initial interactions with endothelial cells usually through the selectin adhesion molecules, which leads to tethering and, due to the force of the blood flow, a rolling along the endothelial cell surface (Fig.1.1, Step a-b). L-selectin is expressed on most leukocytes constitutively and can bind to E-selectin or P-selectin on activated endothelial cells, although some other ligands have also been identified (Vestweber & Blanks, 1999). Adhesion to the endothelium (Fig.1.1, Step d) occurs through the integrin family of adhesion molecules expressed on the endothelium [i.e. LFA-1 ($\alpha L\beta 2$), Mac-1 ($\alpha M\beta 2$), VLA-4 ($\alpha 4\beta 1$) and LPAM-1($\alpha 4\beta 7$)] to members of the immunoglobulin family expressed on leucocytes (ICAM-1, ICAM-2, VCAM-1 and MAdCAM-1) (Harris *et al,* 2000)] (and in some circumstances, the integrins may also be involved in the tethering process). However, integrins on leukocytes are expressed in a state that has low affinity and, therefore, require

activation into a high affinity before adhesion occurs (Fig 1.1, Step c). The presence of endothelial chemokines has a large role in the activation of the integrins (Johnston & Butcher, 2002) allowing adhesion to occur. Furthermore, once adhesion has occurred, chemokines also play an important role in the diapedesis and later migration within a tissue (Fig 1.1, Step e).

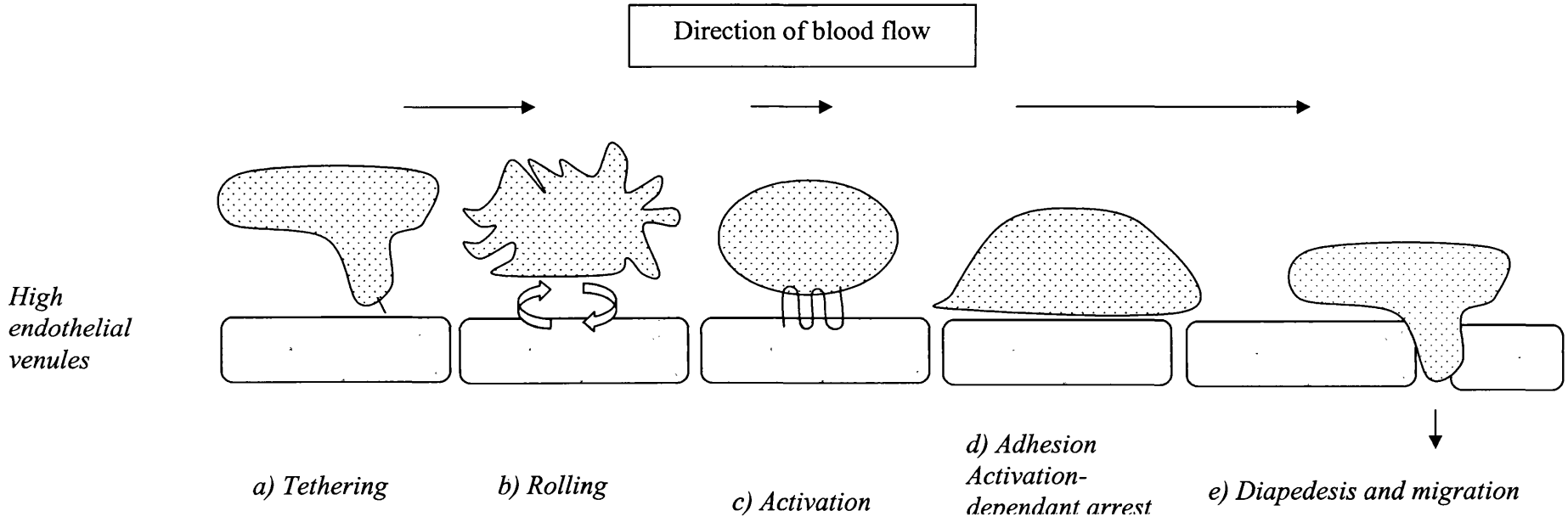


Figure 1.1: The multistep model of endothelial cell-leukocyte interactions involved in the recruitment of leukocytes from the blood. (Adapted from Johnston & Butcher, 2002).

Chemokines

Chemokines are a superfamily of structurally related cytokines, which share an ability to chemotactically attract their target cells along a concentration gradient. It is through this ability that these molecules play an integral role in the migration of immune cells to areas of pathogen challenge. Chemokines also mediate the movements of cells to allow interactions between immune cells which are essential for mounting immune responses (Reviewed by Zlotnik & Yoshie, 2002). As discussed later, other properties have also been described for chemokines (Ward *et al,* 1998) which probably also play an integral role in immune responses.

Chemokine structure

All chemokines are small proteins, ranging in weight from 6-14KDa. There are now over 40 identified chemokines that can be classified into 4 main structural families, dependent upon the position of cysteine residues near the N-terminus. These families are the CC, CXC, C and CX_3C, with the X denoting the number of amino acids between the cysteine residues (Reviewed by Olsen & Ley, 2002). The CC and CXC subfamilies include most of the chemokines identified to date (see Table 1.1). The CXC chemokines can be further subdivided depending upon whether they contain an ELR residue (See Chapter 3), which confers migratory properties for neutrophils (Clark-Lewis *et al,* 1993).

Chemokine receptors

All chemokines bind to a seven-transmembrane receptor that is coupled to a G-protein in a two step process. Firstly, the chemokine binds to a region on the N-terminus of

the receptor, and then residues on the N-terminus of the chemokine bind to a second site on the receptor affecting the switch of the receptor to the active form. It is through the intracellular phosphorylation of Rho proteins that have a role in actin reorganisation that the migratory properties of chemokines are mediated (Worthylake & Burridge, 2001) (Rho proteins are part of a small family of GTPases – GTPases being enzymes that can bind and hydrolyse GTP (Schmitz *et al,* 2000)). The chemokine receptors can be divided into two main families, the CC receptors and CXC receptors, depending upon the chemokines that they bind (see Table 1.1). The binding of chemokine to its receptor is not necessarily exclusive as there is overlap in both the number of chemokines that can bind one receptor and, furthermore, chemokines may be able to bind more than one receptor type (Reviewed by Mantovani, 1999). Furthermore, there can be different potencies of the chemokines that share the same receptor, for example, the ligands of CXCR3 (Lu *et al,* 1999).

Nearly all chemokines are secreted from the site of production and they often bind with glycosaminoglycans (Hoogewerf *et al,* 1997). It is thought that this is the method in which the chemokines form the concentration gradient that target cells migrate towards, as a higher concentration is formed on the connective tissue nearest the area of chemokine production.

The expression of chemokine receptors can be indicative of T cell polarisation, as Th1 cells preferentially express different receptors than those expressed upon Th2 cells (Sallusto *et al,* 1998). The production of the chemokines is thus associated with either Th1 or Th2 mediated conditions.

Table 1.1: The chemokines and their receptors. Names for chemokines were originally given according to their function or the source cell (Common names), however, systemic names have now been assigned to avoid confusion. Chemokines in italics are studied in this thesis. Adapted from Proudfoot, 2002.

Common names for chemokines	**Systemic names for chemokines**	**Chemokine Receptors**
Gro-alpha	CXCL1	CXCR2
Gro-beta	CXCL2	CXCR2
Gro-gamma	CXCL3	CXCR2
ENA78	CXCL5	CXCR2
GCP-2	CXCL6	CXCR1
NAP-2	CXCL7	CXCR2
IL-8	*CXCL8*	*CXCR1, CXCR2*
MIG	*CXCL9*	*CXCR3*
IP-10	*CXCL10*	*CXCR3*
I-TAC	*CXCL11*	*CXCR3*
SDF-1	*CXCL12*	*CXCR4*
BCA-1	CXCL13	CXCR5
	CXCL16	CXCR16
I-309	CCL1	CCR8
MCP-1	CCL2	CCR2
MIP-1alpha	CCL3	CCR1, CCR5
MIP-2alpha	CCL4	CCR5
RANTES	CCL5	CCR1, CCR3, CCR5
MCP-3	CCL7	CCR1, CCR2
MCP-2	CCL8	CCR2
Eotaxin	CCL11	CCR3
MCP-4	CCL13	CCR2, CCR3
TARC	CCL17	CCR4
ELC	CCL19	CCR7
MIP-3alpha	*CCL20*	*CCR6*
SLC	CCL21	CCR7
MDC	CCL22	CCR4
TECK	CCL25	CCR11
CTACK	*CCL27*	*CCR10*
MEC	*CCL28*	*CCR10, CCR3*
Fractalkine	CX_3CL1	CX_3CL
Lymphotactin	XCR1	XCL1

(*BCA-1,* B-cell-attracting chemokine 1; *CTACK*, cutaneous T-cell-attracting chemokine; *ELC*, Epstein–Barr-virus-induced gene 1 ligand chemokine; *ENA78*, epithelial-cell-derived neutrophil-activating peptide 78; *GCP-2*, granulocyte chemotactic protein 2; *Gro,* growth-regulated oncogene; *IL-8,* interleukin 8; *IP-10,* interferon-inducible protein 10; *I-TAC*, interferon-inducible T-cell α chemoattractant; *MCP,* monocyte chemoattractant protein; *MDC*, macrophage-derived chemokine; *MEC,* mucosae-associated epithelial chemokine; *MIG*, monokine induced by interferon-γ; *MIP,* macrophage inflammatory protein; *NAP-2*, neutrophil-activating peptide 2; *RANTES,* regulated on activation, normal T-cell expressed and secreted; *SDF-1,* stromal-cell-derived factor 1; *SLC*, secondary lymphoid-tissue chemokine; *TARC*, thymus and activation-regulated chemokine; *TECK*, thymus-expressed chemokine).

Chemokine expression patterns

The function of chemokines can be subdivided into two main families; those that are induced after inflammatory stimuli, the inflammatory chemokines, and those produced constitutively in tissues, the homing chemokines (Kunkel & Butcher, 2002). There appears to be some overlap between these chemokines as some of the inflammatory chemokines appear to be produced constitutively in some areas of the body (e.g. Izadpanah *et al,* 2001) and some of the chemokines designated as homing chemokines can be upregulated by inflammatory stimuli (e.g. Morales *et al,* 1999).

The inflammatory chemokines

The production of inflammatory chemokines, for example, MIG, IP-10, I-TAC and RANTES, is often associated with inflammatory conditions. They tend to be not expressed or only expressed at low levels during healthy conditions, but are induced / upregulated by pro-inflammatory stimuli, such as pro-inflammatory cytokines (for example, IFN-γ (Sauty *et al,* 1999)).

Homing chemokines

Homing chemokines, for example, MEC, TECK or CTACK, have a role in the surveillance of the tissue for evidence of previous infections. As such they often attract either dendritic cells or T cells that have previously been involved in infection.

It was found recently that effector cells preferentially enter certain tissues connected to the secondary lymphoid organs where antigen was first encountered, partially

through the expression of tissue-specific chemokines and the acquisition of specific receptors for these chemokines (Campbell & Butcher, 2002).

Tissue specific chemokines

Homing chemokines can be expressed in a tissue specific manner and have so far been clearly divided into the skin-homing system and the gut-homing system (Reviewed by Kunkel & Butcher, 2002), although there are also now some chemokines identified that appear to be specific for other areas of the body. The combination of specific expression of adhesion molecules and chemokine receptors thus can specify the tissue-homing capability for a cell. Many of the tissue homing chemokines have been found to be expressed by resident-tissue epithelial cells (for example, TECK in the gut (Kunkel *et al,* 2000), MEC in the colon and salivary gland (Pan *et al,* 2000) and CTACK in the skin (Homey *et al,* 2002)), making chemokine production from this cell type extremely relevant in the recruitment of tissue-specific immune cells.

The fact that chemokines can be expressed in specific areas of the body means that there is a potential for blocking these chemokines in therapeutic treatments (Homey *et al,* 2002). This would mean that the specific tissue affected by inflammation can be treated and the immune response diminished in this area, while, the immune system in other areas of the body would remain unaffected.

Furthermore, it has recently shown that the chemokine and anti-microbial protein mucosae-associated epithelial chemokine (MEC) is produced in saliva (Hieshima *et al,* 2003), in common with other mucosal secretions such as breast milk (Hieshima *et*

al, 2003) and is also present in the colon (Pan *et al,* 2000). In addition MEC has found to have anti-microbial functions to oral bacterium tested (Hieshima *et al,* 2003).

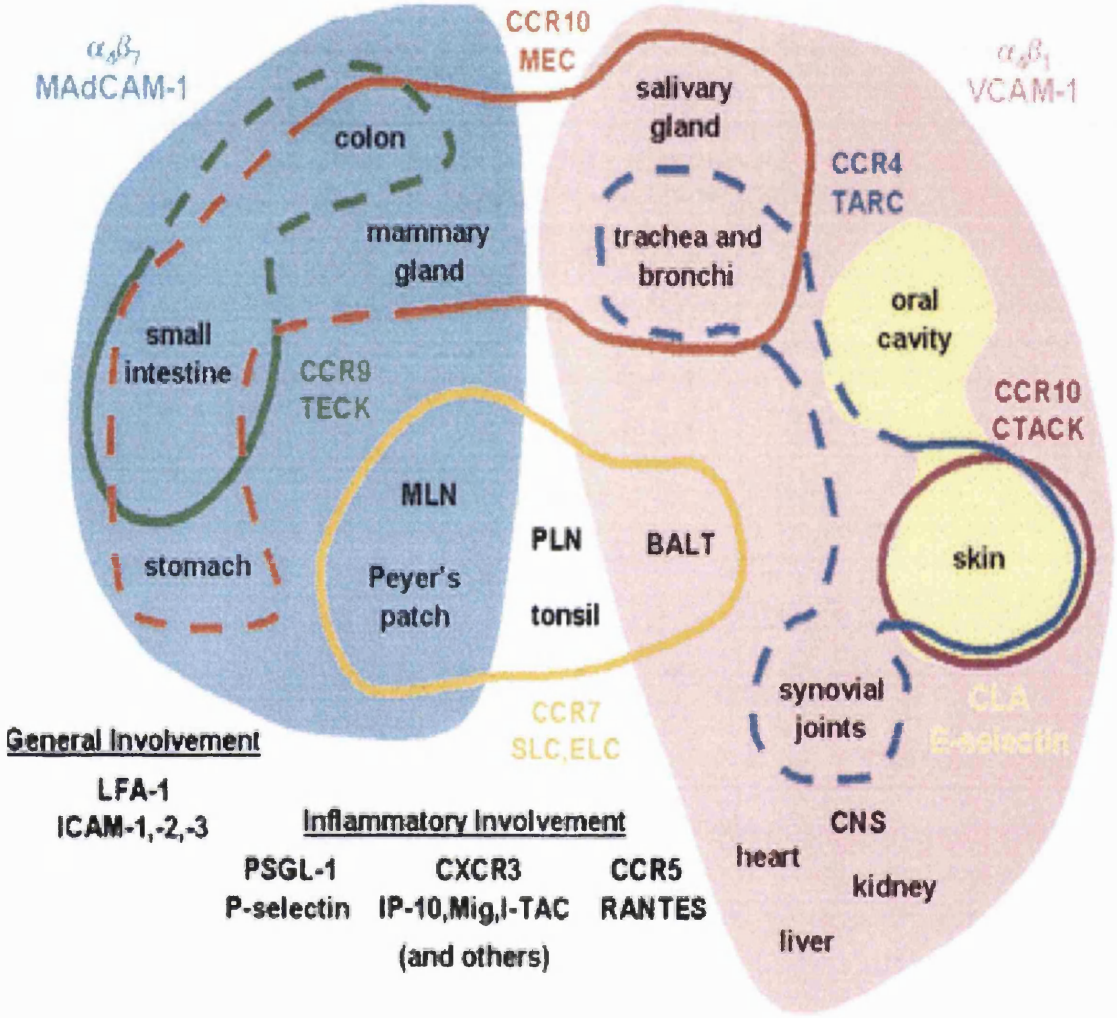


Figure 1.2: The known tissue specific chemokines and adhesion molecules in lymphocyte homing (Kunkel & Butcher, 2002). The large pink and blue solid areas represent the two main areas of tissue-specific adhesion molecule expression; either VCAM-1 or MAdCAM in the bronchial/ skin system-associated or the gut-associated system respectively, whilst the solid yellow area represents tissues where it is known that CLA+ cells are present. The solid lines represent areas where tissue-specific chemokines are strongly expressed and dashed lines where the same chemokines are expressed but to a lesser extent.

1.6 Relevant aspects of the oral mucosa

The skin and oral mucosa consists of the epidermis and dermis or epithelium and lamina propria (LP) respectively, separated by a basement membrane. The epidermis consists of stratified squamous epithelium composed of several cell layers. Individual epidermal cells in the skin and oral mucosa are known as keratinocytes. Upon the basement membrane is the basal layer of keratinocytes, which stratifies to give rise to differentiated cell layers of the spinous layer, granular layer and, only in skin, the stratum corneum. The epidermis is non-vascularised and receives its nutrients from blood vessels in the underlying dermis / lamina propria (Fuchs & Raghavan, 2002). As bacterial colonisation is particularly dense in the oral cavity and lower gastrointestinal tract (Loesche, 1994), the immune response is probably adapted to the specific requirements of these areas. Keratinocytes have recently found to play an important role in immune responses, and are capable of producing and responding to many different cytokines (Reviewed by Grone, 2002), including pro-inflammatory cytokines.

Although the oral mucosal membranes shares some homing characteristics with the skin system, such as the presence of cutaneous lymphocyte antigen (CLA)-positive homing cells (Tonetti *et al,* 1995; Walton *et al,* 1997; Brown *et al,* 1999) (see Fig 1.2) it is unknown whether the oral mucosa also shares other similarities with the skin, such as production of the chemokine CTACK by resident epithelial cells (see Chapter 6).

In contrast to the skin, the oral mucosal membranes share certain characteristics with the common mucosal immune system. This system is characterised by immunisation

at a particular mucosal site conferring protection at a diverse mucosal site. This protection is characterised by production of secretory IgA (sIgA) (Czerkinsky *et al,* 1987) which is regulated in a different manner to serum IgA responses (Gardby *et al,* 2003). For example, immunisation of ovalbumin (and adjuvant) by the oral route can lead to specific sIgA in vaginal, nasal, gut washings, as well as in saliva (Challacombe *et al,* 1997) and specific salivary sIgA antibodies have also been detected after intra-nasal immunisation with antigen (and adjuvant) (Russell *et al,* 1996) or intra-rectal immunisation with *Salmonella typhi* vaccine (Kantele *et al,* 1998). sIgA has been found to be particularly effective at virus inhibition and neutralisation (Renegar *et al,* 1998) in a process known as immune exclusion (Reviewed by Brandtzaeg *et al,* 1999) which is thought to prevent adhesion / invasion of potential pathogens to epithelial cells. Thus epithelial cells in mucosal environments have different surface protective mechanisms to those found in the skin, and these mechanisms are linked to other mucosal sites. This 'mucosal' link may suggest that there are specific differences in the manner that oral epithelial cells react to immune triggers compared to skin epithelial cells and this may be related to cells at mucosal sites.

Epithelial cells derived from the oral mucosal membranes have been found previously to differ from skin-derived epithelial cells in immune function. Adherence of Group A streptococci isolated from the skin adhere to skin epithelial cells in greater numbers compared to oral cells from the buccal epithelial cells and the opposite is the case for streptococcal bacteria isolated from the throat (Alkan *et al,* 1977), suggesting that there are adaptions of both the local bacterial and epithelial responses in these two environments. In fact, oral and skin epithelial cells have been previously been shown to differ in response to cytokine treatments. After cytokine treatment, oral epithelial

cells display HLA-DR, Class I HLA (Li *et al,* 1996[1]) and I-CAM (Li *et al,* 1996[2]) to a greater degree and production of IL-6 and IL-8 (Li *et al,* 1996[3]) is greater in comparison to skin keratinocytes. It has also been previously shown that oral keratinocytes produce a chemokine (GRO-alpha) to a significantly higher level compared to skin epithelial cells (after a number of cytokine stimuli) compared to skin keratinocytes (Li *et al,* 2000). These findings suggests the oral mucosal epithelium differs in response to inflammatory stimuli compared to skin keratinocytes and thus it appears important to determine the chemokine production of oral epithelial cells even in cases where the response of skin epithelial cells has been previously studied.

MHC Class-II expression on keratinocytes

MHC-II expression is a common feature upon epidermal keratinocytes during contact hypersensitivity (Roberts *et al,* 1985) and inflammatory disorders, including psoriasis (Kaneko *et al,* 1990). Presence of HLA-DR on these cells can be induced by IFN-γ (Messadi *et al,* 1988). As yet, the significance of this expression is unknown; HLA-DR4 positive keratinocytes, presenting specific antigen to antigen-restricted T-cells, fail to induce IL-2 production, whereas other studies have reported antigen-specific T-cell activation after encountering with HLA-D positive keratinocytes (de Bueger *et al,* 1993; Mutis *et al,* 1993). Thus, it remains unclear whether keratinocytes are capable of delivering antigen-specific presentation.

Defensins

Defensins can be produced either by cells during phagocytosis or by epithelial cells constitutively or during inflammation. Many of the antimicrobial peptides found in humans are the defensins, 3-6kDa beta-sheet peptides that contain three disulfide

bonds and are encoded by related genes (Reviewed by Ganz & Lehrer, 1998). They are cationic molecules with spatially separated hydrophobic and charged regions. This arrangement allows them to insert into phospholipid membranes, therefore preferentially inserting into bacterial membranes which are rich in negatively charged phospholipids. These molecules can be further classified depending upon the specific pattern of their cysteine spacing and disulfide connections into the alpha and beta defensins (Reviewed by Ganz, 1999). Recently chemotactic properties have also been defined for these molecules (Reviewed in Yang *et al,* 2002).

1.7 Oral lichen planus (OLP)

Lichen planus is a mucocutaneous disorder, but as yet has an unknown cause. It is characterised by a lesional band-like lymphocytic infiltrate within the lamina propria (Kirby *et al,* 1995). This is a chronic condition which can persist for many years, and can eventually evolve into focal erosion of basal layer keratinocytes (Triantafyllou, 1996, Farthing *et al,* 1990). This condition can affect all areas of stratified epithelia, but commonly affects the oral mucosa or the skin. Individuals may exhibit concurrent expression of cutaneous lichen planus and OLP, and in a recent study concurrent expression was present in 16.8% of all OLP patients (Bhattacharya *et al,* 2000), however this percentage is variable (Kleinman *et al,* 1991). This suggests that there may be some link between the pathological processes at the two sites, at least in some cases. However it is striking that cutaneous LP seems to spontaneously resolve or burn out after a few years, while OLP is generally a lifelong disorder (reviewed by Scully *et al,* 1998). This may suggest that a local persistent factor, such as dental plaque, may continue to exert a local immunological stimulus upon the oral mucosa.

Oral lichen planus (OLP) is a common disorder. It affects 1-2% of all adult population, tends to occur in middle to late life, and is more common in females than males (de Moura Castro Jacques *et al,* 2003. It manifests as white patches of the oral mucosa, sometimes with areas of redness, ulceration and rarely blistering (Table 1.2). The disease typically arises bilaterally and the most commonly affected sites are the buccal mucosae, dorsum of tongue and gingivae (manifesting as desquamative gingivitis). Unlike its cutaneous counterpart, the oral lesions of lichen planus tend to be lifelong, which may reflect different immunological mechanisms in the oral cavity and skin (as previously discussed) or different stimuli occurring at the two different sites. In addition it remains controversial if OLP has a malignant potential (Mattsson *et al,* 2002).

Types of oral mucosal and gingival lichen planus*
Reticular
Papular
Plaque-like
Erosive
Ulcerative
Bullous

* Affected patients often have more than one type of intra-oral lesion.

Table 1.2: The clinical forms of oral mucosal and gingival lichen planus.

Oral lichen planus is believed strongly to be immunologically-mediated (see later), topical corticosteroids (Hegarty *et al,* 2002), topical immunosupressants (Kaliokatsou *et al,* 2002) being the main therapeutic approaches for the painful disease.

Disease variations

There are a number of idiopathic lichen planus variants which share a similar pathology. There are also forms of the oral disease which share identical clinical appearances to lichen planus, the lichenoid-like reactions. In these conditions, the trigger causing the disease has been recognised; however, the mechanisms of the ensuing pathological reaction have not been fully resolved. The fact that different triggers may culminate in a lichenoid condition suggests that the idiopathic type of OLP could also be caused by different triggers.

Amalgam-associated lichenoid reaction

A lichenoid-like lesion can arise in proximity of dental restorations (Kallus & Mjor, 1991). These lesions may resolve when the restorative material (usually amalgam) is removed from the site of disease (Scalf *et al,* 2001; Kallus & Mjor, 1991). Sensitisation to dental metals is more common in patients with such lichenoid lesion than in the general population but is not always a reliable predictor of possible amalgam-associated OLP (Scalf *et al,* 2001). The histotypical features of lichenoid reactions to amalgam share many common features with contact hypersensitivity reactions.

Drug-associated lichenoid reaction

A wide range of drugs (e.g. antimalarial, sulphanylureas, some non-steroidal anti-inflammatory drugs (NSAIDS), gold and penicillamines) can induce disease similar to cutaneous and/or oral lichen planus, known as lichenoid drug eruptions (Reviewed by McCartan & McCreary, 1997). However, it has sometimes been difficult to

demonstrate an exact correlation, as OLP does not always subside on cessation of the suspected drug therapy (Reviewed by McCartan & McCreary, 1997; Savage, 1997). The reaction in these patients that occurs to cause a lichenoid reaction is also currently unknown, although in contrast to perhaps "idiopathic" OLP some, but not all (Ingafou *et al,* 1997) patients with oral or cutaneous LP or lichenoid drug eruptions may have a number of circulating anti-epithelial antibodies (Van Joost, 1974; McQueen & Behan, 1982), in particular basal cell cytoplasmic autoantibodies (Lamey *et al,* 1995; McCartan & Lamey, 1998) seem to be distinct to lichenoid drug eruptions. Furthermore lichenoid drug eruptions may have lower levels of intralesional activated Langerhans cells than idiopathic OLP (McCartan & Lamey, 1997) also suggesting that lichenoid drug eruptions may have different pathogenic mechanisms to those of idiopathic OLP. This also may indicate that there may be systemic influences that give rise to localised LP-like reactions of the oral mucosa.

Immunological significance of lichen planus

An immunological basis to lichen planus is implicated by the histopathology of the disease which consists of a large inflammatory cell influx of predominantly lymphocytes and as a consequence the immunology of this disease condition has been widely researched in recent years.

Immunological infiltrate of lichen planus

The immunological features of OLP principally consist of aspects of cell mediated immunity. Abnormalities of humoral immunity have been described, but these may not be central to the immunopathogensis in idiopathic disease – for example anti-epithelial antibodies (such as basal cell cytoplasmic antibodies) are not a constant

feature (as detailed above), and the lymphocyte infiltrate has not been documented to contain large numbers of B lymphocytes. Interestingly, all classes of IgA and IgG are upregulated in saliva during oral lichen planus (Sistig *et al,* 2002) suggesting that the salivary gland is activated during this condition and that these components may be reacting against as yet unidentified pathogens.

The lesional inflammatory infiltrate of OLP mainly consists of lymphocytes, however, peripheral lymphocytes are within the normal range of healthy individuals (Chiappelli *et al,* 1997).

T cell immunology of OLP

T-lymphocytes are numerous within OLP and cutaneous lichen planus (Simon & Gruschwitz, 1997), being especially located at the dermal/epidermal interface, and often infiltrating into the oral epithelium (Kirby *et al,* 1995).

Almost all infiltrating lymphocytes in OLP lesional tissue are CD3-positive. Histopathologically there is greater than 500 CD3+ lymphocytes per high-power field in OLP lesions compared to only 15-24 cells in normal samples (Bramanti *et al,* 1995). In active cutaneous lesions, there are progressively more CD3-positive cells which progressively increased in amount from the suprabasal epidermis, to the dermo-epidermal junction, to the dermis (Akasu *et al,* 1993).

Most studies concur that CD4+ T-cells are more prevalent than CD8+ve in the CD3 infiltrate in both skin (Akasu *et al,* 1993; Shimuzu *et al,* 1997) and oral lichen planus (Walton *et al,* 1998; Simark-Mattson *et al,* 1994), although CD8+ve cells predominate

at the dermal-epidermal junction in cutaneous lichen planus (Shimuzu *et al,* 1997; Akasu *et al,* 1993) and can be found intraepithelially in oral lesions (Eversole *et al,* 1994).

A recent study has suggested that OLP is dominated by Th1 cytokines (Little *et al,* 2003), however, other studies have suggested that neither Th1 or Th2 cytokines dominate OLP lesions (Simark-Mattson *et al,* 1999). Furthermore, there are immunosuppressive cytokines present, such as TGF-β (Simark-Mattson *et al,* 1999), which may be produced by CD4+CD25+ cells.

T-cells have been observed by electron microscopy attached to keratinocytes and in one cutaneous lichen planus sample T-cells were shown to lyse autolygous keratinocytes, suggesting the presence of specific cytotoxic mechanisms (Gadenne *et al,* 1994). In addition, the presence of Colloid/ Civatte bodies in lichen planus is a characteristic sign of apoptosis resulting from granzyme. In one study, dermally infiltrating cells in cutaneous lichen planus were positive for granzyme granules, with most of these cells expressing the CD8 molecule (Shimizu *et al,* 1997). Futhermore, there is evidence of TNF-mediated receptors (ligation of TNF-mediated receptors by CD8+ cells can cause apoptosis) in cutaneous (Simon & Gruschitz, 1997) and oral (Dekker *et al,* 1997) lichen planus suggesting that an immune reaction maybe mediated through both mechanisms against keratinocytes.

In contrast in a study of 6 OLP patients, cells cultured and cloned were mostly CD8+ve and exhibited an increased suppressor activity (Sugerman *et al,* 1994). These suppressor-type cells may correlate to previous studies demonstrating a cell

population which are seen to be producing TGF-β in oral lichen planus (Simark-Mattson *et al,* 1999), and/or the CTLA-4-positive cells witnessed in lichen planus (Alaibac *et al,* 2000). However, the authors suggest that these cloned cells may not be wholly representative of the lesional cells due to the culturing and cloning technique utilised (Sugerman *et al,* 1994).

In lesional OLP, 24% of helper cells have been shown to be CD45RA+ and 67% which are CD45RO+. In contrast there are no CD45RA+ve cells located in normal oral mucosa (Walton *et al,* 1998). There was a higher number of circulating memory (CD4+CD45RO+ or CD4+CD45RA-) cells in OLP patients compared to normal controls (Walton *et al,* 1998), and in erosive OLP cases compared to non-erosive (Chiappelli *et al,* 1997). This suggests that OLP response is part of a secondary response and is likely to follow the pattern of a DTH reaction.

Within T cell lines derived from OLP (Zhou *et al,* 1996) and *in situ* oral lesional T cells (Simark-Mattsson *et al,* 1994) there appears to be a restricted TCR phenotype, suggesting an oligoclonal expansion of T cells. This would indicate that OLP is perhaps driven by a specific antigen-derived source, rather than a superantigen or mitogen.

Gamma-delta T-cells in lichen planus

Although the number of T cells located intraepithelially increases during OLP, there appears to be no specific recruitment in T cells bearing the $\gamma\delta$ receptor (Walton *et al,* 1996). The constant number of these cells does not necessarily indicate their lack of involvement in the disease process.

NK cells in lichen planus

Small numbers of CD56+ve NK cells are observed in both the dermis and epidermis of cutaneous LP (Shimizu *et al,* 1997). Only rare natural killer cells are present in the sub-mucosa of OLP (Eversole *et al,* 1994). Systemic NK cells of OLP patients show a similar level of activity as healthy controls, compared by specific K562 cell lysis assay (Ueta *et al,* 1993). Therefore, there has been no evidence that NK cells significantly influence the outcome of lichen planus.

Lymphocyte homing in OLP

There is a significantly increased vascularity in OLP lesions compared to normal mucosa (Eversole *et al,* 1994). Selectins are expressed upon the endothelium in OLP; P-selectin is highly expressed, however there is also a relative decrease in expression at the sites of dense infiltrate (Regezi *et al,* 1996). Furthermore, there is strong E-selectin expression by endothelial cells in both OLP and cutaneous lichen planus (Walton *et al,* 1999). Integrin molecules are also expressed by endothelial cells in OLP; VCAM-1 shows variable staining on endothelial cells (Walton *et al,* 1994) but is especially upregulated in areas of dense infiltrate (Regezi *et al,* 1996). Furthermore, in OLP, endothelial cells in infiltrate-associated vessels stain strongly positively for ICAM-1 compared to normal controls (Walton *et al,* 1994; Regezi *et al,* 1996). The expression of these selectin and integrin molecules would suggest that there is an active tethering and adhesion to endothelial cells in this disease which would probably lead to potential cell diapedesis.

The large upregulation of selectin and integrin adhesion molecules on endothelial cells in OLP is indicates that these cells are activated and involved in the accumulation of immune cells in OLP lesions. Subsequent adhesion may allow certain cells to migrate into the lesion. However, the cause of the subsequent diapedesis of immune cells in the lesional band witnessed in OLP is unclear. Supernatants derived from OLP-derived keratinocytes are capable of inducing peripheral blood cell migration (Yamamoto *et al,* 1994). Recent evidence shows that mast cells produce the chemokine RANTES in OLP (Zhou *et al,* 2001) suggesting that these cells are producing chemokines. Finally, a very recent study has demonstrated that RANTES is indeed produced by keratinocytes in OLP (Little *et al,* 2003).

Phagocytes in OLP

Neutrophils in lichen planus

Systemic PMNs of OLP patients do not show decreased phagocytosis, but O_2-generation in the same cells is significantly reduced compared to controls (Ueta *et al,* 1993). It is unclear how this reduction is involved in the pathogenesis of OLP.

Macrophages in lichen planus

The proportion of cells staining for macrophage markers appears to be much greater within the sub-epithelial tissue of OLP lesions compared with healthy tissue (Regezi *et al,* 1994). Furthermore, macrophages are most prominent within lesional areas of T-cell accumulation (Regezi *et al,* 1994) and these cells are also present in the lower areas of epithelium near sites of basal layer damage (Kirby *et al,* 1995). In fact, one study located granular cells in OLP lesions, which demonstrate weak lysosome activity, suggesting that these cells are macrophages that are disposing of damaged

keratinocyte material (Triantafyllou, 1996). Furthermore, in active cutaneous lesions, there are moderate numbers of lysosome-rich cells in the dermis (Akasu *et al,* 1993). Therefore, macrophages numbers appear to increase during this disease and these cells may play an important role in disease progression and in the phagocytosis of material, including destroyed keratinocytes.

Langerhans cells in lichen planus

If OLP is being initiated by an external – and presumably oral – antigen, it would be expected that the APCs would be increased both in number and activity. There appears to be a large variation in the findings of LC density in OLP. In some studies, there are no overall differences in numbers of CD1a+ve cells (Farthing *et al,* 1990; Farthing *et al,* 1992; Pitigala-Arachchi *et al,* 1989) or HLA-DR+ve cells (Farthing *et al,* 1992, Pitigala-Arachichi *et al,* 1989) in lichen planus compared to normal controls. In fact, according to Chou *et al,* 1993, there is actually a significant reduction in HLA-DR staining in comparison to normal controls. However, other studies in oral lichen planus found Langerhans cells in the epithelium of every biopsy staining more intensely and in greater numbers than in normal tissue or leukoplakia samples (Rich *et al,* 1989; Regezi *et al,* 1994). Of note, as mentioned previously, there may be an increased number of HLA-DR+ve cells in idiopathic OLP as compared with oral lichenoid drug eruptions (McCartan & Lamey, 1996).

Whereas in other cases, despite no changes in overall numbers (Farthing *et al,* 1990), the cells can appear more dendritic, suggesting that they are activated. In some cases they form an extensive network (Rich *et al,* 1989; Farthing *et al,* 1990). However, in other cases, Langerhans cells tend to localise to the bottom of the epithelium

compared to normal controls (Chou *et al,* 1993), even extending into the basal epithelium (Rich *et al,* 1989). Despite an increase in CD4+ CD1a+ dendritic cell numbers in areas of HLA-DR+ve keratinocytes (Farthing *et al,* 1992), the number of Langerhans cells present has no significant influence upon the thickness of the epithelium (Walsh *et al,* 1989).

Although ICAM-1, HLA-DR and CD44 isoform expression is low in Langerhans cells, there is a suggestion of some degree of activation through the expression of HLA-DP and -DQ. It has been shown that only a few mature DCs are required to provoke a potent T-cell response (Reviewed by Rescigno *et al,* 1999).

There is a significant increase in HLA-DP and HLA-DQ expression (Farthing *et al,* 1990; Farthing *et al,* 1992; Chou *et al,* 1993) within lesions. In addition, in lichen planus it has been shown that there is a significantly greater number of HLA-DQ-positive to T-6-positive cells, and significantly less numbers of HLA-DR +cells compared to T-6+ve cells present (Chou *et al,* 1993).

Lesional adhesion molecules in OLP

LFA-1 expression in lichen planus

As LFA-1 is expressed constitutively by T-cells, a correspondingly large proportion of the lymphocytic infiltrate in lichen planus express this molecule (Konter *et al,* 1990; Regezi *et al,* 1996; Eversole *et al,* 1994). Moreover, LFA-1 staining is also common in intra-epithelial cells (Verdict *et al,* 1992; Eversole *et al,* 1994).

ICAM-1 expression in lichen planus

There appears to be little expression of this molecule by LCs in OLP - less than half of the examined OLP lesions have CD1a-positive LCs that simultaneously express ICAM-1. When present however these cells are localised in areas of cellular damage. ICAM-1 expression is also present on macrophages within the lesion (Walton *et al,* 1994) and infiltrating lymphocytes (Eversole *et al,* 1994). Expression occurs focally on basal keratinocytes at the site of the cellular infiltration in lichen planus (Konter *et al,* 1990; Eversole *et al,* 1994; Bennion *et al,* 1995; Walton *et al,* 1994, Regezi *et al,* 1994), and expression can extend into the suprabasal layers. In areas of keratinocyte ICAM-1 expression there is increased infiltrate of dendritic and mononuclear cells counts in both OLP (Walton *et al,* 1998; Eversole *et al,* 1994) and in cutaneous lichen planus (Wantzin *et al,* 1998). There appears to be no recruitment of immature dendritic cells to the lesional area. This could be due to the fact that in chronic lesions LCs have previously undergone migration from the area to the lymph nodes. However, some studies suggest an adjustment in location of the LCs in OLP in most cases (Rich *et al,* 1989).

LFA-3 expression in lichen planus

LFA-3 is expressed in association with macrophage-like cells, although in most cases only cytoplasmically, and by CD14+ve dendritic cells in the infiltrate (Kirby *et al,* 1995). There is surface staining of LFA-3 in T-cells within the infiltrate (Kirby *et al,* 1995). Furthermore, there is surface-associated LFA-3 expression on keratinocytes, most evident on cells directly above the basal layer and there is expression associated with the extra-cellular matrix near the basal layer (Kirby *et al,* 1995). Therefore, this

variable expression of LFA-3 indicates that this molecule may be linked with the variable severity of this condition.

Keratinocyte expression of molecules associated with antigen presentation

Lesional keratinocytes in most OLP cases express HLA-DR (Takeuchi *et al,* 1988; Farthing *et al,* 1989; Pitgala-Arachi *et al,* 1989; Farthing *et al,* 1990; Farthing *et al,* 1992; Walsh *et al,* 1990), in some areas extending to all suprabasal keratinocytes, although these studies disagree whether the degree of staining is related to the intensity of the cellular infiltrate. As mentioned previously, the relevance of HLA-DR on keratinocytes in currently unknown, but maybe involved in the presentation of antigen to T cells.

Keratinocytes within the lesion express a large number of molecules normally associated with antigen presentation, but whether the 'signals' present activate or cause anergy in T-cells is undetermined. It is likely that IFN-γ plays a crucial role in keratinocyte activation in this manner as it is capable of inducing both ICAM-1 and HLA-DR expression on these cells. Furthermore, in mice that are transgenetically engineered to constitutively express CD86 (B7-1) upon keratinocytes, cutaneous application of a contact sensitisor produces a significantly stronger primary inflammatory response and delayed resolution than control mice (Williams *et al,* 1994). In addition, when there is an exclusive re-challenge at another different site a similar inflammatory response occurs in the previously sensitised sites (Williams *et al,* 1994). A similarly exaggerated immune response occurs when B7.2 transgenic mice are cutaneously treated with *C.albicans* haptens. In fact, a single hapten

application can cause ear-swelling with duration of longer than 6 weeks compared to 72 hours in control mice (Gaspari *et al*, 1998). Therefore, it can be shown that the induction of B7 molecule expression on keratinocytes can cause an increased immunogenecity to a number of different antigenic stimulants, causing a large influx of stimulated T-cells. This increase in the delayed-type hypersensitivity (DTH) response to re-encountered antigens witnessed in B7 transgenic mice displays many similarities to the pathogenic mechanisms visible in OLP, including the large T-cell influx and the chronic nature of this disease. If it were found that CD86 were expressed on keratinocytes in OLP, these cells could be involved in the increased immunogenicity to antigens, including common oral commensals, such as *Candida.* Furthermore, the expression of CD86 on oral epithelium may also be relevant to cell migration, as CD28 ligation of CD4+ cells can alter chemokine receptor expression (Secchiero *et al,* 2000).

The expression of CD86 in OLP is unknown. Simon *et al*, 1994 investigated the expression of CD28 and B7 in cutaneous lichen planus lesions discovering that B7-1 molecule was focally expressed on keratinocytes within the lesion. However, it been subsequently been shown that that the antibody used to detect B7-1 cross-reacted with the MHC-Class II-associated invariant chain (CD74) (Freeman *et al,* 1998) and it was likely that it was this latter molecule that was detected in the previous study. Furthermore, the CD86 mRNA upregulation in expression witnessed in this study may not necessarily extend to an increase in protein expression.

Furthermore, CTLA-4 and CD28 binding is known to affect Th1/ Th2 differentiation, but this would appear to have a larger effect in naïve cells, than for memory cell

interactions (Fontenot *et al,* 2003 & discussed in Salomon & Bluestone, 2001). As these latter interactions appear to predominate in OLP, then the role of CTLA-4 and CD28 binding may be minimal in this condition.

It has been proposed that keratinocytes may act as non-professional antigen presenting cells in OLP (Thornhill, 2001). However, despite keratinocytes possessing the genes necessary for antigen presentation (Albanesi *et al,* 1998), presumably if oral keratinocytes were capable of antigen presentation, it would have to be immunogenic antigen presentation in OLP to cause the reaction witnessed opposed to antigen presentation that induced tolerance. However, it has proved difficult to assess the factors that provide even professional antigen presenting cells with either tolerogenic or activating signals to T cells in the periphery (Reviewed in Walker & Abbas, 2002).

Colonic epithelium also express CD86 (Nagazawa *et al,* 1999) and has been shown to be capable of stimulating T cell responses (Hershberg *et al,* 1997). In contrast, duodenal epithelium expresses the molecular components required for antigen presentation but they lack co-stimulatory molecule expression, and do not induce the activation of T cells (Byrne *et al,* 2002). This suggests that despite the capability of antigen presentation in these cells they cannot achieve activation. This may result in anergy of local T cells, leading to tolerance to these antigens (Reviewed by Hershberg & Meyer, 2000). This may reflect the different requirement for antigen sampling in the two environments as the colonic epithelium has a large resident microflora, similar to the oral mucosa, whereas the duodenal epithelium is a relatively sterile environment. It may be the case that the oral epithelium is capable of antigen-specific presentation, but only in times of inflammation when there is an increase in the

expression of co-stimulatory molecules. Interestingly, bronchial epithelium is capable of inducing T cell proliferation in an IFN-γ and CD40 dependant mechanism (Tanaka *et al,* 2001).

If this antigen sampling and presentation process occurs in the oral mucosa, it is tempting to suggest that those antigens most often sampled would be present at high doses and thus more likely to elicit an effective T cell response (reviewed by Lanzavecchia & Sallusto, 2001). Such antigens found in the oral cavity at constantly localised high levels in the oral mucosa, such as betel nut antigens in persistent users or amalgam antigens in patients with these fillings. If these antigens were combined with inflammatory signals they may produce active antigen presentation which may produce an immunogenic rather than a tolerogenic response to these antigens.

The proposed activation / tolerance theories for dendritic cells proposes that stimulatory function is either enhanced by toll-like receptors (TLR) on dendritic cells that recognise microbial products and upregulate co-stimulatory molecules (Reviewed by Medzhitov & Janeway, 2002) or that damage in other cells, and the production and liberation of substances, such as heat shock proteins, act as 'danger signals' (Basu *et al,* 2000) to activate dendritic cells. Such theories may also be true for epithelial cells. It is interesting to note that TLR have been discovered upon keratinocytes (Kong *et al,* 2002) (see Chapter 4) and are increased on these cells during psoriasis (Curry *et al,* 2003). Moreover, heat-shock protein expression has been implicated in the pathogenesis of OLP (Chaiyarit *et al,* 1999).

The induction of co-stimulatory molecules upon epithelial cells does not necessarily result in T cell activation. These molecules may actually bind to CTLA-4, which is thought to be an important molecule in providing tolerance (Perez *et al,* 1997). Interestingly, CTLA-4 knockout mice demonstrate a systemic autoimmune condition and anti-CTLA-4 can cause an increase in the pathology of autoimmune conditions (Karandikar *et al,* 1996). It may well be that dysregulation of CTLA-4 in OLP patients is responsible for a breakdown in tolerance for oral keratinocyte antigens. In cutaneous lichen planus tissue there is a clear expression of CTLA-4 cells in most samples, whereas there is no expression in a variety of other skin inflammatory disorders (Alaibac *et al,* 2000). The implications of this expression are unclear. The findings that many infiltrating T cells in lichen planus express CD28 (Simon *et al,* 1994), suggests that these cells could interact with CD86-expressing keratinocytes leading to specific clonal activation of these cells. In fact, in salivary gland epithelial cells, CD86 was expressed and found to interact with CD28, with reduced binding to CTLA-4 (Kapsogeorgou *et al,* 2001). This may also prove to be the case in OLP, suggesting the CD28+ T cells witnessed in lichen planus patients are more relevant to the disease process than the CTLA-4 positive cells present. This pattern of CD28 infiltration of OLP was again similar in contact hypersensitivity reactions (Simon *et al,* 1994). This suggests that there may be similar mechanisms of T cell infiltration in the two pathologies.

A subset of CD4+ T cells that express CD25 represent regulatory T cells that are proposed to be involved in the suppression of autoimmunity (Reviewed by Shivach *et al,* 2002). Interestingly, CD25+ cells are upregulated in OLP (Hasseus *et al,* 2001). These cells may also cause persistence of infection, perhaps in order to permit long-

term immunity (Belkaid *et al,* 2001). Therefore, it could be proposed that these cells are involved in causing the chronicity of diseases, with a low-level (perhaps non-detectable) infection occurring. The chronic nature and presence of CD25+ cells in OLP may therefore be related.

CD25+ regulatory T cells may produce some of immuno-suppressive effects by the production of transforming growth factor-1alpha (TGF-1α) (Reviewed by Levings *et al,* 2002). mRNA for the immunosuppressive cytokine TGF-1α was expressed in all OLP lesions studied (Simark-Mattson *et al,* 1999), adding to the evidence that there is some form of suppression occurring.

Interestingly, CD25+ cells constitutively express CTLA-4 (Read *et al,* 2000), although the role that this plays in the suppressor qualities of these cells is not known. In fact, the CTLA-4 cells located in cutaneous lichen planus may correspond to the CD25+ cells found in OLP. Nevertheless, the presence of CD25+ cells in OLP and the upregulation of CTLA-4 cells in lichen planus suggests that some form of immune regulation is occurring, but perhaps to an insufficient extent or, in fact, the suppression is in some way contributing to the pathology of OLP.

Presumably, if oral keratinocytes are capable of antigen presentation it would be 'non-professional' antigen presentation i.e. these cells would not be able migrate to the lymph nodes to stimulate naïve T cells. This would suggest that predominantly only memory cells could be activated in this manner, in fact, memory T cells are activated by a range of different APC and have less requirement of co-stimulatory function than naïve cells (Croft *et al,* 1994). It is interesting to note that memory T cells constitute

the majority of infiltrating cells in OLP lesions (Walton *et al,* 1998) and are more prevalent in the blood of OLP patients (Sugerman *et al,* 1992), suggesting that a secondary immune reaction is occurring in OLP. Furthermore, a possible increase in chemokine production that may occur by T cell ligation of keratinocytes in OLP (Alternberg *et al,* 1999) would presumably promote the migration of further memory cells into this area. This suggests that Langerhans cells have an important role in initiating naïve T cell responses, whereas in the secondary response keratinocytes may play a role in re-activating memory T cell responses. However, resident tissue APCs are implicated in presenting self-antigen to Th1 cells during auto-immune conditions (Katz-Levy *et al,* 1999), and thus keratinocytes may also play a role in initiating the presentation process to Th1 cells.

The reaction against oral keratinocytes in OLP suggests that may be a break-down in tolerance to self-antigens of oral epithelial cells. There may be many mechanisms influencing potential breakdown of self-tolerance in the oral mucosa. Although still debated as a theory, breakdown of tolerant T cells in the periphery may be caused by a process known as molecular mimicry (Reviewed in Ohashi, 2002). This involves the presence of pathogens expressing antigens that are similar to host antigens, thus activating, through presentation by mature dendritic cells (and possibly activated epithelial cells), auto-reactive T cells to initiate organ-specific autoimmunity. Although it is not known if this process occurs in OLP, the association of this condition with viruses such as hepatitis C (Reviewed by Carrozzo & Gandolfo, 2003) suggest there is some link between the two conditions. If OLP were caused by such a mechanism, it may explain different severity of OLP lesions between patients and the chronic nature it presents. Furthermore, there are many other proposed links between

infectious bacteria and viruses and the onset of autoimmune diseases (reviewed by Wucherpfennig, 2001), for example, murine models of myocarditis (induced by peptides from *Chlamydia*) (Bachmaier *et al,* 1999) and herpes simplex keratitis, (a T cell-mediated inflammatory disease induced by application of herpes simplex virus) (Zhao *et al,* 1998).

Another proposed theory of the breakdown of tolerance is epitope spreading. This occurs when the normal immune response to selective epitopes in a disease spread during the course of an immune reaction to include other epitopes, which is thought to a have a protective role in normal immune responses. However, regardless of the initiating antigenic factor, this process can eventually lead, after tissue damage, to include self-antigens, therefore, causing an auto-immune condition (Reviewed by Vanderlugt & Miller, 2002). Due to the number of agents associated with the onset of OLP and the chronicity of the disease, which may be caused by different shift of epitopes detected in the disease, epitope spreading appears a possible candidate for the pathogenesis of this disease. Furthermore, CD80/86 blockade (Vanderlugt *et al,* 2000) or CD40-CD154 blockade (Howard *et al,* 1999) can inhibit epitope spreading and ease ongoing autoimmunity in animal models. The fact that CTLA-4+ve cells are a positive factor in easing epitope spreading (Karandikar *et al,* 2000), suggests that the CD25+ cells witnessed in OLP may cause a down-regulation in the immune reaction, however, perhaps further epitope changes promote a wave of inflammatory T cells. As epitope spreading is implicated in chronic diseases like multiple sclerosis (Reviewed in Croxford *et al,* 2002), where there is relapses and remission this suggests this pathogenic pattern could occur in OLP.

However, in OLP despite the large number of CD4+ cells present in the lesions, it appears that it is CD8+ cells that are in the proximity to the epithelial area. This suggests that it is actually MHC-class I restricted presentation that is occurring within lesions. However, it may be that prior class II presentation by keratinocytes assist in initiating a CTL reaction. Interestingly, CD40 ligation of antigen presenting cells plays an important role in the generation of CTL cells (Ito *et al,* 2000; Lefrancois *et al,* 2000). Therefore, maybe if keratinocytes are presenting antigen through the MHC-Class II pathway, the ligation of CD40, which is classically associated with antibody-mediated reactions, not only amplifies the inflammation of the area, but may be also involved in the generation of specific CTL.

However, there are a group of CD8+ effector memory cells that are preferentially located in non-lymphoid tissue, that rapidly expand after activation (Masopust *et al,* 2001), thus may not require further T helper cells. Furthermore, CD8+ memory cells also have a limited requirement for B7 co-stimulatory signals. However, as autoreactive CD4+ and CD8+ T cells often have a weaker affinity to antigen, they may actually require B7-stimulation (Reviewed in Salomon & Bluestone, 2001) thus the expression on oral epithelium may still be relevant.

It appears that a process called cross-presentation is important in maintaining tolerance of CD8+ cytotoxic T cells. This process involves antigen presenting cells presenting exogenous antigens (that are traditionally considered to be processed and presented on MHC-II molecules) that are actually processed through the MHC-Class I pathway and presented to CD8+ T cells by MHC-I (Reviewed by Heath & Carbone, 2001). This mechanism is thought to provide tolerance to self-antigen for CD8+ T

cells, which are not activated after this process but undergo deletion. The presentation process is thought to be carried out mainly by a specialised group of CD8+ dendritic cells (Scultz *et al,* 2002).

The process of cross-tolerance is thought to be important in gaining self-tolerance to apoptotic cells. In fact, there is a subset of intestinal dendritic cells that have been shown to transport apoptotic epithelial cells to the lymph nodes (Huang *et al,* 2000), in a process thought to provide exposure and induction of tolerance to 'self' antigen. Furthermore, Langerhans cells have been shown to be capable of phagocytosis of vaginal apoptotic epithelial cells (Parr *et al,* 1991). These findings are of particular interest as it is thought that there are apoptotic keratinocytes in OLP; there are increased serum levels of apoptosis related molecules (Fas and Bcl-2) in OLP patients (Sklavounou-Andrikipoulou *et al,* 2004) and evidence of lesional apoptotic keratincoytes (Tobon-Arroyave *et al,* 2004; Hirota *et al,* 2002; Neppelberg *et al,* 2001). In addition there are increased levels of granzyme-B and perforin positive T cells in OLP compared to the cutaneous variant of the disease (Santoro *et al,* 2004) and the apoptotic index of keratinocytes is higher in the former disorder (Santoro *et al,* 2004). It may be that the process of transport of antigen from these cells to the lymph nodes may in fact induce an immunogenic response to these antigens instead of the tolerising effect. In fact, in an experimental model of autoimmunity, cross-presented CD8+ T cells that are normally tolerant to self-antigen can cause increased aggressiveness in the presence of virus or CD40 ligation (Vezys *et al,* 2003), as well as causing an increased production of both IFN-γ and TNF-α.

It could be speculated that this process would produce auto-reactive T cells to keratinocytes, however, there has been no evidence to suggest these specialised dendritic cells occur in the oral cavity. Furthermore, in experimental models the degree of cross-presentation can be age-related process (Rafii-Tabar & Czitrom, 1986) depending upon the environment which it occurs, therefore, perhaps increasing the potential for a break-down in tolerance to occur in this process in older patients.

Also, molecular mimicry and epitope spreading is also thought to occur in Class I restricted antigens (Misko *et al,* 1999) as well as Class II antigens, so it may an effect of direct MHC-Class I presentation that may cause the cytotoxic effect in OLP. There is increasing evidence from the presence of molecules on epithelial cells that they may in some circumstances be capable of antigen presentation. Whether this antigen presentation produces a tolerogenic or immunogenic response remains to be seen, although the presence of MHC-II and the potential of CD86 expression on oral epithelial cells, and CD28 in the T cell infiltrate of OLP suggests that 'activating' antigen presentation could occur. The antigen presentation may take the form of 'sampling' antigens on the oral mucosa, such as bacterial antigens. The implications for antigen presentation may be very important in conditions such as OLP as there appears to be a breakdown in the tolerance for 'self' keratinocyte antigens. This breakdown could occur by a number of mechanisms, including molecular mimicry or epitope spreading. However, CD8+ cells appear to be acting cytotoxically in OLP, therefore, perhaps the Class I pathway is more important in the disease process. Perhaps the presence of inflammatory signals present during cross-presentation of apoptotic keratinocytes causes normal tolerogenic CD8+ cells to become auto-reactive for these cells. If antigen presentation is occurring in this disease it is important to

assess which chemokines may be produced by the keratinocytes, which may bring T cells in contact with the oral epithelial cells.

Antimicrobial peptides in oral lichen planus

Interestingly, there appears to be an upregulation of antimicrobial peptide production in OLP patients. Defensin-1 (HNP-1) was present in significantly higher concentrations in the saliva of OLP patients than normal controls (Mizukawa *et al,* 1999), human beta defensin-2 was produced by epithelial cells in OLP (Abiko *et al,* 2002) and calprotectin, an anti-microbial/ fungal component was identified in lichen planus with intense and pervasive staining (Eversole *et al,* 1993). The presence of these products is perhaps surprising as there are no findings of bacterial pathogenesis in OLP, but maybe reflects the activation state of the epithelium in OLP or an underlying bacterial association in this condition.

Conclusions

Overall, there appears to be increasing evidence accumulating that OLP is caused by a cytotoxic reaction towards keratinocytes. This reaction appears to be mediated by memory T cells in a delayed-type reaction, however, the method of cytotoxic killing appears to differ in different cases, with evidence of TNF-R mediated killing mechanisms, as well as granzyme and perforin mediated killing (Santoro *et al,* 2004). The source of any supposed autoimmune antigen is not known, although the presence of similar conditions in response to localised antigens, such as amalgams or systemic sources, such as certain drugs suggest that the antigen could derive from a number of sources and not necessarily the same for each case of idiopathic lichen planus. Thornhill, (2001) suggested that diverse sources such as food or local microflora may

be possible triggers for this disease. However, what causes the migration of the pathogenic auto-immune cells to the lesional areas is relatively unknown in OLP.

Aims

Oral lichen planus is a disease characterised by a chronic inflammatory infiltrate, localised in a band-like infiltrate beneath the oral epithelium. Furthermore, oral keratinocytes are capable of producing chemokines and it is known that OLP keratinocytes produce chemotactic factors. This study aims to investigate the production and expression of 5 different chemokines in this oral inflammatory disorder. The chemokines selected for this study have been shown to induce the migration of memory (CD45RO+) T cells, previously shown to be the most numerous T cell phenotype within the infiltrate of oral lichen planus. In addition, due to the location of this large infiltrate directly beneath the epithelium in oral lichen planus, the production of these chemokines in oral epithelium was examined, in order to determine whether oral keratinocyte production of the selected chemokines is possible and thus could be partially responsible for the T cell recruitment in OLP. The production of all selected chemokines was tested both after non-stimulatory and pro-inflammatory conditions, thus assessing whether the pro-inflammatory conditions witnessed in OLP can effect the production of T-cell attractant chemokines from the oral epithelium. Other factors cited to influence the production of these chemokines were also investigated; CD40 expression on epithelial cells, and bacterial factors.

Furthermore, blocking the functions of chemokines has potential therapeutic value (reviewed by Proudfoot *et al,* 2003), particularly the tissue-specific chemokines (Mackay, 2003). Therefore, another aim of this study was to evaluate the potential of

blocking tissue-specific chemokine function from oral keratinocytes in order to prevent T cell migration. Thus the possible value in this treatment of oral inflammation, and especially OLP can be evaluated.

Overall, the aims for these studies were to 1) characterise the expression and/or production of these chemokines in oral epithelial cells and oral (lichen planus) tissue, 2) investigate the effect and/or presence of possible influences that may affect the production of these chemokines in oral lichen planus, 3) determine the potential effects of blocking the production of chemokines from oral epithelial cells.

The specific chemokines and the aspects to be studied are:

- The mRNA expression and protein production of the inflammatory CXC ELR-chemokines, tissue-specific chemokine CTACK and LC-attractant chemokines, MIP-3α by oral epithelial cells
- mRNA expression levels of the above chemokines in normal oral mucosa and oral lichen planus
- Profiles of peripheral blood cells chemo-attracted to studied chemokines and supernatants derived from oral epithelial cell cultures
- The antimicrobial effects of the studied chemokines on a typical oral bacterium

Chapter 2: Materials & Methods

2.1 Patients

Oral lichen planus (OLP) lesional tissue was collected from patients (8 female, 4 male, median age 59, range 37-74 years) with clinical and histopathological features of oral lichen planus (WHO, 1978) were attending the Department of Oral Medicine Unit of the Eastman Dental Institute, University College London, UK. A description of each source patient is given in Table 2.1. All patients had undergone an incisional biopsy under local anaesthesia for the diagnosis of lichen planus. None of the patients had received treatment for their oral lichen planus or were taking medication likely to give rise to lichen planus. There were no amalgams situated near to the site of biopsy.

Normal oral buccal mucosa was obtained from patients attending the Department of Oral Surgery Unit of the Eastman Dental Institute, University College London, UK. These patients were of a similar age and gender distribution, but not identical as those with oral lichen planus (Table 1.1), and were not taking medication likely to give rise to lichen planus (Lamey *et al,* 1995).

Ethical approval was obtained from the local relevant committee for the use of these tissues.

Table 2.1: Patients with clinical and histopathological features of oral lichen planus and normal control patients.

	Age (years)	**Gender**	**Duration of oral lichen planus**	**Site of oral lichen planus**
1	60	F	2 years	Tongue
2	61	F	6 months	Buccal mucosa
3	37	M	6 months	Tongue
4	53	F	6 months	Buccal mucosa
5	58	F	5 years	Buccal mucosa
6	49	M	5 years	Buccal mucosa
7	56	M	3 years	Buccal mucosa
8	63	F	3 years	Tongue
9	72	F	Unknown	Buccal mucosa
10	55	F	7 years	Buccal mucosa
11	74	F	1 year	Buccal mucosa
12	66	M	Unknown	Buccal mucosa
1	29	M	Not applicable	Not applicable
2	43	F	Not applicable	Not applicable
3	49	F	Not applicable	Not applicable
4	19	F	Not applicable	Not applicable
5	39	M	Not applicable	Not applicable
6	19	F	Not applicable	Not applicable
7	30	F	Not applicable	Not applicable
8	52	F	Not applicable	Not applicable

2.2 Immunohistochemical methods

2.2.1 Tissue section preparation

a) Paraffin section preparation

Sections of archivial paraffin-embedded formalin-fixed (PEFF) oral lichen planus and lymph node tissue were employed in these studies.

Paraffin blocks of biopsy tissue were cut using a Shandon microtome (Anglia Scientific, Cambridge, UK) to obtain sections of a 3μm thickness. The sections were then mounted onto 3-aminopropyltriethoxysilane (APS) (Sigma, Poole, UK)-coated slides (approx. 2 sections per slide), which were then incubated at 37°C overnight in a heated cabinet (Merck, Lutterworth, UK). Slides were then covered and stored at room temperature until required.

The paraffin sections were de-waxed in xylene (BDH, Poole, England) for 10mins, followed by rinsing with descending concentrations of ethanol (100 to 70%) to aid tissue hydration. The tissue sections were then used for antigen retrieval and detection (2.2.2 and 2.2.3).

b) Frozen section preparation

Frozen sections of OLP tissue were prepared by orienting fresh OLP biopsy material (2.1) on a cork disc and mounting in OCT compound (Lamb Ltd, Eastbourne, UK). The biopsies were then snap-frozen by immersion in liquid nitrogen. The frozen samples were then mounted on a chuck using OCT and placed in the cryostat (Bright, Huntingdon, UK) for sectioning. Samples were trimmed until there was an equal face

and sections of 9μm thickness were then obtained by cutting using a cryostat blade. The process was repeated until satisfactory sections were obtained and then the sections were mounted upon Superfrost glass slides (BDH, Lutterworth, UK). A fresh cryostat blade was used for each tissue sample.

The slides were stored at -70°C in airtight containers, until required, when they were air-dried at room temperature for 1 hour after removal. The slides were then fixed in ice-cold acetone (BDH, Lutterworth, UK) for 10 minutes and washed 3 times in phosphate buffered saline (PBS). The slides were thus prepared for antibody detection of antigen (2.2.3).

2.2.2 Antigen retrieval methods

Antigen retrieval was achieved by placing slides with sections in a volume of target retrieval solution (Dako, Cambridge, UK) sufficient to cover the slides, and then heated in a microwave for 20mins at full power. Following this time the solution was allowed to cool and the slides removed.

2.2.3 Chromogenic antigen detection

In this procedure, all incubations were carried out at room temperature using a humidified chamber.

To block endogenous peroxidase in the tissue, the slides were firstly incubated for 10mins in 1% peroxidase solution (1ml of H_2O_2 (BDH, Lutterworth, UK) in 100ml methanol (BDH, Lutterworth, UK)) and washed 3 times in PBS. Before adding the primary antibody, the slides were incubated with 5% bovine serum albumin (BSA),

Fraction V (Sigma, Poole, Dorset) in PBS for at least 15mins to block non-specific protein binding, following which the excess solution was removed by gently tapping the slides onto absorbent tissue. The area around the tissue was then carefully blotted. The primary antibody, diluted to an appropriate concentration (Section 3.2.8), was then placed on the tissue section and incubated for 1 hour. Incubation of a serially-cut tissue section with an isotype-matched antibody at the same concentration was also carried out as a negative control for the primary antibody. The slides were washed 3 times in PBS following which 100µl of goat anti-mouse-HRP-conjugated antibody (Vector, Peterborough, UK) (diluted 1:600 in PBS) was added, and the slides incubated for a further 1 hour. The slides were then washed 3 times in PBS.

Labelled antigens were visualised by using 3', 3'-diaminobenzide (DAB), as prepared from the SigmaFast DAB Peroxidase substrate set (Sigma, Poole, UK), with an incubation for 10 minutes. The reaction was terminated by washing 3 times in PBS. The slides were then counterstained in Harris' haemotoxylin (BDH, Poole, UK), followed by running in tap-water for 10mins. Sections were then dehydrated through ascending concentrations of ethanol (70% to100%), mounted in DePeX (BDH, Poole, UK) and coverslipped.

2.3 Cell culture techniques

All mammalian cell culture was performed in a Class II Flow Cabinet (MAT Ltd, Manchester, UK), with aseptic techniques.

2.3.1 Primary oral keratinocyte cell culture

Normal oral mucosal was immediately placed in transport medium (keratinocyte basal medium-2 (KGM) containing recommended growth supplements (Biowittaker, Wokingham, UK) and 5% fungizone (Gibco, Paisley, UK)) to maintain the tissue integrity and phenotype.

Primary oral keratinocytes were established by separating the epithelium from the connective tissue of the excised normal tissue in a sterile plastic Petri dish (Starstedt, Leicester, UK) by using forceps and scapel, then dissecting material approximately $1mm^3$ pieces and culturing at 37°C, 5% CO_2 in small volumes (but sufficient to cover the tissue samples) of keratinocyte basal medium-2 (KGM) containing the recommended growth supplements (Biowittaker, Wokingham, UK) and 5% fungizone (Gibco, Paisley, UK). The cells were left undisturbed in culture medium for 30mins to allow attachment of the epithelial cells to the flask, before adding 5mls of KGM and incubating undisturbed for one week until epithelial outgrowth occurred. The epithelial cells were then detached using 0.25% trypsin-1mM EDTA (Sigma, Poole, UK). The viability of the keratinocytes was then confirmed by trypan blue exclusion.

Established keratinocytes were fed with fresh KGM every 3-4 days and maintained at 37°C, 5%CO_2. Passage, thawing and freezing of these cells were carried out as described in Sections 2.3.4 and 2.3.5. These primary cells were only used up to passage 3 in all experiments.

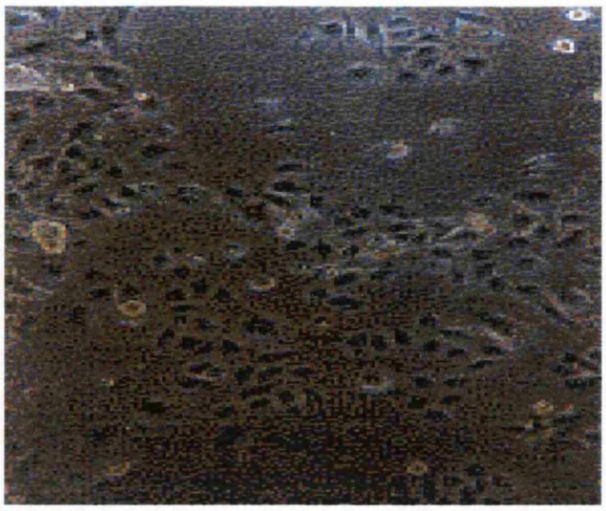


Figure 2.1: Representative established primary oral keratinocyte cell line in cell culture. Original magnification x10.

2.3.2 H357 cell culture

The oral squamous cell carcinoma cell line, H357, as established by Prime *et al* (1990), from a primary explant of a tongue squamous cell carcinoma was employed. These cells were found not to be contaminated with other cell types (Prime *et al,* 1990). Frozen cell stocks were recovered by thawing (as detailed in 2.3.5) and the cell line was maintained at 37°C, 5% CO_2 in KGM (as described in 2.3.1). The cells were fed with fresh medium every 2 to 3 days and passaged every 5 to 7 days.

Figure 2.2: Representative sample of H357 cells grown in culture. These cells were maintained as described in 2.3.2. Original magnification x10.

2.3.3 UP cell culture

The UP cell line is a cutaneous epithelial cell line. It was established by transfection of the pSV2neo/16 plasmid into normal human epidermal keratinocytes (Pei *et al*, 1991). This plasmid carries the human papilloma virus 16 (HPV-16) genome and confers immortality to normal human epidermal keratinocytes. The medium and conditions for growth were as previously described for primary cell culture (Section 2.3.1).

2.3.4 Passage of epithelial cells

All medium was removed from the cell culture flask and the adherent cells washed twice with PBS. A volume of trypsin-EDTA (warmed to 37°C) (Gibco, Paisley, UK) sufficient to cover the cell culture was then added to the flask, which was then incubated at 37°C in humidified air for 5-10 minutes. After this time, the cells were

examined under the microscope for evidence of rounding-up, and then an equal volume of fresh KGM was then added to the flask. The cell suspension was washed once with PBS, resuspended in KGM and the cell number was adjusted to a suitable density (e.g.$1x10^5$/ml) before reseeding in a $25cm^2$ tissue culture flask (Starstedt, Leicester, UK). Incubation of the cells continued at 37°C, 5% CO_2.

2.3.5 Mammalian cell freezing and thawing

a) Cell freezing

Cells were frozen at a concentration of approximately $2x10^6$/ml. The adherent cells were trypsinised (as described in 2.3.4), washed by centrifugation at 1500rpm for 10 mins, and resuspended in freezing medium [90% foetal calf serum (FCS) (Gibco, Paisley UK) and 10% DMSO (Sigma, Poole, Dorset)]. Cells were then aliquoted into Nalgene 1.5ml cryovials (VWR International, Lutterworth, UK), placed in a Nalgene freezing vessel (VWR International, Lutterworth, UK), which had been previously filled with isopropanol (VWR International, Lutterworth, UK). The container was stored at -70°C for 24 hours and the vials then transferred to liquid nitrogen storage.

b) Cell thawing

The vials of frozen cells were carefully removed from liquid nitrogen storage and placed in a water bath at 37°C. Once thawed, the vials were sprayed with alcohol and transferred to the flow cabinet where they were carefully opened and the contents added to 10mls of pre-warmed (to 37°C) KGM. The cells were washed in KGM and resuspended in fresh growth medium. The cells were then seeded at an appropriate density in $25cm^2$ flasks and maintained as described (Sections 2.3.1., 2.3.2 and 2.3.3).

2.3.6 Cell treatment assay

a) Cytokine treatment assay

In a modification of the method utilised by Altenberg *et al,* 1999, the H357, UP cells or primary oral epithelial cells (at 2nd or 3rd passage) were seeded at a density of $8x10^4$cells/ well in a Falcon 6 well plate (Becton Dickinson, Oxford, UK) with 3mls of KBM medium containing all recommended supplements (except hydrocortisone – a known inhibitor of T cell activation (Goodwin *et al,* 1986)). The cells were incubated for at least 2-5 days until cell culture was 60-80% confluent. Fresh medium containing a known concentration of recombinant human cytokine/s were added to 3 wells; cell culture medium only was added to the remaining 3 wells, as untreated control samples. The cells were incubated at 37°C, 5% CO_2 for an allocated time (see 3.2, 6.2, 7.2 for description of cytokines and times utilised). The supernatant was then extracted, centrifuged and stored at -70°C.

The adherent cells were then washed with PBS (Gibco Life Technologies, Paisley, UK) and 0.5mls TriReagent (Sigma-Aldrich, Poole, Dorset) was added to each well. The cells were aspirated 10 times, transferred to a sterile Eppendorf (Starstedt, Leicester, UK) and stored at -70°C. The RNA was isolated as described in Section 2.9.2.

b) LPS treatment assay

H357 cells were seeded at 1.5 $x10^5$ cells /well in a Falcon 6 well plate (Becton Dickinson, Oxford, UK) with 3mls KGM containing 5% foetal calf serum (FCS). These cells were incubated for approximately 2 days until the culture was 80%

confluent. At this point the cells were stimulated with 1μg/ml *E.coli* LPS (055: B5) (Sigma, Poole, UK) for 2, 4, 6 or 8hours. The supernatant was removed, the cells washed in PBS, and the RNA extracted from the adherent cells using the phenol / chloroform method (Chomzynski and Sacchi, 1987).

2.3.7 Bacterial cell culture

All bacterial stocks were maintained frozen at -70°C in trypticase soy broth (TSB) (Becton Dickinson, Oxford, UK) supplemented with 0.6% yeast extract (YE) (Oxoid, Basingstoke,UK) and 10% glycerol (BDH). Approximately every four weeks fresh stocks were grown from frozen supplies. Cultures were checked weekly both visually and by Gram-staining for contamination with other bacteria. Stocks of *E.coli* NCTC JM22 and *S.sanguis* NCTC 10904 (provided by Dr.Rod McNab, Department of Oral Microbiology, Eastman Dental Institute, University College London, UK) bacterium were plated on agar plates containing 3% trypicase soy broth (TSB). The bacteria were grown for 48 hours at 37°C, 5% CO_2 and maintained by twice weekly sub-culture on TSB agar plates.

2.4 Enzyme-linked immunosorbant assay (ELISA) for detection of chemokines

The ELISA for the specific protein was first optimised by using a standard concentration of specific protein in combination with serial dilutions of the primary and biotinylated antibody, to assess the concentration of antibodies to be utilised. The concentration of the protein in cell culture supernatants was then measured as detailed below. A Nunc 96 well maxisorp-surface immunoplate (BDH, Lutterworth, UK) was coated overnight with a monoclonal antibody against the human protein to be studied.

The plate was then washed 3 times with wash buffer (see Appendix 1) and thoroughly blotted on absorbent tissue.

100µl of cell supernatant or positive control (normally recombinant chemokine in a range of dilutions to obtain a standard curve) was added and incubated for 2 hours at room temperature, then washed as detailed above. A biotinylated antibody was used as a secondary antibody; 100µl of this antibody, diluted to the appropriate optimised concentration, was added to each well. The plate was sealed and incubated for 1 hour at room temperature, then washed 3 times. Bound secondary antibody was detected by adding 100µl avidin-HRP (Dako, Denmark) [diluted 1:4000 in PBS] and incubating for 30 minutes at room temperature. To detect bound antigen, 25µl H_2O_2 was added to OPD (1 tablet of o-phenyl diaminazadine (Sigma, Poole, Dorset) in 25mls of 34.7mM citric acid, 66.7mM Na_2HPO_4) and 100µl of this solution was dispensed to each well immediately and incubated at room temperature for 15mins. The reaction was stopped by adding 100µl of 1M sulphuric acid to the wells and the absorbances measured at 490nM with an ELISA plate reader (Dynex Technologies, Virginia, US).

Chemokine concentration in the supernatant was then extrapolated from the standard curve generated from standards using Revelation software (Dynex Technologies, Virginia, US).

Specific antibodies and standards utilised for each ELISA are detailed in the relevant sections (3.2, 6.2 and 7.2).

2.5 Separation of peripheral blood lymphocytes

Venous blood was obtained from healthy volunteers by vene-puncture from the antecubital fossa into lithium heparin-coated tubes (Becton-Dickinson, Oxford, UK) and transferred to a tissue culture cabinet. Appropriate ethical approval had been obtained for the use of blood.

Peripheral blood mononuclear (PBM) cell separation was then carried out by density centrifugation using Ficoll-paque Plus (Amersham Biosciences, Chalfont St.Giles, UK). Firstly, the blood was diluted in equal amounts of Dulbecco's PBS (Gibco Life Technologies, Paisley, UK) and layered carefully upon the same volume of Ficoll-paque. The solution was then centrifuged for 30mins at 1700rpm. The PBM cells were then visible as a translucent band at the interface of the solutions (the lower layer containing Ficoll-paque and red blood cells and the upper layer containing serum). The interface layer was carefully transferred to another tube using a Pasteur pipette. At least 3 times the volume of PBS was then added to the transferred PBM and centrifuged at 1700rpm for a further 30mins, when the supernatant was removed and the process repeated.

To deplete adherent cells, such as monocytes, PBMC obtained were incubated for 1hr in RPMI-1640 (Gibco Life Technologies, Paisley, UK) plus 5% foetal bovine serum (Sigma, Poole, UK) and those cells remaining in suspension (predominately peripheral blood lymphocytes (PBL)) (as described in Kunkel *et al,* 2002) were adjusted to a density of $0.5x10^6$cells/100µl. These cells were then prepared for migration in the transwell migration assay (Section 2.6).

2.6 Transwell migration assay

600µl of cell culture supernatant obtained from the cytokine treatment assays (2.3.6) (defrosted at room temperature) or recombinant human chemokine was added to the bottom chamber of Corning Co-star 5µm pore transwells (BDH, Lutterworth, UK) in triplicate. The insert was then carefully placed in the well and 100µl of PBL ($0.5x10^6$ cells) (section 2.5) added to the top chamber. The transwells were then incubated for 3 hours at 37°C in an atmosphere containing 5% CO_2. Following migration, the cells that passed through the membrane were collected and 5µl of 15µM polybeads (Polysciences, Germany) added to each tube.

The collected cells were then incubated with the appropriate concentration of fluorescently-labelled antibodies against specific human T cell markers (sections 3.2 and 6.2) for 30mins at 4°C. Following incubation, the cells were washed in PBS, resuspended in 1% paraformaldehyde (Sigma, Poole, UK) in PBS and stored at 4°C in the dark.

2.7 Flow cytometry

2.7.1 Fluorescent activated cell sorter (FACS) acquisition and analysis

The flow cytometer (Becton Dickinson, Oxford, UK) was prepared according to the manufacturer's recommendations. Colour compensation for the different fluorescent labelled-antibodies to be studied was executed (2.7.2) and the labelled migrated cell populations (2.6) were acquired using CellQuest software (Becton Dickinson, Oxford, UK).

Analysis was carried out using CellQuest software (Becton Dickinson, Oxford, UK). Firstly, by using forward and side scatter, initial gatings of peripheral blood lymphocytes and polybead populations were set, and all further analysis as described carried out from within the gated peripheral blood lymphocyte population (Figure 2.3). After acquisition, dot-plots of the gated population were drawn displaying the florescence levels of one type of labelled cells against different antibody-labelled cells (e.g. FITC-labelled cells on the X-axis and PE-labelled cells on the Y-axis). Those cells that labelled with FITC would be represented as a higher florescence along the X-axis, and those with labelled with PE would be higher on the Y-axis. Thus cell populations with high or low expression for the antigens studied were gated.

To standardise for the flow rate of the flow cytometer all gated populations measured were then calculated as a percentage of the polybead population.

2.7.2 Colour compensation of fluorescent activated cell sorter (FACS) machine

When carrying out this assay, it was essential to set the colour compensation accurately on the FACS machine as fluorescent dyes have significant overlaps in their spectrum. The compensation levels were set by running control PBL cells labelled with each florescent dye to be tested singly and adjusting the compensation settings until there was no crossover of different fluorescent dyes (i.e. no singly-labelled cells should be positive for a different coloured dye). After these settings had been adjusted for all 3 colours, the relevant study cell populations were then analysed.

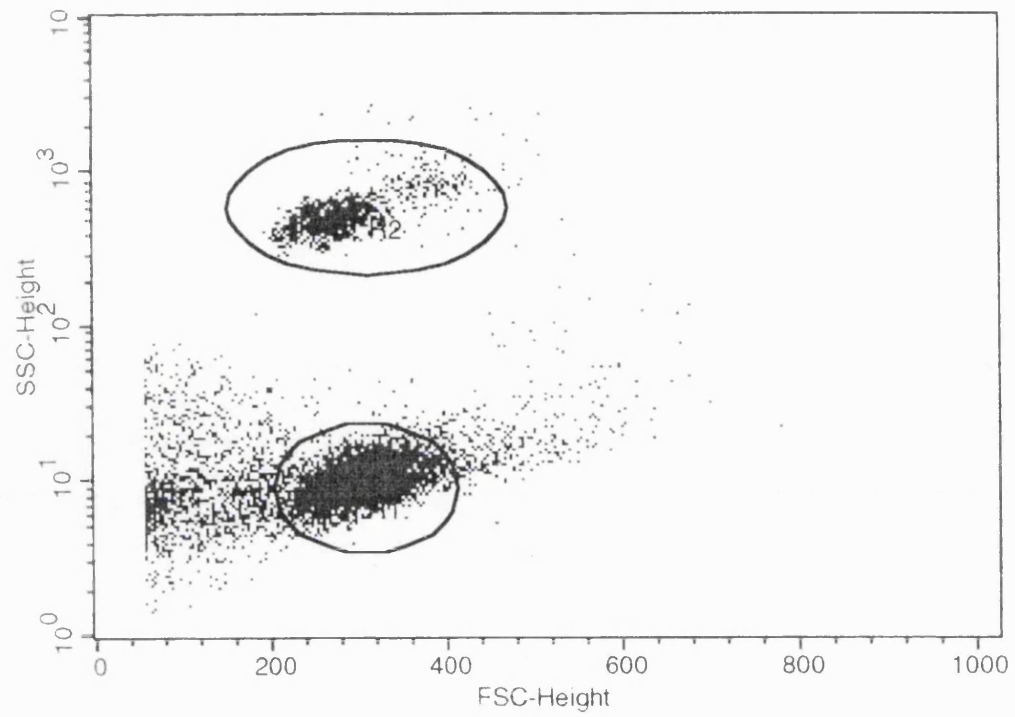


Figure 2.3: FACS plots of peripheral blood lymphocytes and polybead populations. A representative sample of the PBL gated population (ringed cells in lower area of graph) and the polybeads (ringed cells in upper area of graph), as determined after forward- (FSC) and side-scatter (SSC) are applied to the cells.

2.8 Antimicrobial assays

2.8.1 Radial diffusion antimicrobial assays

This assay was adapted from the method of Steiner *et al*, 1997. This sensitive method assesses the antimicrobial activity of substances using target microbes. Firstly, one colony of *E.coli* was selected from a culture plate (Section 2.3.7) with a sterile inoculation hoop and resuspended in 50mls of 3% TSB. For the oral streptococci species, 3 streaks of a culture plate were isolated with a sterile inoculation hoop and resuspended into 10mls of 3% TSB. Cultures were then shaken at 250rpm at 37°C for 15-18 hours. 50µl of the *E.coli* culture or 2mls of the oral streptococci species culture was transferred into 50mls or 10mls of 3% TSB respectively. This culture was shaken at 250rpm for 3.5 hours at 37°C to obtain the log phase of the bacterial cell culture

growth. Following this incubation, the cultures were centrifuged at 1500rpm (4°C) for 10mins, washed in ice-cold, sterile 10mM sodium phosphate buffer and resuspended in 5mls of the same buffer. 1ml of this solution was transferred to a cuvette and the optical density was adjusted to 1.0 at 620nm, by adding sodium phosphate buffer to the bacterial cultures if required.

Following this, 8µl of *E.coli* or 16µl of the streptococcal species was added to 5mls of molten underlay (incubated in a 60°C waterbath) and placed in a Petri dish (Starstedt, Leicester, UK), using a levelling tray (Bio-Rad, Hemel Hempstead, UK) to ensure an equal distribution of bacteria. This underlay was allowed to set for 5-10mins at room temperature, and then 3mm holes were punched in the gel using the base of a 10ml pipette (Starstedt, Leicester, UK). 5µl of chemokine (recombinant human MIG or IP-10 both from Peprotech, London, UK) or tetracycline diluted in 0.01% acetic acid was added to the wells.

The plates were covered, turned gel-side up and incubated for 3 hours at 37°C in a heated chamber (Merck, Lutterworth, UK), before 5mls of molten overlay (incubated in a 60°C waterbath) was added to the plate, allowed to set at room temperature for 5-10mins, and incubated at 37°C overnight. The zones of bacterial culture depletion around the wells were then measured from 3 different points to the nearest 0.1mm, the diameter of the well was subtracted and the differences were multiplied by 10 to convert the zone diameter to units. Images of the plates were recorded using AlphaImager software (AlphaInnotech Corp., Cannock, UK).

2.9 Molecular biology techniques

2.9.1 Preparation of tissue for RNA isolation

Oral lichen planus tissue or normal oral mucosal tissue was collected (as detailed previously in Section 2.1) and stored in 1ml RNAlater (Ambion, Huntingdon, UK) at -20°C, until required.

To prepare the tissue, the RNAlater solution was defrosted to room temperature and the oral mucosal tissue sample was removed. The tissue was transferred to a sterile Petri dish (Starstedt, Leicester, UK), rinsed in PBS (Gibco, Paisley, UK), and chopped finely with a sterile scalpel. The sample was then placed in 1ml of TriReagent (Sigma, Poole, UK), aspirated and transferred into a sterile tube. The sample was then stored at -70°C in airtight containers.

2.9.2 RNA isolation

Firstly, work surfaces were prepared with RNAaseZAP (Ambion, Huntingdon, UK) to remove any endogenous RNAase.

The TriReagent solution, containing sample material, was incubated for 5mins (after defrosting on ice, if previously frozen) at room temperature, then 0.2µl of chloroform (BDH)/1 ml of TriReagent added to the sample. The sample was covered, shaken for 15secs and incubated at room temperature for 15mins before centrifuging at 12200rpm for 15mins at 4°C. The top aqueous phase obtained after centrifugation containing RNA was transferred to a new tube, ensuring the interface layer was not disturbed.

0.5µl of isopropanol (BDH, Lutterworth, UK) was then added to the sample and was incubated for 5-10 minutes at room temperature before centrifuging for 12200rpm for 15 minutes at 4-8°C, utilising 2µl Pellet paint Co-precipitant (Novagen, Nottingham, UK) to visualize the RNA pellet. The supernatant was removed and the pellet washed in 1ml 75% ethanol (BDH, Lutterworth, UK), vortexed and centrifuged at 9700rpm for 5mins. The supernatant was removed and the pellet allowed to air dry for approximately 10mins.

25µl of DEPC water (Ambion, Huntingdon, UK) was then added to the pellet, and the solution heated at 60°C for 15mins. If not required immediately, this RNA solution was stored at -70°C in an airtight container.

2.9.3 cDNA Single strand synthesis

To synthesise single strand cDNA the following procedure was undertaken. 2µl of RNA was added to 4µl deoxynucleotides (dNTPs) (2.5mM) (Sigma, Poole, UK), 2µl of random hexamers (50µm) (Ambion, Huntingdon, UK) and 9.5µl nuclease-free water (Ambion, Huntingdon, UK). This was incubated at 70°C for 3 minutes and allowed to cool to room temperature. Then 1µl of RNAaseIN (Ambion, Huntingdon, UK), 2µl 10x Moloney murine leukaemia virus reverse transcriptase (M-MuLVRT) buffer and 0.5µl M-MuLVRT (200U/µl) (Roche, Lewes, UK) was added and incubated at 42°C for 1 hour. cDNA samples were then stored at -20°C until required.

As a negative control, RNA samples were incubated in this reaction as described, but without the addition of the Mu-LVRT enzyme. Thus, any of the negative RNA control

samples that provided a 'positive' band in the RT-PCR reactions were treated with DNA-free (Ambion, Huntingdon, UK), according to the manufacturer's instructions to eliminate DNA contamination from the sample.

2.9.4 RT-PCR

Primers to be utilised in RT-PCR reactions were designed using Primer3 software (http://www.basic.nwu.edu/biotools/Primer3.html) on specific mRNA sequences located in Genebank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

The general RT-PCR protocol that was followed is described:

1µl of cDNA (see 2.9.3) was added to 4µl dNTP (2.5mM) (Sigma, Poole, UK), 5µl 10x buffer, 0.225µl AmpliTaq (5.0U/µl) (Roche, Lewes, UK), 4µl of each specific primer (5µM), 1-4mM MgCl in each reaction. Nuclease-free H_2O (Ambion, Huntingdon, UK) was then added to give a final volume of 50µl.

The thermocycler (Techne Genius; Cambridge, UK) parameters utilised varied depending upon the primers used, as the annealing temperature of the reaction is dependant upon the guanidine-cytosine content of the primers. The general parameters used were (unless otherwise stated):

94°C for 45secs

(57°C-60°C) annealing temperature for 45secs

72°C for 45secs

Repeat for 35 cycles

The products were separated on a 2% agarose (GibcoBRL Life Technologies, Paisley, UK) gel, stained with ethidium bromide (Sigma, Poole, UK) and specific bands visualised by ultra-violet trans-illumination in a MultiImage Light Cabinet (AlphaInnotech Corp., Cannock, UK). Digital images of the gels were acquired and stored using AlphaImager Software (AlphaInnotech Corp., Cannock, UK). Unless otherwise stated, the primers utilised for the study of housekeeping expression encoded a region of 18S ribosomal RNA (5'- TTTCGGAACTGAGGCCATGA-3', 5'-GCATGCCAGAGTCTCGTTCG -3').

2.9.5 Semi-quantitative RT-PCR

Semi-quantatitive RT-PCR was undertaken to analyse the level of mRNA expression in a given sample.

For each specific primer to be studied, the linear range was determined by repeating the RT-PCR reaction with optimised magnesium concentration for each primer and stopping the reaction every 2 cycles. The mid-point of each linear range was determined by using intensity analysis of the bands with AlphaImager software, and this cycle length was utilised for each primer in subsequent reactions.

18S primer and 18S competitor primers (Ambion, Huntingdon, UK) were combined to ratios 1:9, 2:8 and 3:7 respectively. For each of the primers to be analysed by semi-quantatitive RT-PCR, 4µl of 18S primer:competitor mix (either 1:9, 2:8 or 3:7) was also added to the RT-PCR reaction (2.9.4). The band intensity obtained from the specific primer reactions containing the 18S: competitor primers were compared to the reaction containing the specific primer without any 18S primer:competitor mix.

The reaction with a similar level of specific primer expression as that without a ratio 18S primer:competitor added was selected for quantification. The band intensity of 18S (with competitor) and the specific primer was quantified in each sample using Phoretix 1D software (Phoretix, Newcastle, UK) (Fig. 2.4).

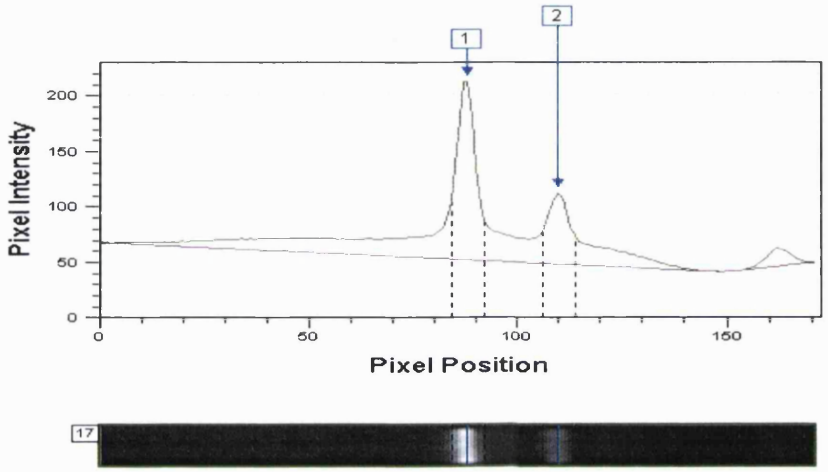


Figure 2.4: A representative diagram of band intensity analysis using Phoretix ID software. The specific bands to be analysed have background intensity removed and the volume intensity of the band is measured. This specific volume can then be calculated relative to the intensity determined for the 18S house-keeping gene.

2.10 Statistical analysis

All p values in studies included in this thesis were obtained by executing Mann-Whitney U test, unless otherwise stated. The data used to compose the Tables and Figures within the results of this thesis are detailed in Appendix 2.

Chapter 3: The CXC ELR- chemokines in oral epithelial cells and oral lichen planus

3.1 Introduction

Monokine induced by interferon-gamma (MIG) (CXCL9), interferon-induced protein-10 (IP-10) (CXCL10) and interferon-gamma induced T-cell attractant chemokine (I-TAC) (CXCL11) all belong to the CXC family of chemokines and are termed CXC ELR- chemokines. They can be distinguished from other CXC chemokines by the lack of a Glu-leu-arg (ELR) motif that mediates neutrophil migration (Clark-Lewis *et al,* 1993). Instead, these three chemokines all bind the CXCR3 receptor (Loetscher *et al,* 1998; Tensen *et al,* 1999; Cox *et al,* 2001), predominately expressed on activated/memory CD4/CD8 cells (Rabin *et al,* 1999; Qin *et al,* 1998; Loetscher *et al,* 1998), that are associated with a Th1 phenotype (Sallusto *et al,* 1998).

The 3 chemokines are expressed by a number of different cell types including macrophages (Meyer *et al,* 2001) and neutrophils (Gasperini *et al,* 1999) and non-haemopoetic cells; endothelial cells (Mazenet *et al,* 2000), fibroblasts (Meyer *et al,* 2001) and epithelial cells (Romagnini *et al,* 2002; Sauty *et al,* 1999) including keratinocytes (Albanesi *et al,* 2001; Tensen *et al,* 1999). Interferon-gamma (IFN-γ) can induce most of the above cell types to produce these chemokines, but other factors are also known to either induce or act synergistically with interferon-γ, such as TNF-α (Sauty *et al,* 1999), lipopolysaccharide (LPS) (Kawai *et al,* 2001), CD40 ligation (Altenberg *et al,* 2001), IFN-alpha (IFN-α) or beta (IFN-β) (Ogaswara *et al,* 2002), IL-4 (Albanesi *et al,* 2002) and vascular endothelium growth factor (VEGF) (Lin *et al,* 2002) to stimulate their production.

The production of MIG, IP-10 and I-TAC and the presence of CXCR3 cells are associated with many T-cell mediated disorders including allograft rejection (Miura *et al,* 2001; Meyer *et al,* 2001), autoimmune diseases (e.g. an animal model of Sjogren's syndrome) (Tornwall *et al,* 1999), rheumatoid arthritis (Qin *et al,* 1998), ulcerative colitis (Qin *et al,* 1998) and airway inflammation (in an animal model of asthma) (Medoff *et al,* 2002). These chemokines are also expressed in some cutaneous inflammatory disorders such as contact hypersensitivity (Goebeler *et al,* 2001; Tokuriki *et al,* 2002; Sebastiani *et al,* 2002; Flier *et al,* 2001) and lichen planus (Tensen *et al,* 1999; Spandau *et al,* 1999; Flier *et al,* 2001), where the chemokines are produced in abundance by involved keratinocytes. The identification of these chemokines in oral inflammation (Gemmel *et al,* 2001), suggests that they may also be influential in the infiltration of T cells into oral mucosa.

Keratinocytes in the epithelial layer are capable of producing many different cytokines (Formanek *et al,* 1999; Li *et al,* 1996, Uchi *et al,* 2000), including chemokines (Li *et al*[2], 1996; Bickel *et al,* 1999; Li *et al,* 2000; Uchi *et al,* 2000) likely to influence local immune reactions (Steinoff *et al,* 2001). However, it is known the dynamics of cytokine production can differ between oral and cutaneous keratinocytes (Li *et al,* 1996). There is no data of the expression of MIG, IP-10 or I-TAC in oral keratinocytes *in vitro*.

CD40 can be expressed on a number of different epithelial cells including basal peripheral corneal epithelium (Iwata *et al,* 2002), ovarian epithelial carcinoma cell lines (Gallacher *et al,* 2002), salivary gland epithelium (Dimitriou *et al,* 2002),

bronchial epithelium (Tanaki *et al,* 2001) as well as buccal oral epithelium *in vitro* (Farmer *et al,* 2001).

IFN-γ can cause an increase of CD40 expression on a number of different epithelial cell lines, including buccal oral epithelium (Farmer *et al,* 2001). TNF-α can also enhance CD40 expression on some cell types, such as corneal epithelium (Iwata *et al,* 2001). However, there appears to be no effect by Th2 cytokines, such as IL-4 or IL-5, on bronchial epithelial CD40 expression (Tanaka *et al,* 2001).

The ligand for CD40 is CD154, a cell surface molecule mainly expressed upon activated T cells. There is an increased production of pro-inflammatory cytokines IL-15 (Weiler *et al,* 2001) and IL-6 (Gallacher *et al,* 2001) in tubular and ovarian carcinoma epithelium respectively after ligation of CD40. There is also an effect upon chemokine production after CD40 ligation; IL-8 (Li *et al,* 2002; Gallacher *et al,* 2001), MCP-1 (Li *et al,* 2001) and RANTES (Propst *et al,* 2000) can be stimulated in various epithelial cells. Furthermore, production of MIG and IP-10 could be synergistically enhanced (with IFN-γ stimulation) after CD40 ligation (Altenberg *et al,* 1999).

Due to the increase in CD40 expression after pro-inflammatory cytokine treatment, it is maybe not surprising that epithelial CD40 expression is enhanced in some inflammatory diseases. The expression is increased on bronchial epithelium during non-treated corticosteroid-dependent asthma (Vignola *et al,* 2001) and is increased on salivary gland epithelium during Sjogren's syndrome (Dimitriou *et al,* 2002). Furthermore, the blockade of CD40-CD40L interactions in inflamed bronchial

epithelium inhibits the proliferation of CD4+ cells, suggesting that CD40 has an important role in T cell activation at epithelial surfaces (Tanaka *et al,* 2001). Furthermore, an agonistic anti-CD40 antibody can increase inflammation in graft-versus-host-disease (Buhlmann *et al,* 1999).

As discussed in Chapter 1, oral lichen planus is histopathologically characterised by a large infiltrate of memory T cells (Scully *et al,* 1998) localised in a band-like pattern directly beneath the basal epithelium. It might thus be expected that epithelial cell-derived chemokines, such as MIG, IP-10 and I-TAC would play an important role in this T cell recruitment - especially as CXC ELR- chemokines are associated with other inflammatory disorders involving memory T cell immunopathogenesis. Hence, the aim of the present section was to determine if MIG, IP-10 and I-TAC are expressed in healthy oral mucosa and oral lichen planus. Furthermore, there is increasing evidence that the expression of CD40 on epithelium plays a role in immune responses in a number of different ways. The ligation of this molecule causes the induction or enhancement of the production of a number of cytokines and chemokines, including IP-10. Therefore, the expression of CD40 by oral epithelial cells was investigated *in vitro* and by immunohistochemistry of OLP tissue.

Specifically :

- To determine if primary oral epithelial cell supernatants can generate IP-10, MIG and IL-8 with and without IFN-γ stimulation. IL-8 is a CXC ELR+ chemokine, previously reported not to undergo enhancement with IFN-γ (Li *et al,* 1996) and was thus studied to be an appropriate control.

- Determine the level of CXC ELR- chemokine mRNA expression in primary oral epithelium, as assessed by RT-PCR.

- Determine temporal effects of IFN-γ stimulation upon MIG, IP-10 and IL-8 production by an oral squamous cell carcinoma cell line (H357).

- Examine the temporal effects of IFN-γ stimulation upon mRNA expression of MIG, IP-10, I-TAC and IL-8 by an oral carcinoma cell line in response to stimulation with or without IFN-γ.

- Determine the profile of peripheral blood cells that migrate to recombinant human IP-10, using markers for T cells (CD3) and a memory cell marker (CD45RO).

- Assess levels of IFN-γ and relative levels of CXC ELR- chemokine mRNA in oral lichen planus and normal oral mucosal tissue.

- To investigate the effect of IFN-γ treatment on CD40 mRNA expression by oral epithelial cells and study the expression of CD40 and CD154 expression in OLP tissue by immunohistochemistry.

The results of these experiments would thus determine if oral epithelial cells are capable of expressing and secreting CXC ELR- chemokines, and thus of potential importance in the immunological aspects of OLP.

3.2 Materials & Methods

3.2.1 IP-10, MIG and IL-8 production by primary oral epithelial cells

Primary oral epithelial cells (Section 2.3.1) were incubated as described previously (Section 2.3.6) with 1000U/ml IFN-γ (Sigma-Aldrich, Poole Dorset) for 48hrs. The general aspects of the ELISA procedure are described in Section 2.4.

ELISA for IP-10

To determine the concentration of IP-10 protein in cell culture supernatants, a monoclonal antibody against human IP-10 (Clone 33036.211, R+D systems, Abingdon, UK) diluted to 4μg/ml in PBS was utilised as the coating antibody. As a standard, recombinant IP-10 protein (R&D systems, Abingdon, UK) was diluted to concentrations ranging 2000pg/ml–30 pg/ml. A biotinylated mouse anti-human IP-10 antibody (R&D systems, Abingdon, UK) (diluted to 200μg/ml) was employed as a secondary antibody.

ELISA for MIG

The concentration of MIG protein in cell culture supernatants was detected using primary antibody monoclonal anti-human MIG (Clone 49106.11) (R&D systems, Abingdon, UK) at 6μg/ml in PBS. A monoclonal biotinylated anti-human MIG antibody (R&D systems, Abingdon, UK) was employed as the secondary antibody and recombinant human MIG (R&D systems, Abingdon, UK) as an internal standard.

ELISA for IL-8

To assess the levels of IL-8 in supernatants, the coating antibody was an immunoaffinity-purified polyclonal antibody from sheep anti-human IL-8 serum S333/BM. A stock solution of coating antibody was diluted to 2μg/ml using phosphate buffered saline (Gibco, Paisley, UK). The detection antibody utilised was a biotinylated immunoaffinity-purified polyclonal antibody from sheep anti-human IL-8 serum S333/BM (All IL-8 specific reagents provided by Dr. Steve Poole, National Institute for Biological Standards & Controls, Potters Bar, UK).

3.2.2 Determination of level of MIG, IP-10, I-TAC and IL-8 mRNA expression by primary oral epithelial cells

RNA isolation and cDNA synthesis from primary oral keratinocyte cells stimulated with 1000U/ml IFN-γ (Sigma-Aldrich, Poole Dorset) for 48hrs (Section 2.3.6) was carried out as described in Sections 2.9.2 and 2.9.3. RT-PCR for MIG, IP-10, I-TAC and IL-8 was undertaken as detailed in Section 2.9.4. The following primers were generated for use in this study (Genosys-Sigma, Poole, UK):

MIG (5'- CCAACACCCCACAGAAGTGC -3', 5'- GCCAGCACCTGCTCTGAGAC -3')

IP-10 (5'- GCCAATTTTGTCCACGTGTTG -3', 5'- AAAGAATTTGGGCCCCTTGG -3')

I-TAC (5'- GGCTTCCCCATGTTCAAAG -3', 5'- CAGATGCCCTTTTCCAGGAC -3')

IL-8 (5'- ATGACTTCCAAGCTGGCCGTGGCT -3', 5'- TCTCAGCCCTCTTCAAAAACTTCTC-3')

The thermocycler (Techne Genius, Cambridge, UK) parameters utilised for MIG, IP-10 and I-TAC were 94°C for 45secs, 57°C for 45secs and 72°C for 45secs, the linear range of cycle number for these primers was utilised (2.9.5). IL-8 parameters were a

primary step of 95°C for 5mins then 95°C for 1min and 60°C for 2.5mins for a total of 32 cycles.

3.2.3 Determination of temporal effects of IFN-γ stimulation upon MIG, IP-10 and IL-8 production by H357 oral squamous cell carcinoma cells

To assess the temporal effects of IFN-γ treatment upon H357 cells (Section 2.3.2), these cells were incubated with 1000U/ml IFN-γ for 3hrs, 6hrs, 9hrs, 24hrs, 48hrs and 72hrs. The supernatants were then were extracted and the production of MIG, IP-10 and IL-8 in the cell culture was determined by ELISA of IP-10, MIG and IL-8 as detailed for the primary cells.

3.2.4 Determination of temporal effects of IFN-γ stimulation upon mRNA expression of MIG, IP-10, I-TAC and IL-8 by H357 oral squamous cell carcinoma cells

RNA isolation and cDNA synthesis from H357 cells incubated with 1000U/ml IFN-γ for 3hrs, 6hrs, 9hrs, 24hrs, 48hrs and 72hrs (Section 2.3.2) was carried out as described in Sections 2.9.2 and 2.9.3. RT-PCR for MIG, IP-10, I-TAC and IL-8 was then undertaken as detailed in Section 2.9.4. The primers and conditions are stated in RT-PCR of the primary cells.

3.2.5 T cell chemotactic profiles in response to IP-10 and stromal derived factor-1alpha (SDF-1α)

Peripheral blood lymphocytes (PBL) were prepared as described in Section 2.5. The migration of the cells towards 1μg/ml recombinant human IP-10 (R+Dsystems, Minneapolis, US) or 100mM stromal-derived factor-1alpha (SDF-1α) (R+Dsystems,

Minneapolis, US) was assessed using the transwell migration assay (Section 2.6). SDF-1α was employed as a control chemotactic agent as this is a potent CXC chemokine. The collected cells were incubated with allophycocyanin-conjugated anti-human CD45RA (Clone HI100) and Cy-chrome conjugated mouse anti-human CD4 (Clone RPA-T4) (Both BDPharmingen, San Diego, US) at a concentration of 3µl / 10^6cells. FACS acquisition and analysis was carried out as previously detailed (2.7).

3.2.6 Expression of IFN-γ, MIG, IP-10 and I-TAC mRNA in oral lichen planus and normal oral mucosa

RNA isolation and cDNA synthesis from OLP and NOM tissue was carried out as described in Sections 2.9.2 and 2.9.3. IFN-γ expression was measured using a gene-specific RT-PCR kit (Ambion, Huntingdon, UK).

MIG, IP-10 and I-TAC primers (as described above) were utilised with QuantamRNA 18S internal standards (Ambion, Huntingdon, UK) for semi-quantitative analysis. The procedure was then carried out as described as described in Section 2.9.5. The idv (integrated density value) of MIG mRNA specific bands after removing the RT-PCR reactions at different cycle numbers is shown in Fig.3.1. The midpoint of the linear ranges of MIG, IP-10 and I-TAC (Fig 3.2) was calculated to be 23, 21 and 30 respectively, therefore these cycle lengths were utilised for the following semi-quantitative study. The ratio of 18S: competitor mix utilised in all the semi-quantitative analysis was 2:8 respectively as this ratio was found to demonstrate equivalent specific intensities to those without 18S added (Fig 3.3).

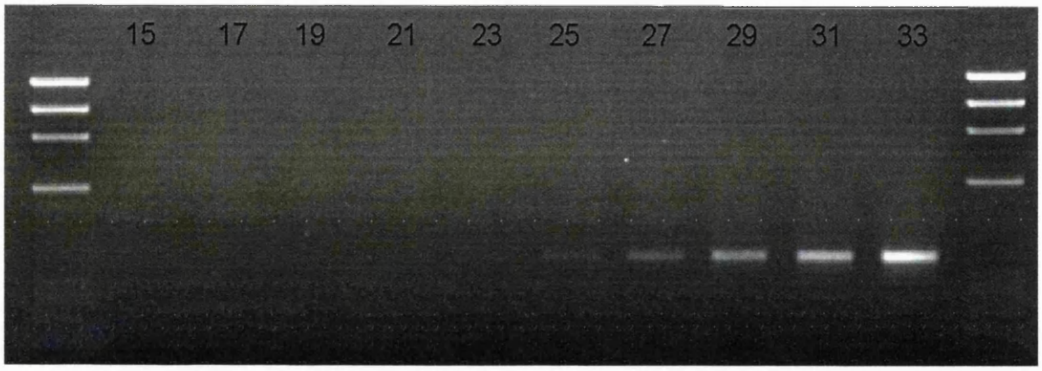


Figure 3.1: The linear range of MIG in a representative OLP sample. The samples were removed from the PCR machine every 2 cycles (from cycle 15-33). MIG expression is located at 351 base pairs (bp). The same process was performed for IP-10 and I-TAC primers.

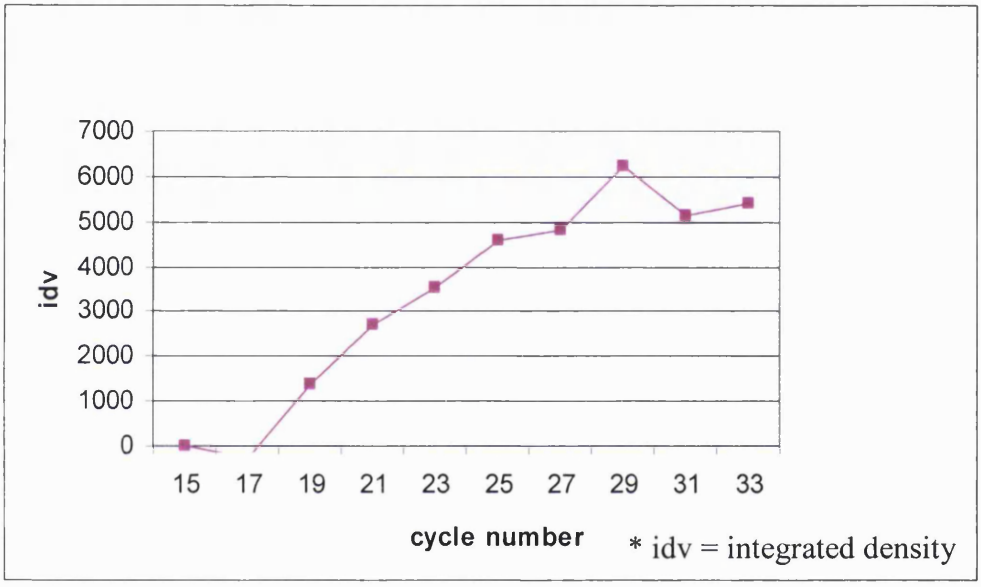


Figure 3.2: The integrated density values (idv) of MIG in a representative OLP sample. Densitometric analysis of the linear range (Fig 3.1) was performed, and the cycle number that corresponded to the mid-point of the linear range was selected for further analysis. The same process was performed for IP-10 and I-TAC primers.

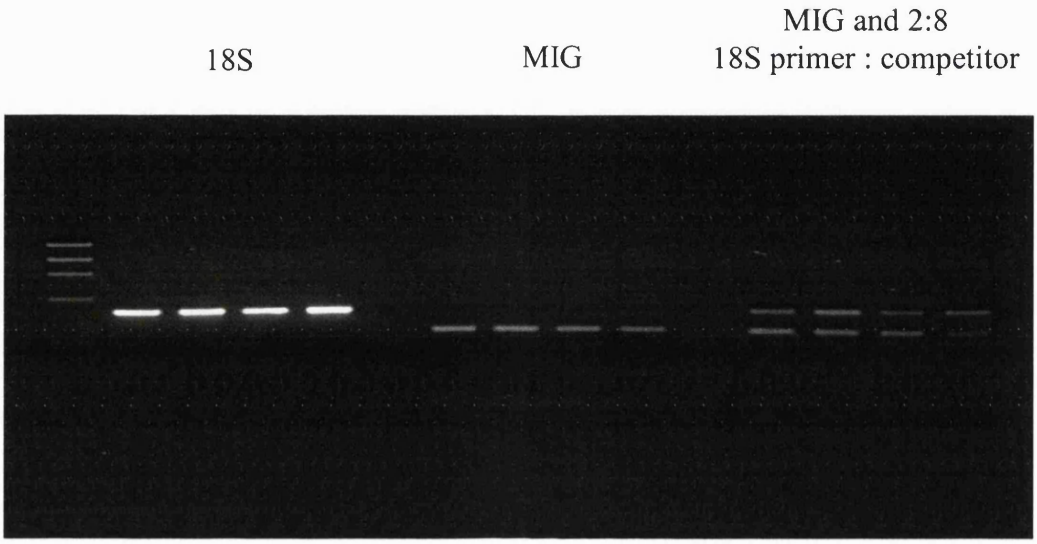


Figure 3.3: A representative example of the 2:8 ratio of 18S primer/ competitor expression in the analysis of MIG expression in 4 different OLP tissue samples.

3.2.7 The expression of CD40 mRNA by IFN-γ stimulated primary epithelial cells

mRNA derived (2.9.2, 2.9.3) from primary oral epithelial cells (2.3.1) treated with 1000U/ml IFN-γ for 48 hours (2.3.6) was investigated. Primers specific for human CD40 mRNA were generated for this reaction:

CD40 (5' - CTGGGCTAGCGATACAGGAG -3', 5'- GGAATTTCTG TTGGCCAAATCCA -3') (Genosys-Sigma, Poole, UK)

RT-PCR was then performed as previously described (2.9.4).

3.2.8 The expression of CD40 and CD154 in OLP tissue

Immunohistochemistry for CD40 in PEFF OLP tissue (2.2.1a) was performed by using a target retrieval solution for antigen retrieval (2.2.2) and chromogenic detection (2.2.3). The antibody monoclonal anti-CD40 (Serotec, Oxford, UK) was used at a dilution of 1:20 in PBS. Frozen OLP sections (2.2.1b) were utilised for the study of CD154, with incubation of primary antibody anti-CD154 (1:100) (Alexis, Nottingham, UK) and subsequent antigen detection using chromogenic staining methods (2.2.3).

3.3 Results

3.3.1 IP-10, MIG and IL-8 production by primary oral epithelial cells

In the absence of IFN-γ there was almost no IP-10 production and negligible production of MIG (<25pg/ml) by primary oral epithelial cells, in contrast, IL-8 was produced in relatively large amounts by oral epithelial cells without IFN-γ stimulation (>200pg/ml). IP-10 and MIG production by primary epithelial cells was significantly increased ($p<0.05$) following IFN-γ stimulation (Fig 3.4a and b) although there was a substantially lower concentration of MIG as compared to IP-10 (Fig 3.4b). In contrast, IFN-γ stimulation did not significantly affect IL-8 released from 2 out of the 3 primary cell lines tested (Fig 3.4c), indeed IL-8 production was higher in resting cells than stimulated cells in one of the tested primary epithelial cell cultures.

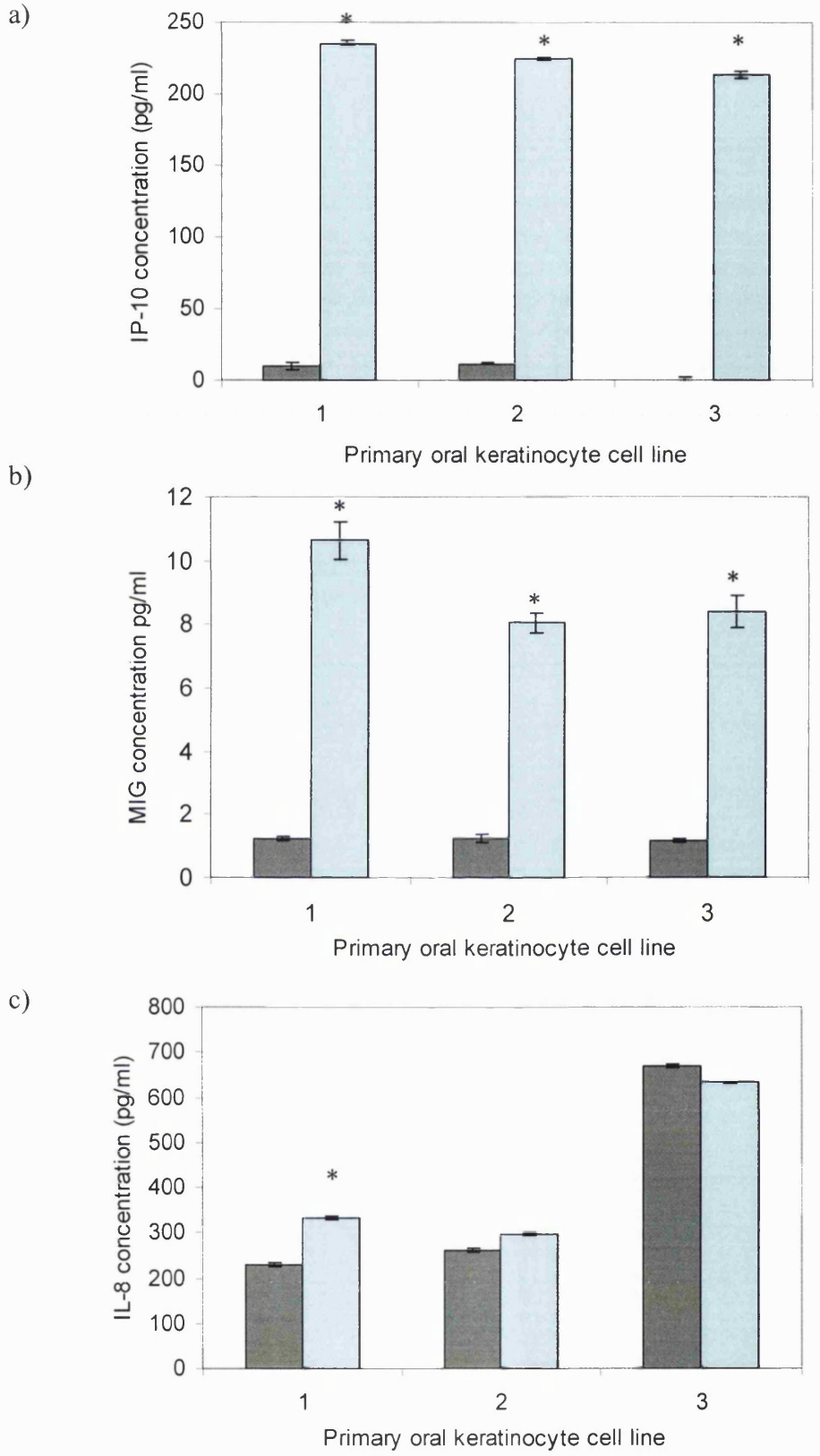


Figure 3.4: Concentration of a) IP-10 b) MIG and c) IL-8 produced by 3 different primary oral keratinocyte cell lines unstimulated (■) and stimulated with IFN-γ (■) for 48 hours. Bars represent ± standard error. Significant difference (* = $p<0.05$) between chemokine produced in cell line treated with IFN-γ and untreated cells at the same time point.

3.3.2 MIG, IP-10, I-TAC and IL-8 mRNA expression by primary oral epithelial cells

mRNA of IP-10, MIG and I-TAC were expressed in low levels by primary oral epithelial cells without IFN-γ stimulation. IFN-γ stimulation led to increased expression of mRNA of all 3 chemokines, although the expression of IP-10 and MIG was greater than that of I-TAC (Fig 3.5). In contrast, IL-8 mRNA was expressed in both unstimulated and stimulated cell lines (Fig 3.5e). These results thus mirror the protein production of IP-10, MIG and IL-8 by primary oral epithelial cells with and without IFN-γ stimulation.

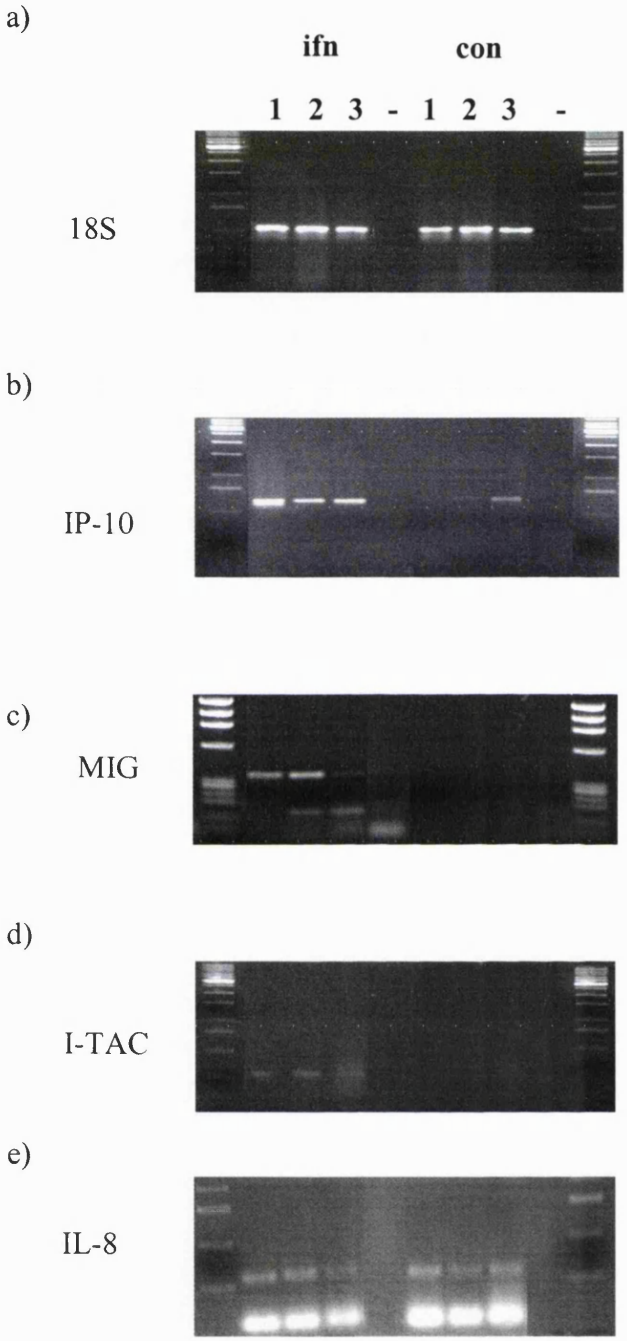


Figure 3.5: a) 18S, b) IP-10, c) MIG d) I-TAC and e) IL-8 mRNA expression in 3 different primary oral epithelial cell lines with (ifn) and without (con) IFN-γ stimulation.

3.3.3 Temporal effects of IFN-γ stimulation upon MIG, IP-10 and IL-8 production by H357 oral squamous cell carcinoma cell line

IP-10

Levels of IP-10 production demonstrated a sharp increase after about 9 hours, reaching a peak of 263.3pg/ml after 48hours of IFN-γ stimulation. Production was significantly higher than that of unstimulated cells ($p<0.05$) at all time-points tested after 3 hours (Fig 3.6a).

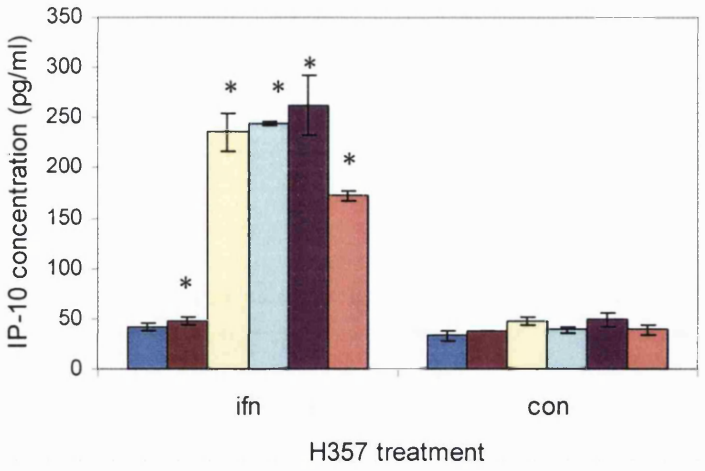
MIG

The concentration of MIG produced by H357 cells also reached a peak after 48 hours of IFN-γ incubation, but in contrast to IP-10 the increase was significantly higher than the control cells only at 9, 24 and 48 hours (Fig 3.6b). The concentration of MIG produced in the IFN-γ-treated cells was lower than IP-10 produced at all time-points and appeared to undergo a slower induction as the concentration of MIG produced was only significantly greater than the control cultures after 9 hours of IFN-γ stimulation.

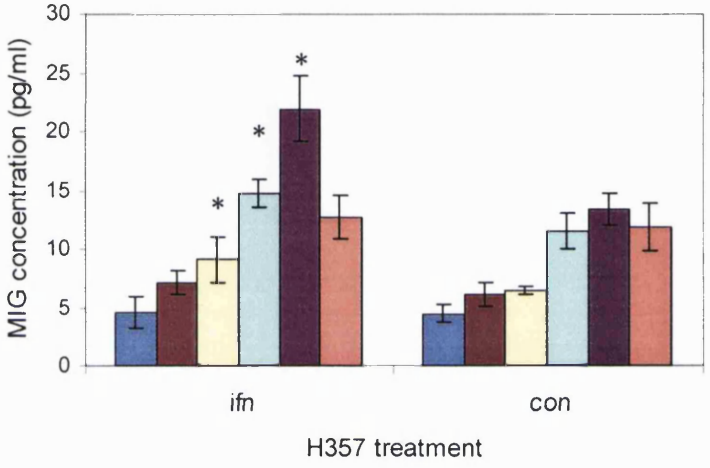
IL-8

In contrast to the above, IFN-γ did not enhance the production of IL-8 by H357 cells at any time-points examined, indeed, the concentration of IL-8 in the control cells was significantly higher than the IFN-γ stimulated cells at 3 and 48hrs (Fig 3.6c). But IL-8 levels generally increased with time of culture with or without IFN-γ.

a)



b)



c)

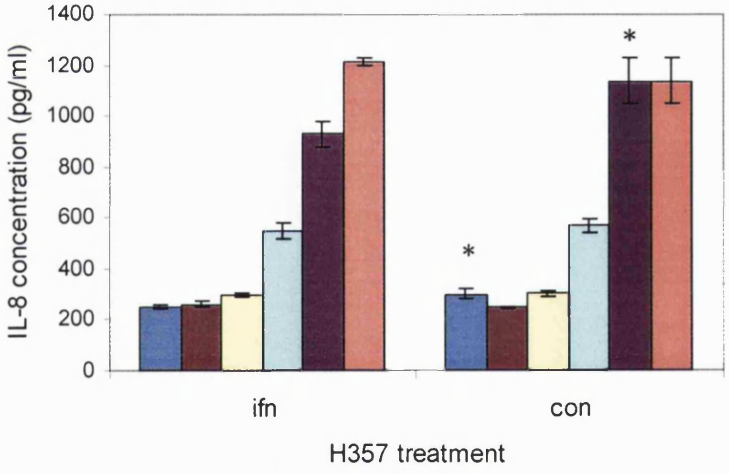


Figure 3.6: Concentration of a) IP-10, b) MIG or c) IL-8 produced by the H357 cell line stimulated with (ifn) or without (con) IFN-γ for 3 (■), 6 (■), 9 (■), 24 (■), 48 (■) and 72 (■) hours. Triplicate experiments were undertaken in all cases, hence the standard deviation (SD) is also denoted. Significant difference (* = $p<0.05$) between chemokine produced in cell line treated with IFN-γ and untreated cells at the same time point.

3.3.4 Temporal effects of IFN-γ stimulation upon MIG, IP-10, I-TAC and IL-8 mRNA expression

IP-10 mRNA in H357 cells was detected after only 3 hours of IFN-γ treatment and mRNA expression appeared to peak at 24 hours. In contrast, there was little expression of IP-10 mRNA by unstimulated cells over the same time period. MIG mRNA transcript expression demonstrated a similar pattern to that of IP-10, with a rapid induction of mRNA with IFN-γ stimulation although this peaked at 48 hours (Fig 3.7). I-TAC expression appeared weaker than MIG and IP-10 transcripts at many time-points, but was detectable at 3 hours and expression did increase up to 24 hours. mRNA expression for IP-10, MIG and I-TAC was, in general, not detected in any unstimulated cell samples. IL-8 mRNA was expressed in both control and IFN-γ stimulated cell lines. However, IL-8 expression in the control cells continued until 72 hours, whereas in the IFN-γ stimulated cells appeared to decrease after 24 hours.

The results of these studies thus demonstrate a similar pattern to the finding of IP-10, MIG and IL-8 protein secretion by the H357 cells.

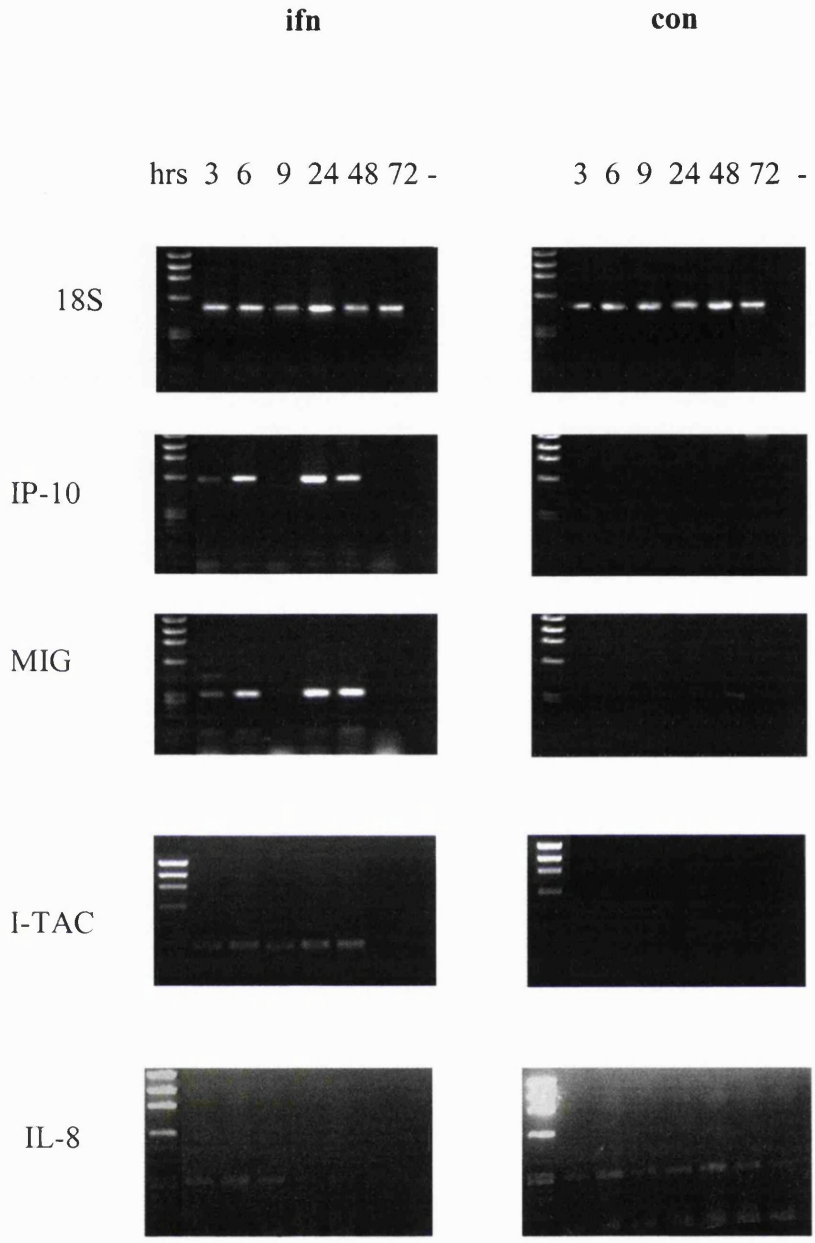


Figure 3.7: 18S, IP-10, MIG and I-TAC mRNA expression in H357 cells treated with IFN-γ for 3, 6, 9, 24, 48 or 72 hours (ifn) and control cells untreated (con) over the same time periods.

3.3.5 The profile of T cell chemotactic profiles in response to IP-10 and stromal derived factor-1alpha (SDF-1α)

The profiles of lymphocytes that demonstrated *in vitro* chemotaxis to IP-10 and a potent CXC chemokine SDF-1α are indicated in Fig.3.8a and b respectively. Both IP-10 and SDF-1α attracted lymphocyte populations above the rate of basal migration, including CD4+CD45RAlo cells (Fig. 3.8c). However, while both CD45RAhi and CD45RAlo cells in the CD4+ population were attracted to SDF-1α, predominately CD4+CD45RAlo cells migrated to IP-10 ($p<0.05$). This result thus suggests that IP-10 is involved in the recruitment of memory (CD45RAlo) CD4+ T cells to sites where it is expressed.

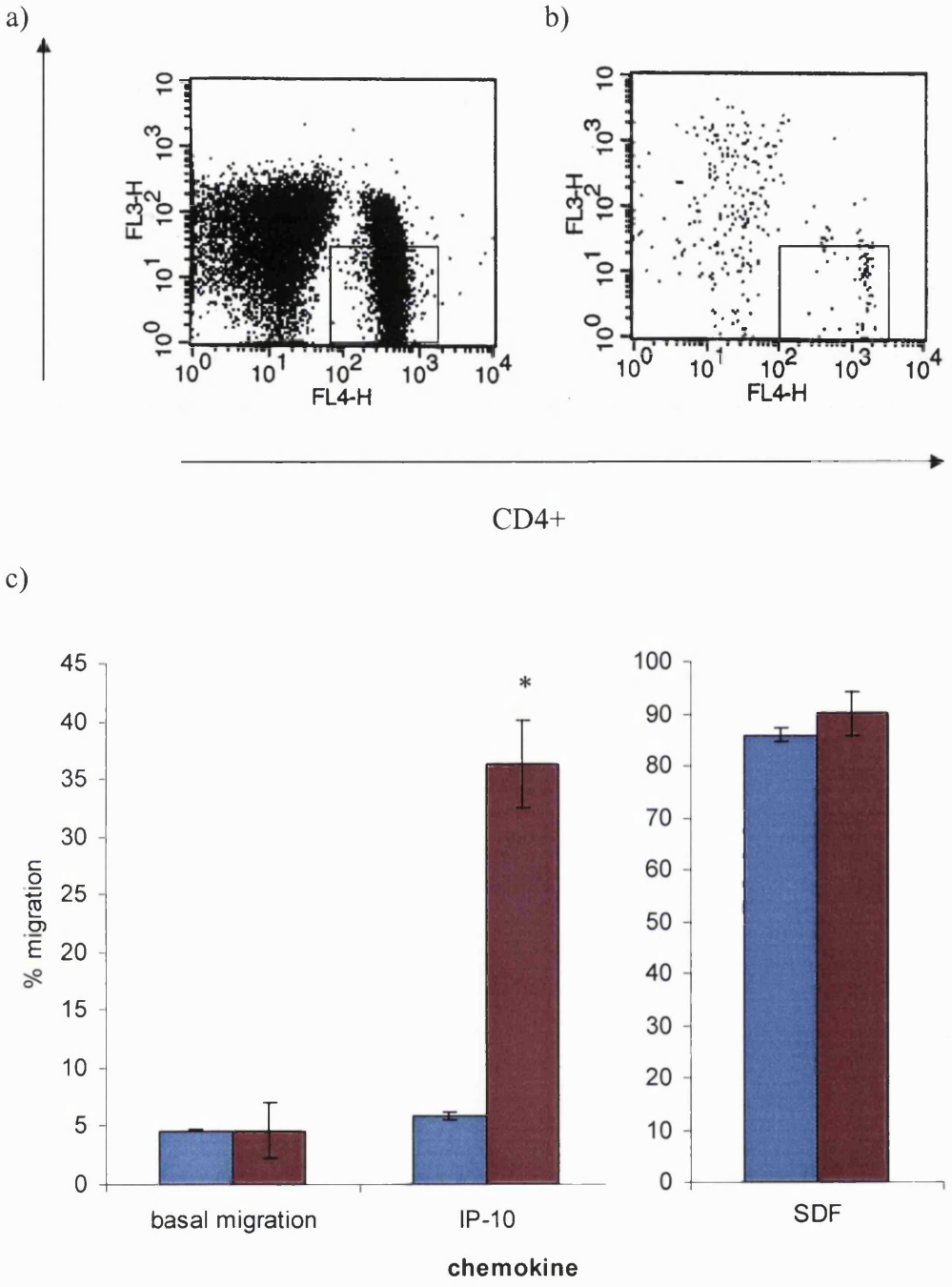


Figure 3.8: T cell chemotactic profile in response to IP-10 and SDF-1α. FACS profile of gated lymphocytes labelled with anti-CD4 and anti-CD45RA migrated to a) 100nM SDF1-α or b) 1μg/ml IP-10. c) The normalised % migration (to input PBMC) of CD45RAhiCD4+ (■) or CD45RAloCD4+ (■) to IP-10 and SDF1-α. Basal migration is the migration to cell culture medium alone. * represents a significant difference ($p<0.05$) between CD4+CD45RAhi and CD4+CD45RAlo.

3.3.6 Levels of IFN-γ, MIG, IP-10 and I-TAC mRNA in normal oral mucosa and oral lichen planus

IFN-γ mRNA

Using RT-PCR, there was no detectable expression of IFN-γ in any of the OLP or NOM tissue tested (Fig.3.9). In contrast the assay did detect the internal IFN-γ control.

MIG, IP-10 and I-TAC mRNA

mRNA of MIG, IP-10 and I-TAC were detected in all samples of OLP and oral mucosa (Fig. 3.10). The ratios of MIG, IP-10 and I-TAC mRNA detected in OLP and NOM tissue samples are shown in Fig 3.11. The greatest mean ratio of the CXC ELR-chemokines in OLP tissue compared to the adjusted 18S was MIG, followed by IP-10; I-TAC was only minimally expressed in OLP (Fig 3.11). All OLP samples expressed I-TAC without 18S / competitor, whereas only faint expression for I-TAC mRNA was detected in normal oral mucosa samples (Fig 3.10f). IP-10 and MIG were detected in healthy oral mucosa. The ratios of detected MIG, IP-10 and I-TAC mRNA were significantly higher in OLP than NOM tissue ($p<0.05$), however, there was considerable variation in expression between the different samples in each tissue group as shown by the large standard deviations measured.

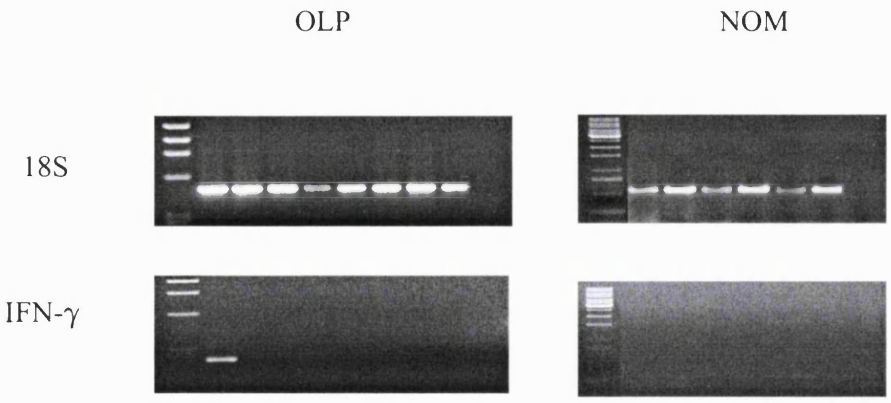


Figure 3.9: IFN-γ expression in OLP tissue (samples 1-7), NOM (samples 1-6) and positive control (+) (IFN-γ positive sample provided by Ambion) as measured by RT-PCR. IFN-γ mRNA amplicon is located at 241bp.

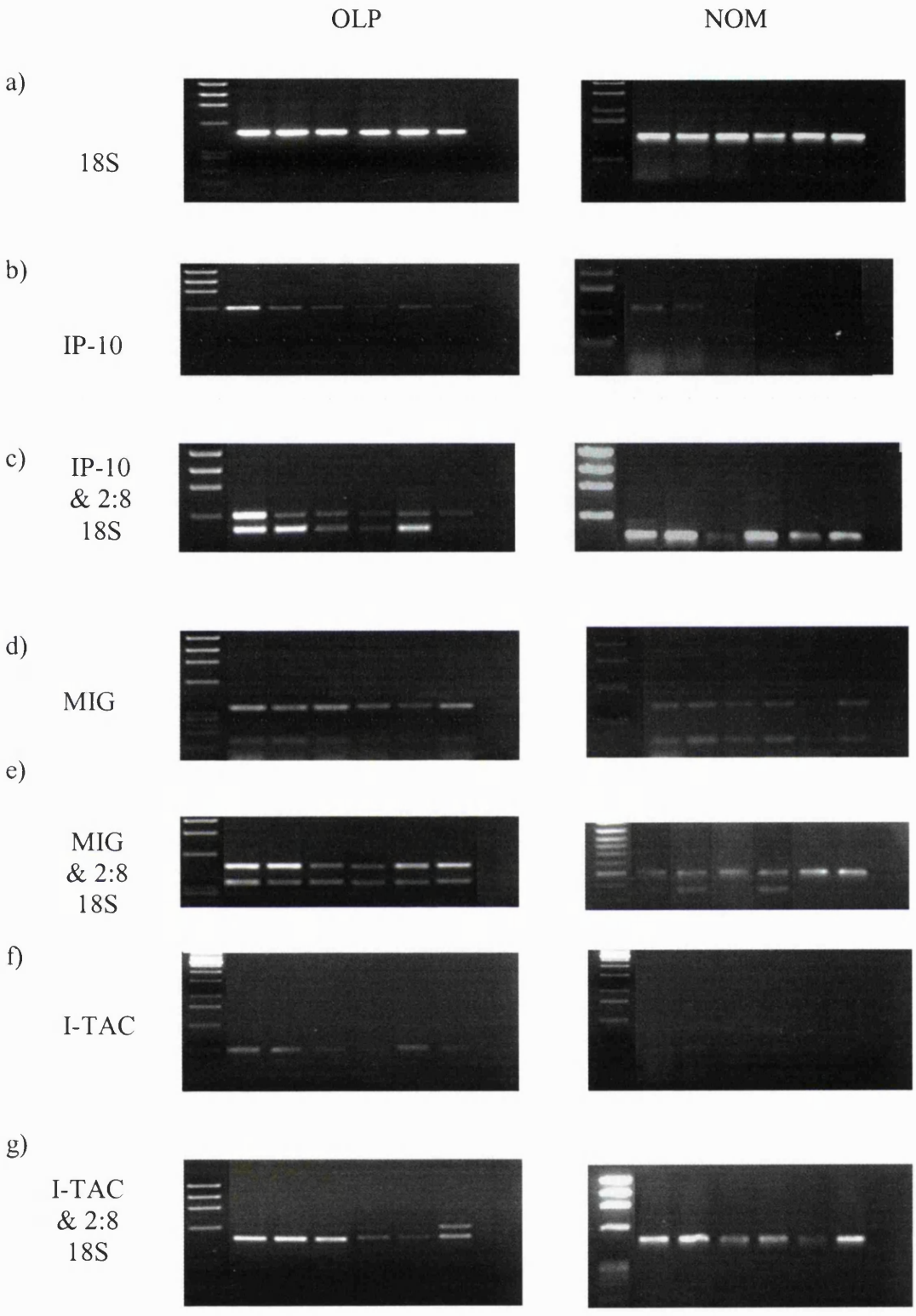


Figure 3.10: MIG, IP-10, I-TAC mRNA in oral lichen planus and normal oral mucosa. Semi-quantification of the CXC ELR- chemokines in oral inflammation. a) 18S, b) IP-10, d) MIG and f) I-TAC mRNA expression in 6 samples of oral lichen planus (OLP) and 6 samples of normal oral mucosa (NOM). c) IP-10, e) MIG and g) I-TAC mRNA expression for the same cases with a 2:8 ratio of 18S:competitor (2:8 18S).

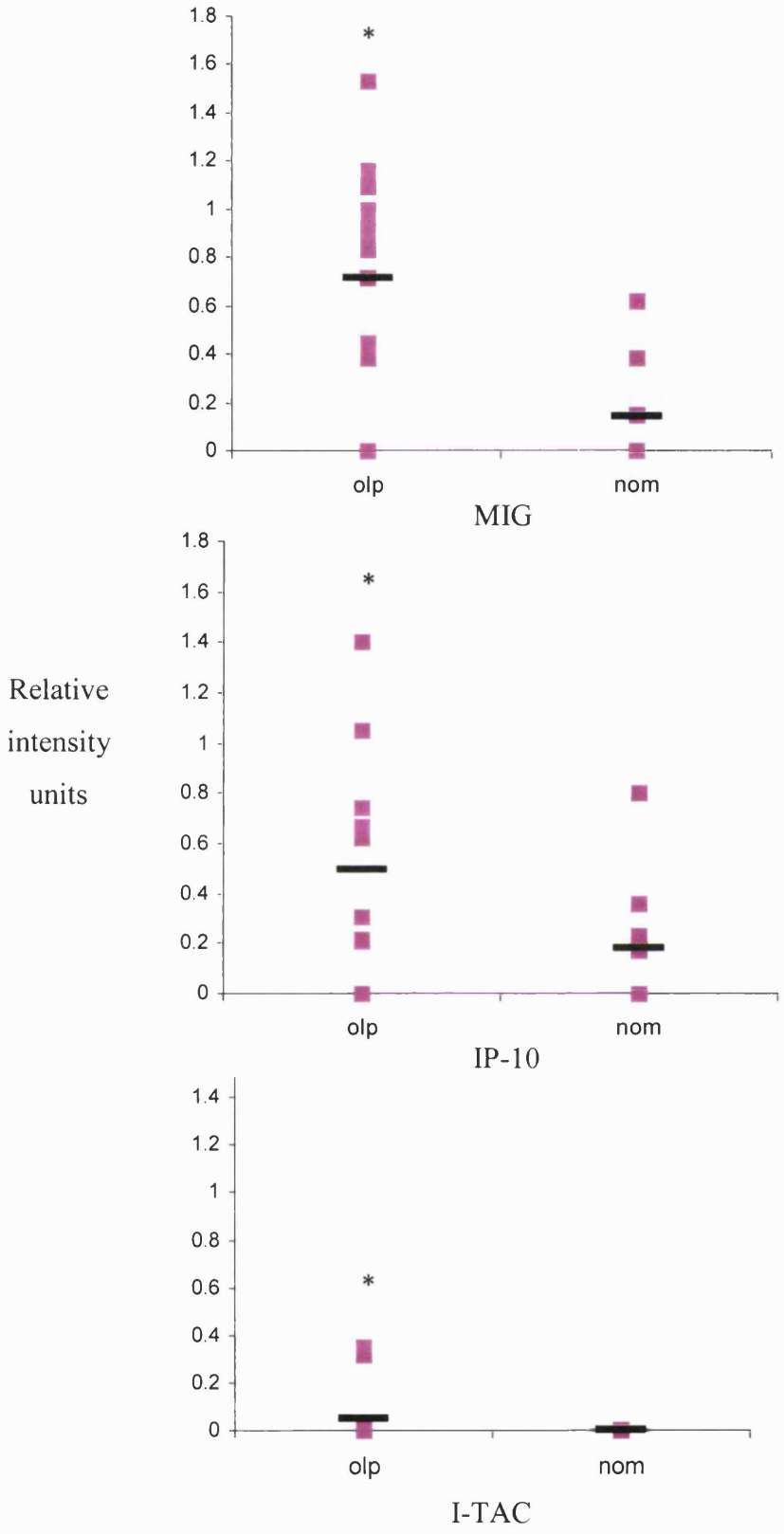


Figure 3.11: The semi-quantitative ratios of MIG, IP-10 and I-TAC mRNA expression in OLP (olp) and NOM (nom) tissue (normalised to 18S / 2:8 competitor mRNA expression). Mean mRNA expression in each tissue type is represented by . ▬ . Standard deviations for OLP: MIG ± 0.47, IP-10 ± 0.23, I-TAC ± 0.12 and NOM: MIG ± 0.24, IP-10 ± 0.27, I-TAC ± 0.0. * represents a significant difference ($p<0.05$) between OLP and NOM tissue.

3.3.7 The expression of CD40 mRNA by IFN-γ stimulated primary epithelial cells

CD40 was expressed constitutively in three different primary oral epithelial cultures tested. IFN-γ stimulation of the primary epithelial cell cultures increases expression of CD40 expression in each case (Fig 3.12).

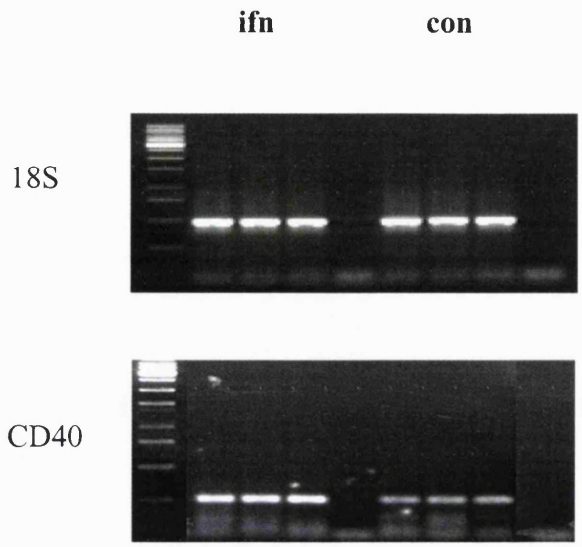


Figure 3.12: The expression of 18S and CD40 mRNA in three cell cultures of primary oral epithelial cells with or (ifn 1-3) without (con 1-3) IFN-γ treatment for 48 hours. – represents the negative control for this experiment.

3.3.8 Expression of CD40 and CD154 expression in OLP tissue

In sections of OLP lesional tissue, a concentrated area of CD40 immunoreactivity is associated with cells within the dense infiltrate, although staining of individual cells in proximity to the epithelium are also visible (Fig 3.13). Individual cells in the oral epithelium also demonstrate cell surface CD40 staining (Fig 3.14). Cells in the basal area appear to express relatively high expression, although positive cells are also present in the supra-basal area. Certain focal areas of the basal epithelium have particularly high expression levels. CD154 is expressed upon infiltrating cells in OLP lesions (Fig 3.15) and positive cells appear to be preferentially located near areas of epithelial cells, especially in focal areas of cell damage. In fact, some cells associated with CD154-posititivity appear to be associated with epithelial cell layers.

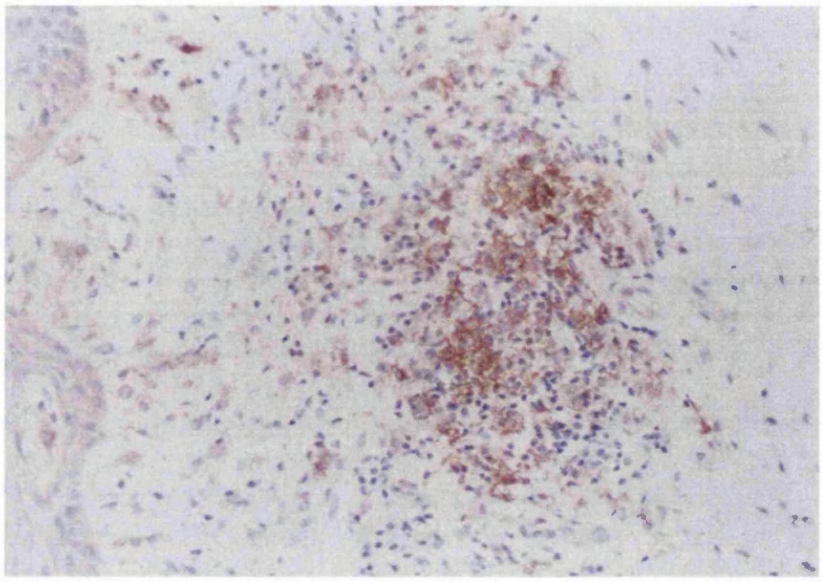


Figure 3.13: CD40 localisation in OLP tissue using peroxidase staining. Intense staining was associated with the dense infiltration of cells, however, staining can also be witnessed upon single cells in proximity to / and infiltrating the epithelial layer. Magnification x10. Isotype-matched antibody control sections demonstrated no staining.

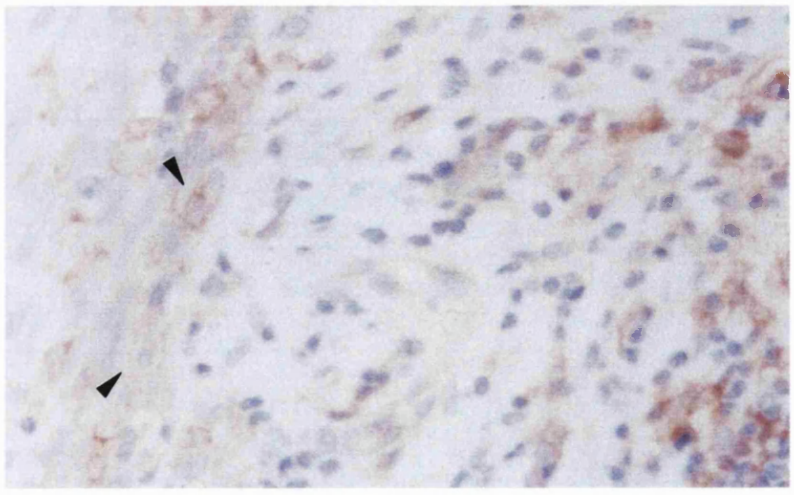


Figure 3.14: CD40 localisation associated with the epithelium in OLP tissue. Epithelial cells within OLP lesions appeared to demonstrate cell surface staining (▲). Magnification x40.

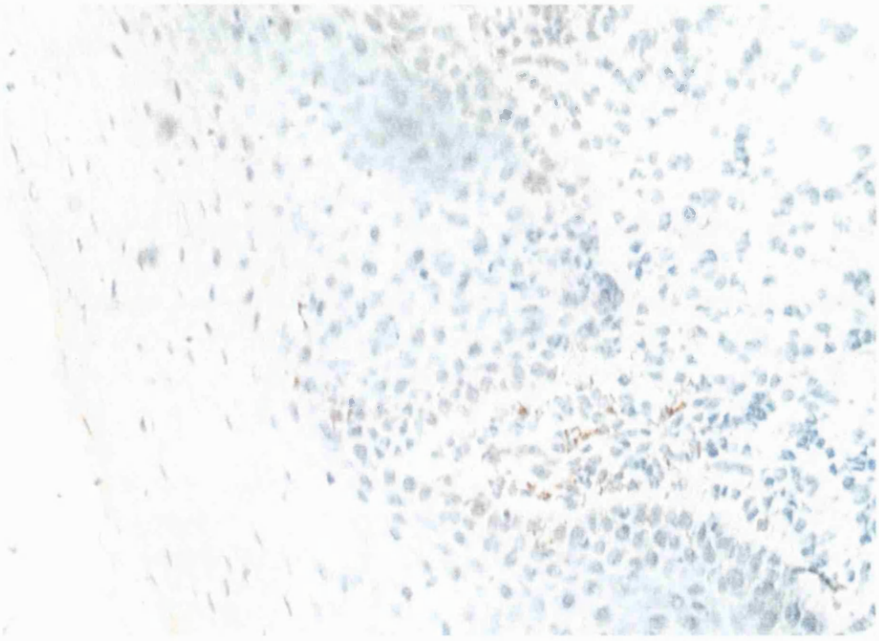


Figure 3.15: CD154 expression in oral lichen planus tissue. Infiltrating cells in proximity to the basal epithelium are associated with anti-CD154 positive staining. Magnification x20.

3.4 Discussion

This study sought to investigate the potential of oral epithelial cells to express and produce mRNA and protein respectively of the CXC ELR- chemokines. In addition it compared the expression of mRNA of MIG, IP-10 and I-TAC in oral mucosa and lesional tissue of oral lichen planus. The present study found in general that oral epithelial cells did not express significant mRNA or synthesise protein of CXC ELR- chemokines unless stimulated with IFN-γ, furthermore there was an upregulation of these chemokines in OLP. These findings may prove important as these chemokines are implicated in other cases of T cell immunopathogenic disorders and may be crucial for the recruitment of T cells that contribute to oral inflammation, including oral lichen planus.

This is the first study to demonstrate that epithelial cells of oral mucosal origin are capable of generation of the CXC ELR- chemokines MIG, IP-10 and I-TAC. Both MIG and IP-10 were highly induced in primary epithelial cells following IFN-γ treatment, however, IL-8, a CXC ELR+ chemokine, was not specifically induced in 2 of the 3 cell lines, thus suggesting that the oral epithelium is capable of directing CXC ELR- chemokine-mediated immune responses.

The present findings are in agreement with studies of other cell types, such as glomular cells (Romagnani *et al,* 2002), polymorphonuclear leucocytes (Gasperini *et al,* 1999), cervical carcinoma cell lines (Altenberg *et al,* 1999), bronchial epithelial cells (Sauty *et al,* 1999), intestinal epithelial cells (Dwinell *et al,* 2002) and cutaneous keratinocytes (Albenesi *et al,* 2000) all of which have been found to release IP-10 under IFN-γ stimulation. MIG production has also been demonstrated in various IFN-

γ-stimulated cell types, including intestinal epithelium (Dwinell *et al,* 2000) and cutaneous keratinocytes (Albanesi *et al,* 2000). Although not tested in this study, Tensen *et al,* 1999 also demonstrated that I-TAC protein can be extracted from stimulated cutaneous keratinocytes. The present study revealed that IFN-γ treatment alone was sufficient to induce IP-10 and MIG release from oral epithelial primary cells and thus accords with studies of other epithelial cell types, but contrasts that of some other cell types, such as neutrophils, which require both IFN-γ and TNF-α stimulation in order to produce MIG (Gasperini *et al,* 1999). The present observations suggest oral epithelial cells are responsive to IFN-γ stimulation only in order to induce CXC chemokine production. Although in comparison to some other studies, the concentration of IFN-γ used in this study was relatively high (1000U/ml) lower doses can be used stimulate the release of these chemokines in cutaneous keratinocytes (Albanesi *et al,* 2000), thus induction of CXC chemokine production by oral epithelial cells may be possible at lower levels of IFN-γ.

The present results demonstrate that the chemokines MIG, IP-10 and I-TAC are only produced to a minimal degree in unstimulated oral epithelial cells, but are induced after IFN-γ stimulation. These observations mirror those found in skin keratinocytes and bronchial epithelial cells (Sauty *et al,* 1999; Albanesi *et al,* 2000; Tensen *et al,* 1999). Intestinal epithelial cells (Dwinell *et al,* 2001) constitutively release these chemokines, hence oral epithelial cells seem to have more in common in terms of CXC ELR- chemokine production with the skin or bronchial epithelium than gut epithelium.

The concentration of IP-10 induced by IFN-γ was significantly higher than the concentration of MIG produced from all of the primary oral epithelial cell lines. This is a pattern similar to that observed in one study of skin keratinocytes stimulated, in which equal amounts of MIG and I-TAC are produced, although levels of IP-10 protein production are 40 times those of the other two CXC ELR- chemokines (Tensen *et al,* 1999). However, in a further investigation, the level of MIG produced by IFN-γ-stimulated cutaneous keratinocytes was slightly lower than IP-10 (Albanesi *et al,* 2000). It would thus appear that oral epithelial cells can produce CXC ELR- chemokines *in vitro* and thus have the potential to play a role in the pathogenesis of OLP.

The premise of CXC ELR- chemokine production by oral epithelial cells was further confirmed by the observation of expression of mRNA of IP-10, MIG and I-TAC by IFN-γ-stimulated oral epithelial cells. mRNA of all 3 CXC ELR- chemokines was induced, or enhanced, in primary oral epithelium by treatment with IFN-γ. In contrast, IL-8 mRNA (a CXC ELR+ chemokine) was not specifically induced nor enhanced. This IFN-γ-enhanced expression of IP-10 and MIG mRNA has also been observed in renal glomelular cells (Romagnani *et al,* 2001), fibroblasts (Meyer *et al,* 1999), bronchial epithelial cells (Sauty *et al,* 1999) and cutaneous epithelial cells (Albanesi *et al,* 2000). Likewise, in the present study I-TAC mRNA was induced in primary oral epithelial cells by IFN-γ, in common with monocytes, astrocytes (Cole *et al,* 1999), endothelial cells (Mazanet *et al,* 2000), cutaneous keratinocytes (Tensen *et al,* 1999; Albenesi *et al,* 2000) and bronchial epithelial cells (Sauty *et al,* 1999). However, I-TAC mRNA appeared to be expressed at a lower level in the IFN-γ stimulated oral primary keratinocytes than MIG and IP-10 mRNA. This differential expression of

CXC ELR- chemokines has been observed with other cell types. In bronchial epithelium I-TAC mRNA is hardly detectable after IFN-γ stimulation alone (Sauty *et al,* 1999) while in murine fibroblasts IP-10 mRNA is expressed at a higher level than I-TAC mRNA (Meyer *et al,* 2001). The comparative lower expression of I-TAC would suggest that I-TAC protein would also be produced at lower levels than IP-10 in oral epithelial cells, in agreement with the observation of cutaneous keratinocyte supernatants (Tensen *et al,* 1999), although not investigated in the present study due to a lack of commercial antibodies for I-TAC.

IP-10 mRNA transcript was detected in oral epithelial cells without IFN-γ stimulation, and only low levels of IP-10 protein were produced. These observations also accord with findings of cutaneous keratinocytes *in vitro* (Boorsma *et al,* 1998).

The observation of CXC ELR- mRNA demonstrates that IFN-γ stimulation does lead to an up-regulation of MIG, IP-10 and I-TAC expression, and along with the observations of enhanced chemokine production reveals that oral epithelial cells are capable of then producing active chemokine proteins. To determine how quickly this IFN-γ stimulation would influence chemokine expression and production the temporal aspects of MIG, IP-10 and I-TAC were examined. As primary oral epithelial cells have a limited passage, H357 oral squamous cell carcinoma cells were utilised for these studies. The concentration of IP-10 and MIG produced by H357 cells was within the range of primary oral epithelial cells production at the same time-point, suggesting these cells were an adequate model for the study of these chemokines. Although, of course, it is possible that the dynamics of production between the two cell types could still differ over time.

A peak of high levels of both IP-10 and MIG protein were generated after 48 hours of IFN-γ stimulation, however, there was an increased production of IP-10 after only 6 hours stimulation. In contrast, IFN-γ incubation did not increase IL-8 production at any time-point studied. This pattern correlates with the protein production by IFN-γ-stimulated bronchial epithelium (Sauty *et al,* 1999). Likewise, IP-10 protein increased after IFN-γ treatment in neutrophils for up to 42 hours (Gasperini *et al,* 1999). In cutaneous keratinocytes, there is an increase of IP-10 and MIG protein in the supernatant up to 72 hours, however, the rate of increase in protein production decreases after a 42 hour time-point (Tensen *et al,* 1999).

In the present study of oral epithelial cells the production of IP-10 after IFN-γ treatment was higher than MIG at all time-points in our study – similar to the findings for the primary cells. The present observations of oral epithelial cells mirror those of cutaneous keratinocytes where MIG is not produced as early as IP-10, and MIG is also produced at lower concentrations at all time-points (Albanesi *et al,* 2000). However in the latter cell type MIG is produced at levels which are closer in relation to IP-10.

The reasons for the lower quantities of MIG production by oral than cutaneous epithelial cells are unclear. There appears to be redundancy within the chemokine / chemokine receptor system, with more than one chemokine being able to bind a single, shared receptor (Devalaraja & Richmond, 1999). MIG, IP-10 and I-TAC all appear to have a similar effect on the chemotaxis of CXCR3-positive cells except from the fact that I-TAC is more potent than IP-10 and MIG (Meyer *et al,* 1999). It

may be that there are different regulatory mechanisms for each of the chemokines in different cell types to prevent the overproduction of all 3 chemokines, which could result in uncontrolled inflammation. Indeed, I-TAC is highly effective at promoting trans-endothelial migration (Mohan *et al,* 2002) and it may be that the expression of I-TAC in endothelial cells (Mazenet *et al,* 2001) is the main role of this chemokine, whereas, from our studies IP-10 appears to be the chemokine that is highly expressed in the oral epithelium and therefore, perhaps plays an important role in the migration within the oral micro-environment.

In the present study IFN-γ did not stimulate IL-8 production in most cell lines tested. Other similar studies have found that IFN-γ does not increase the production of IL-8 from oral epithelial cells (Li *et al,* 1996) nor oral carcinoma cell lines (Watenabe *et al,* 2001). However, IFN-γ can prime or enhance IL-8 production from such cells after further inflammatory signals, such as TNF-α or LPS (Li *et al,* 1996; Uehara *et al,* 2002) and may potentially still play a role in IFN-γ induced inflammation.

In the present study, there was an early induction of IP-10, MIG and I-TAC mRNA in H357 cells after only 3 hours of incubation with IFN-γ, with the expression increasing until 24 hours. Hence, at least *in vitro,* IFN-γ exerts a rapid effect upon relevant chemokine production. Such rapid induction of all 3 CXC ELR- chemokine mRNA has been observed in bronchial and cutaneous epithelial cells (Sauty *et al,* 1999; Albanesi *et al,* 2000; Tensen *et al,* 1999). The observed peak of CXC ELR-chemokine mRNA transcription at 24 hours is likewise in agreement with the findings of IFN-γ-stimulated endothelial cells (Mazenet *et al,* 2002), however, mRNA appears to decrease in bronchial cells at 8 hours and cutaneous keratinocytes after 12 hours of

stimulation (Albanesi *et al,* 2000). However, the study of cutaneous keratinocytes stimulated the cells with only 200U/ml IFN-γ (compared to 1000U/ml in the present study) and hence may account for the increased concentration observed in the present work. Alternatively, this difference may reflect the different sensitivity to IFN-γ in cutaneous and oral epithelium, as previously reported in other studies (Li *et al,* 2000). In agreement with the observations of skin keratinocytes (Albanesi *et al,* 1999), the pattern of MIG expression was similar to that of IP-10.

In the present study I-TAC mRNA expression was lower than that of MIG and IP-10. However, this chemokine has a more potent migratory influence upon CXCR3-bearing T cells than IP-10 and MIG (Tensen *et al,* 1999; Cole *et al,* 1998), and potentially even this limited mRNA expression maybe relevant in inducing migration of CXCR3 cells, and thus influencing local oral inflammation.

To confirm that IP-10 does indeed have a chemotactic action and thus a potential role in recruiting inflammatory cells in oral mucosal disease, such as lichen planus, the potential of IP-10 to induce chemotaxis was evaluated. The present results demonstrate that IP-10 was capable of recruiting a large percentage of CD4+ T cells with memory cell characteristics (CD4+CD45RAlo) from peripheral blood, but has a lesser ability to cause the chemotaxis of 'naïve' CD4+ T cells (CD4+CD45RAhi). These observations confirm those of other studies that found CXCR3 is predominately expressed upon memory CD4 cells (and naïve and memory CD8+ cells) (Rabin *et al,* 1999). Hence, even if IP-10 is only produced in small amounts local IP-10 production in the oral epithelium is likely to attract cells with memory T cell characteristics into the oral epithelium, and thus may contribute to inflammatory conditions which are

characterised by memory CD4+ T cell infiltration, for example, oral lichen planus. Moreover, CXCR3 is predominately expressed on activated memory (CD45RO+) T cells (Loestcher *et al,* 1998), which is also the main phenotype within the T cell infiltrate of OLP (Scully *et al,* 1998). This suggests that IP-10 could indeed play a role in the migration of the immunological infiltrate of OLP.

Also, although not investigated in the present study, IP-10 is effective at attracting CD8+ cells (Sebastini *et al,* 2002), considered in some studies to be the dominant T cell type in OLP (Khan *et al,* 2003), and located adjacent to the epithelial cells. This may crucial in a condition which may be caused by cytotoxic CD8+ cells. In fact, CXCR3 signalling appears to be involved in the activation and proliferation of CD8+ cells, suggesting that these chemokines also play an important role in CD8+ immune responses (Ogasawara *et al,* 2002).

The data thus presented suggests that the oral epithelium is capable of generating chemokines that may affect lymphocyte migration to the oral mucosa to cause inflammation. To establish if such *in vitro* observations are relevant to *in vivo,* the expression of MIG, IP-10 and I-TAC in oral lichen planus lesional tissue and normal oral mucosa was thus examined.

This study found that MIG, IP-10 and I-TAC chemokine expression are all up-regulated in oral lichen planus compared to normal oral mucosal tissue. MIG had the highest level of expression compared to 18S in OLP lesional tissue, whereas I-TAC was weakly expressed compared to MIG and IP-10. This study was the first to

compare these chemokines in OLP using semi-quantitative analysis, and the first study to focus on all 3 CXC ELR- chemokines in oral inflammation.

The present *ex vivo* observations of oral lichen planus accord well with previous immunohistochemical studies of cutaneous lichen planus (Spandau *et al,* 1998; Tensen *et al* 1999; Flier *et al,* 1999), in which MIG was found to be the dominant chemokine present, with I-TAC being only present weakly. In the studies of cutaneous lichen planus MIG was mainly found to be present in the dermal infiltrate, whereas IP-10 and I-TAC was produced in keratinocytes adjacent to the infiltrate. The present observations of chemokine production perhaps mirror those of cutaneous lichen planus as high levels of IP-10 were produced by activated oral keratinocytes whereas MIG was only found at low levels in the same cell type. As previously discussed, despite the fact that the level of I-TAC mRNA is expressed at low levels in both OLP tissue and activated oral keratinocytes, this chemokine may still be relevant in oral inflammation, as it has been found to be more potent than IP-10 or MIG at attracting CXCR3-bearing cells (Tensen *et al,* 1999).

The contribution that chemokines make to different oral disorders may vary. In periodontitis, IP-10 is rarely expressed by oral epithelial cells (Gemmell *et al,* 2001). This differential expression of chemokines in the 2 different oral disorders would suggest there is a recruitment of different cell types, and this is probably reflecting different aetiological factors. However, a study of inflamed gingival tissue found that there were infiltrating CXCR3 cells present (Kabashima *et al,* 2002), suggesting that these cells can play a role in plaque-related oral inflammation.

Of great relevance, the pattern of chemokine expression in OLP observed in the present study is similar to that of allergic contact dermatitis (Qin *et al,* 1998), a reaction similar to the "contact hypersensitivity" reactions of the lichenoid reactions (Scully *et al,* 1998). In addition, IP-10 knock-out mice exhibit an impaired contact hypersensitivity response with reduced inflammatory cell infiltration (Dufour *et al,* 2002). Thus as IP-10 is produced in OLP this disorder may well reflect a hypersensitivity reaction to as yet an unknown trigger. The expression of CXCR3+ T cells appears to be protective against allergies to environmental antigens, such as grass pollen (Campbell *et al,* 2002), so perhaps an unfettered 'protective' Th1 response to an environmental allergen may cause an increase in CXCR3+ cells and causes the response that occurs in OLP. However, there is a decrease in MIG expression in the plasma of individuals with type I allergic disease (Campbell *et al,* 2002). More relevant to OLP, MIG and IP-10 and I-TAC are expressed in inflammatory skin conditions with contact allergens (i.e. type IV hypersensitivity), but not in skin conditions in contact with irritants (Flier *et al,* 1999). In these contact dermatitis conditions, IP-10 is expressed in the epidermis and dermis, whereas MIG is mostly expressed by the cells in the dermis, similar to the condition in lichen planus, and corresponding with our findings in oral epithelium. This suggests that if OLP is caused by contact with a substance, it is likely that this is a contact allergen opposed to an irritant substance.

As recent theories suggest that OLP may have a cell-mediated autoimmune basis, it is interesting to note that the expression of these chemokines have also been associated in other T cell-mediated autoimmune reactions. In a murine model of type IV autoimmune disease the production of these chemokines appears to reduce after 24

days which corresponds to the destruction of the target organs in this condition (Goebeler *et al,* 2001), however, in oral lichen planus these chemokines are highly expressed even after an extended period (see 2.1 for OLP patient profiles) suggesting that the induction of these chemokines is still occurring.

Furthermore, supernatants from OLP-derived keratinocytes are known to induce increased migration from peripheral blood mononuclear cells than keratinocytes from normal patients (Yamamoto *et al,* 2000) and it is possible that some of the chemokines responsible for this migration are the CXC ELR- chemokines. However, the influence of other epithelial-derived chemokines on the migration of other leukocyte subsets in OLP may also play a large role. It is known that mast cells in OLP produce RANTES (Zhao *et al,* 2001) and recently Little *et al,* 2003 found RANTES was produced by keratinocytes in OLP. The production of this chemokine would probably cause the specific migration of cells bearing the chemokine receptor CCR5 to this area. The presence of CCR5+ and CXCR3+ cells are often associated with the same inflammatory reactions (Qin *et al,* 1998), therefore the combination of RANTES with MIG, IP-10 and ITAC in the same disease is not surprising.

The present study thus has demonstrated that there is an up-regulation of MIG, IP-10 and I-TAC in OLP compared to normal oral mucosa, which suggests that these chemokines are playing a role in the continued migration of the memory T cells that infiltrate OLP lesions. Although MIG was the dominant chemokine expressed in these lesions, I-TAC is the most potent of the three chemokines at attracting CXCR3 cells and therefore is perhaps still influential in this disease. Furthermore, IP-10 was produced in large quantities by the oral epithelial cells and suggesting that this

chemokine could have a role in the accumulation of T cells in the band-like infiltrate below the oral epithelium (Fig 3.16).

The induction of these chemokines appears to be induced by a small level of IFN-γ, or by another stimulant, as it could not be detected in OLP tissue in this study. However, only small doses of IFN-γ are required to induce these chemokines experimentally in bronchial epithelial cells (Sauty *et al,* 1998) and it may be that small amounts are present, but are below the detectable level of RT-PCR (minimum dose of IFN-γ detectable ranges from 0.5 - 1.5 amol/mL (R&Dsystems)).

In a murine model of autoimmune disease the expression of IFN-γ did not correspond with the onset of MIG and IP-10 expression, suggesting that either other factors are responsible, or that in both cases the assay utilised is not responsive enough to detect the IFN-γ produced (Meyer *et al,* 2001). A study quantifying IFN-γ mRNA in murine experimental allografts showed that IFN-γ expression was at least 1,000 fold less than the three CXCR3 binding chemokines (Meyer *et al,* 2001). Furthermore, a study by Simark-Mattson *et al,* discovered that less than 1% of the OLP infiltrate produced IFN-γ, however, these cells were located crucially at the dermal/ epidermal interface, where they may be in a position to induce CXC ELR- chemokine production from oral keratinocytes. Indeed there is evidence of the presence of IFN-γ in this area due to induction of ICAM-1 and HLA-D on oral keratinocytes in OLP (Farthing *et al,* 1992).

Despite a finding that mononuclear cells expressed IFN-γ in OLP (Khan *et al,* 2003), in cutaneous oral lichen planus, it was found that all the IFN-γ producing cells were

CD3+. As IFN-γ production is a characteristic feature of Th1 cells, the fact that Th1 type cells are located in this region leads to the possibility that these cells are CXCR3+ cells recruited by the CXC ELR- chemokines. Furthermore, IP-10, MIG and I-TAC have shown to be antagonists for CCR3-bearing cells (Loetscher *et al,* 2001), a receptor associated with Th2 cells, which could further skew the reaction in OLP to a Th1 reaction. These recruited Th1 cells could in turn induce the production of more chemokines through IFN-γ production leading to the exacerbation of the lesions. Interestingly, IL-8 is not expressed by keratinocytes in OLP (Little *et al,* 2003) and as present results confirm that IFN-γ does not upregulate this chemokine in oral keratinocytes, the lack of this chemokine in OLP further implicates IFN-γ in the induction of chemokines in OLP.

Furthermore, as IFN-γ is induced by IL-12 stimulation (Chatterjee *et al,* 1995), the production of this cytokine may play a key role in the pathogenesis of OLP. IL-12 can be produced by antigen presenting cells and keratinocytes in allergic contact dermatitis (Yawalkar *et al,* 2000), and although expression has not been investigated in OLP it would appear likely it is also expressed by these cell types in OLP. In addition, IL-18, a cytokine produced by keratinocytes (Koizumi *et al,* 2001) can enhance IL-12 production mediated IFN-γ production in lymphocytes. Therefore, in OLP the production of IL-18 by keratinocytes may cause a localised enhanced production of IFN-gamma in vicinity to the epithelium. Furthermore, neutralisation of IL-12 in murine leishmania could reduce IP-10 production (presumably by reducing IFN-gamma production) (Zaph & Scott, 2003) and may thus may provide a possible therapy in OLP.

Another study suggested that OLP was not characterised by Th1 cytokines, but contained a mixture of Th1/ Th2 cytokine-producing cells (Simark-Mattson *et al,* 1999). In fact, TARC, a chemokine which is associated with the migration of Th2 cells, is expressed by the endothelium in cutaneous lichen planus (Campbell *et al,* 1999) (although it is not known if this chemokine can be expressed in the oral cavity). It may be that the specific recruitment of Th1 cells from within the T cell infiltrate to the epithelial border may be crucial to the further induction of IP-10 and I-TAC production by the epithelium, although recent studies have also shown that Th2 derived supernatant can also induce the production of IP-10 from keratinocytes (Albanesi *et al,* 2001), although to a lesser degree.

The other possibility is that these chemokines are being up-regulated in the presence of a different stimulant, as it has been reported that IL-4 (Albenesi *et al,* 2000) or TNFα and IL-1β (both without IFN-γ) are sufficient to generate these chemokines in some cell types, although TNF-α and IL-1β treatment was not sufficient for the stimulation of IP-10 mRNA in keratinocytes (Boorsma *et al* 1998). The presence of these cytokines has also been reported in OLP (Simark-Mattson *et al,* 1999), but it remains to be investigated whether the CXC ELR- chemokines could be induced in the oral cavity without the presence of IFN-γ.

Interestingly, IP-10 and MIG have been found to have additional roles aside from the promotion of chemotaxis. They are especially noted for their angiostatic properties (Angiolillo *et al,* 1995; Streiter *et al,* 1995), whereas there is proposed to be an increased vascularity in oral lichen planus (Regezi *et al,* 1995). This may be caused by the influence of other cytokines, such as vascular-endothelial growth factor

(VEGF), which reported to have an effect upon the vascularity (Deitmar *et al*, 1998). However, the presence of VEGF in OLP has never been reported previously. Incidentally, the production of IP-10 and MIG has been correlated with a regression of tumours in mice partially through the reduced blood supply to the tumour (Oppenheim *et al,* 1997). The fact that we have shown that these chemokines can be induced by an oral carcinoma cell line, suggests that the upregulation of these chemokines could be a potential therapy for oral epithelial cancers.

These chemokines are also noted to have antimicrobial activity, in a mechanism similar to that of the defensins (Cole *et al,* 2001). As Thornhill, 2001 proposes that an autoimmune reaction could possibly be caused by local food antigens or the microbial flora, it is interesting to speculate that these chemokines maybe induced initially for their function as antimicrobial agents in OLP, which, however, through the chemotactic effect of these chemokines leads to the large T cell infiltrate witnessed.

IP-10 and MIG are also proposed to have a role in the latter stages of wound healing (Engelhardt *et al,* 1998), correlating with a lymphocyte infiltration peaking at 14 days after wound damage. It is possible that these chemokines are expressed in a wound healing process of damaged keratinocytes in OLP. If wound healing was the cause of IP-10 and MIG upregulation in OLP, there must be either continued damage to keratinocytes by some method, such as cytotoxic autoreactive T cells, or unregulated production of these chemokines. The fact that RANTES is not associated with the wound healing process (Engelhardt *et al,* 1998), whereas it is expressed in OLP (Zhao *et al,* 2001) suggests that other factors rather than participation in wound healing is causing CXC ELR- chemokine up-regulation.

Recently there has been shown to be suppression of adjuvant arthritis after blocking IP-10 function (Salomon *et al,* 2002), and therefore there could be potential reduction in inflammation in diseases where these chemokines are upregulated by blocking these chemokines.

In contrast to oral epithelial cells, the present results indicate that there is constitutive CXC ELR- mRNA expression in normal oral mucosa. Perhaps other cell types in the mucosa express these chemokines or there is an induced production in oral epithelium from a few different stimuli. However, the presence of these chemokines in normal oral tissue is perhaps not surprising as findings these chemokines have been detected in uninflamed oral gingival tissue (Gemmell *et al,* 2001) and the mouth has a significant microbial flora which may be capable of inducing inflammation with great ease.

There is a strong intensity of CD40 staining in OLP especially upon cells within the infiltrate. Furthermore, CD40 expression can be witnessed in association with oral epithelial cells, this expression appears to be upon epithelial cells but is not confirmed in this study. CD40 has been reported in other epithelial cell types (Iwata *et al,* 2002; Gallacher *et al,* 2002; Dimitriou *et al,* 2002; Tanaki *et al,* 2001) and does appear to increase during other inflammatory disorders. Furthermore, it could be demonstrated that CD40 expression is increased on primary oral keratinocytes after IFN-γ incubation, in accordance with other studies (Farmer *et al,* 2001), again implicating this cytokine in the stimulation and inflammation of oral epithelium.

T cells in OLP appear to be in an activated state and would thus be expected to express CD154 (Ford *et al,* 1999), the ligand for CD40. The proximity of infiltrating T cells and epithelium in OLP, which can extend to interactions between T cells and the epithelium in some cases, would suggest that ligation of CD40 and CD154 would occur in this disease. This ligation has been shown to induce / enhance a number of effects in epithelial cells, including evidence of an increase in the production of pro-inflammatory cytokines (Weiler *et al,* 2001; Gallacher *et al,* 2001) and chemokines (Li *et al,* 2002; Gallacher *et al,* 2001; Li *et al,* 2001; Propst *et al,* 2000). It is likely that an increased production of these molecules from the epithelial area in OLP would increase the inflammation that occurs. Specifically, the increased expression of the CXC ELR- chemokines witnessed in OLP (see Fig 3.11) may be partially due to the ligation of CD40 on oral epithelial cells, as previously demonstrated by cervical carcinoma cells (Altenberg *et al,* 2001). Furthermore, the increased production of RANTES by keratinocytes observed in OLP (Little *et al,* 2003) could also be influenced by CD40 ligation (Propst *et al,* 2000; Woltman *et al,* 2000).

IFN-γ may also play an important role in contributing to inflammation in oral epithelium as it can increase the expression of CD40 on epithelial cells, as well as inducing the expression of chemokines such as IP-10. The production of the CXC ELR- chemokines presumably promotes the migration of activated T cells into the area, which may interact with epithelial cells and bind CD40. This would in turn synergise to produce more chemokines and cause an increase in the inflammatory status of conditions such as OLP (Fig 3.16). In fact, CD40 engagement of keratinocytes in murine models of contact hypersensitivity display an exacerbated

response (Fuller *et al,* 2002), perhaps through the increased pro-inflammatory cytokine and chemokine production that occurs after ligation.

Additionally, ligation of CD40 on epithelial cells causes growth inhibition and increased apoptosis of cells in ovarian carcinoma cell lines (Gallacher *et al,* 2001), which is interesting due to the increased apoptosis of keratinocytes in OLP. Therefore, CD40 ligation may be involved in this process.

Interestingly, CD40 ligation of antigen presenting cells plays an important role in the generation of CTL cells (Ito *et al,* 2000; Lefrancois *et al,* 2000). Therefore, maybe if keratinocytes are presenting antigen through the MHC-Class II pathway, the ligation of CD40, which is classically associated with antibody-mediated reactions, not only amplifies the inflammation of the area, but may be also involved in the generation of specific CTL. As OLP is currently proposed to be caused by a CD8+ T cell mediated auto-reactive reaction, this ligation may be crucial.

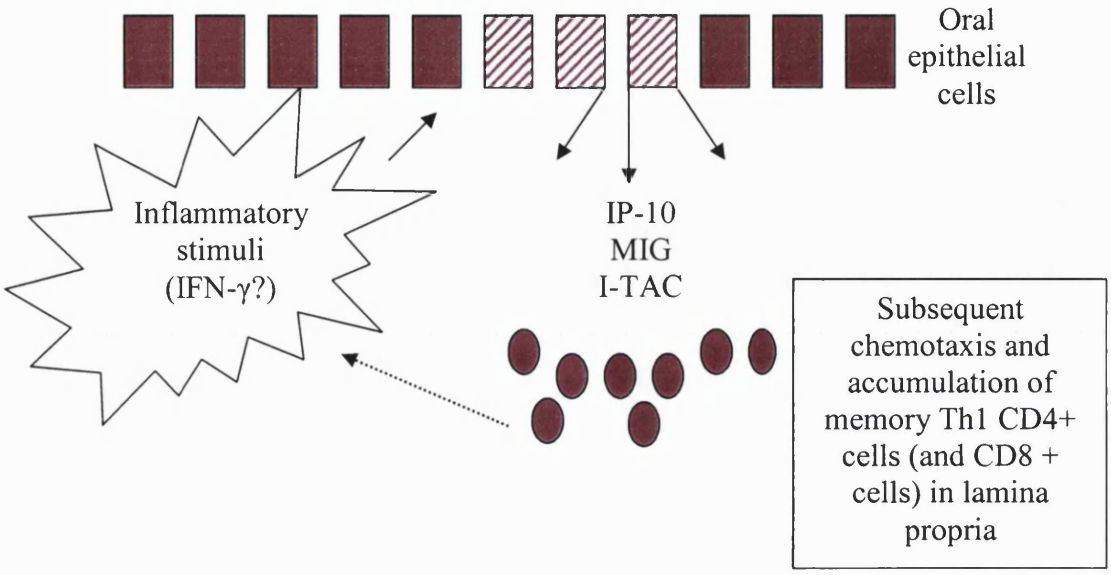


Figure 3.16: The potential role of epithelial cell-produced CXC ELR- chemokines in OLP. The oral disorder is perhaps initially triggered by an inflammatory stimuli which induces IFN-γ production. This, in turn may induce CXC ELR- chemokine production from affected oral epithelial cells ▨ (particularly IP-10?) which would presumably recruit large numbers of memory Th1 CD4+ cells (and CD8+ cells) in a band-like pattern in the oral lamina propria. Th1 cells are capable of producing inflammatory cytokines, such as IFN-γ, which may further stimulate the release of these chemokines from the epithelium, causing an exacerbation of the lesions.

Chapter 4: The antimicrobial activity of IP-10 and MIG in the oral mucosa

4.1 Introduction

The role of chemokines in directing the migration of specific cell populations has been well established (see Chapter 3). It has recently been demonstrated that some chemokines may also exhibit anti-bacterial and / or anti-fungal activities (Reviewed by Dürr & Peschel, 2002). Unlike some CXC ELR+ chemokines, all of the CXC ELR- chemokines have been shown to display antimicrobial effects upon the bacteria *Escherichia coli* and *Listeria monocytogenes* (Cole *et al,* 2001). RANTES, a CXC ELR+ chemokine, also has antimicrobial properties (*E. coli* and *Staphylococcus aureus*) (Tang *et al,* 2001) and also may be antifungal (for example, towards *Candida albicans*) (Tang *et al,* 2001). Furthermore, it has been recently shown that the tissue-specific chemokine MEC shares this antimicrobial / fungal activity against a range of bacteria, and despite the structurally similar CTACK acting less effectively against bacteria it could also kill *Candida* at high concentrations (Hieshima *et al,* 2003). Antimicrobial properties have also been previously described for MIP-3α (against *E.coli* and *S.aureus*) (Hoover *et al,* 2002).

The mechanism of antimicrobial action is still debatable, whereas the CXC ELR- chemokines share structural similarities with the defensins (Cole *et al,* 1999), MEC and CTACK contain structural similarities with histatin-5 (Hieshima *et al,* 2003), an anti-candidal protein found in saliva. However, the finding that MIP-3α, a chemokine which does not share these structural aspects, also has anti-microbial properties casts doubts on whether these structural similarities are involved in the antimicrobial action (Hoover *et al,* 2002). However, it has been demonstrated that incubation with MEC

produces pores in *Candida* (Hieshima *et al,* 2003), thus killing these organisms in a defensin-like manner.

Bacteria colonise all surfaces of humans, but are particularly dense in the oral cavity and lower gastrointestinal tract (Loesche, 1994). It has previously been shown that bacteria can adhere and invade (for example, *Actinobacillus actinomycetemcomitans* (Meyer *et al,* 1991)) host oral epithelial cells, furthermore, there are bacterial receptors present in saliva that can be absorbed onto oral mucosal surfaces (Henderson *et al,* 1998).

The toll like receptors (TLR) expressed on host cells are involved in the recognition of conserved bacterial patterns (Akira *et al,* 2001), for example, the cell wall component of gram negative bacteria, lipopolysaccharide (LPS) (Erridge *et al,* 2002). It is also known that LPS is capable of inducing a range of cytokines and chemokines from epithelial cells (Rouablina *et al,* 2002), predominately by signalling through TLR-4 (Fig 4.1). Furthermore, it is known that the CXC ELR- chemokines can be induced in some cell types by LPS, such as macrophages (Meyer *et al,* 2001) and that these chemokines are expressed during bacterial infections (Lauw *et al,* 2000). Indeed, signalling through TLRs has been implicated in allergic and non-allergic chronic inflammatory disorders (Sabroe *et al,* 2002).

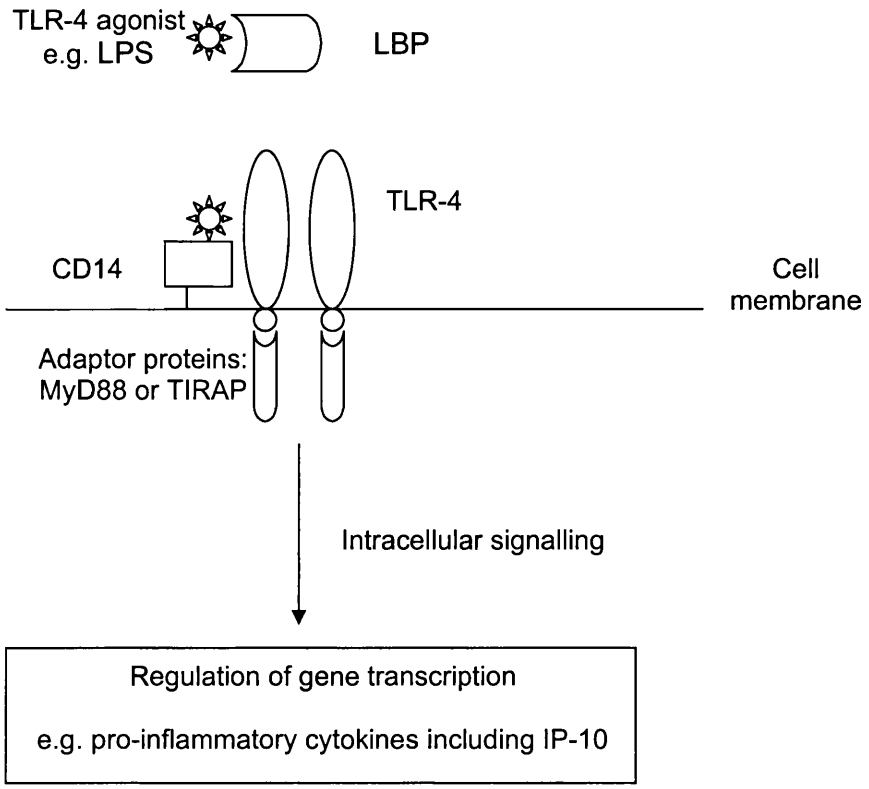


Figure 4.1: LPS signalling through Toll-like receptor-4 (TLR-4). LPS is bound by LPS-binding protein (LBP), a plasma-based protein which then associates with CD14. The CD14 complex then binds to TLR-4 and intracellular signalling is induced after ligation of adaptor molecules MyD88 or TIRAP. Intracellular pathways can lead to the transcription of a number of genes, including some proinflammatory cytokines such as IP-10.

In order to counter bacterially-mediated tissue damage, secretions by various cells of the skin and mucosa contain a number of antibacterial molecules, including the defensins. Depending upon the positioning of cysteine residues, defensin molecules can be divided into 2 main structural groups, alpha or beta defensins. Whereas alpha-defensins are produced mainly by phagocytes, production of the beta-defensins is restricted to epithelial cells (Ganz *et al,* 1999). Human beta defensin-2 (HBD-2) can be induced in response to proinflammatory cytokine treatment (Harder *et al,* 1997) in epithelial cells in a similar manner to inflammatory chemokines. Indeed, human beta defensin-2 (HBD-2) has a proven ability to bind to the chemokine receptor CCR6 and

elucidate chemotaxis of cells bearing this receptor, demonstrating that there is an increasing overlap in antimicrobial and chemotactic functions. Interestingly, HBD-2 can be produced by the oral mucosa and has been reported to be upregulated in OLP tissue (Abiko *et al,* 2002), suggesting there may be an underlying bacterial cause to OLP.

The few studies that have been undertaken have focussed on the ability of platelets and macrophages to produce antimicrobial chemokines (Cole *et al,* 2001; Tang *et al,* 2001), however, epithelial cells can produce defensins suggesting that these cells play an important role in antimicrobial protection. As the oral epithelial cells are known to produce chemokines, these may be playing a role in microbial defence. The present work has demonstrated that MIP-3α, CTACK, MIG and IP-10 chemokines (see Chapter 6, 5 and 3 respectively) can be produced in oral epithelial cells. The induction of these chemokines (and the defensins) may be induced partially to response to an alteration in the microbial flora, which could in turn cause an ensuing immune cell infiltration, perhaps akin to the response witnessed in OLP. Moreover, CXC ELR- chemokines are upregulated in OLP (Chapter 3), and as they also appear to have a role in antimicrobial activity, these chemokines may be upregulated by, and functioning against resident bacterial flora in this disease. In fact, it has been recently proposed that the OLP reaction may be triggered by a reaction to the microbial flora (Thornhill, 2001). Therefore, to explore this notion further the aim of this section was to investigate CXC ELR- chemokine expression in an oral keratinocyte cell line after LPS stimulation and the potential of these chemokines to mediate anti-microbial activity on oral bacteria, utilising *Streptococci sanguis* as a model. Streptococci comprise a large proportion of the resident oral microflora and are the most common

bacteria isolated from the buccal mucosa (Schuster, 1990). *Escherichia coli* was also tested as a positive control, but also proves an interesting contrast to the oral gram-positive bacteria, as *E.coli* is a commensal of the gastrointestinal system as well as being a gram-negative bacteria.

The specific aims of this section were:

- To assess whether MIG or IP-10 could be induced /enhanced in oral epithelial cell line in response to LPS
- To assess the antimicrobial effect of MIG, IP-10, CTACK or MEC upon oral specific gram-positive *S.sanguis* and gram-negative *E.coli.*

4.2 Materials & Methods

4.2.1 MIG and IP-10 mRNA transcripts in an oral epithelial cell line in response to lipopolysaccharide (LPS)

RNA was isolated from H357 cells treated with LPS as described previously (Section 2.3.6). Single strand cDNA synthesis was performed (Section 2.9.3) and RT-PCR for 18S, MIG and IP-10 utilised with conditions as described in Section 3.2.

4.2.2 Antimicrobial effect of MIG, IP-10, CTACK or MEC upon S. sanguis and E.coli

Reagents for the antimicrobial assay were prepared as described in Appendix 1. Radial diffusion assays (as detailed in Section 2.8) were then performed, adding 5μl of either recombinant human MIG, IP-10, MEC or CTACK (all Peprotech EC, London, UK) or 0.01% acetic acid to the wells before incubating the plates as previously described (Section 2.8). 3 wells for each test chemokine were studied. 100μM tetracycline was used as a positive control for the assay, an example of which is demonstrated in Fig 4.2.

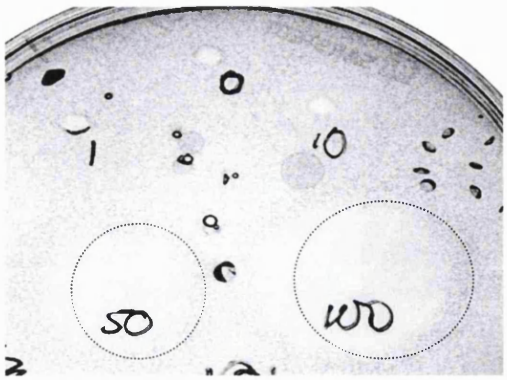


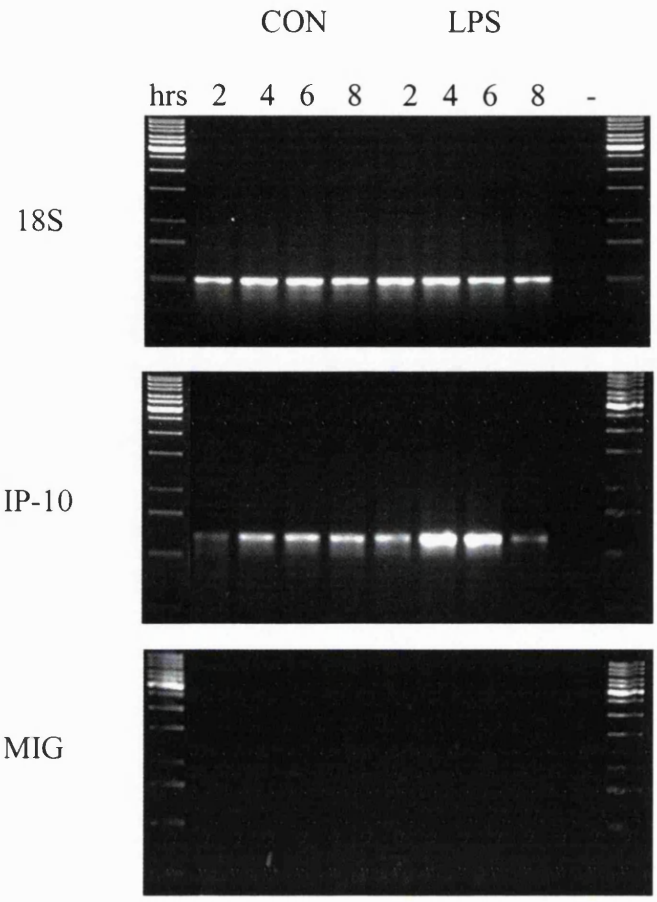
Figure 4.2: The antimicrobial activity of tetracycline on *E.coli* at 1, 10, 50 and 100μM. The clear zones represent areas where bacterial growth has been restricted by the addition of various concentrations of tetracycline to the wells. A higher concentration caused a larger zone of depletion.

4.3 Results

4.3.1 MIG and IP-10 mRNA transcripts in an oral epithelial cell line in response to lipopolysaccharide (LPS)

IP-10 mRNA was expressed in control cells (without LPS exposure), but was enhanced in response to LPS stimulation after 2, 4 and 6 hours incubation ($p<0.05$). This expression subsequently decreased after 8 hours of stimulation ($p<0.05$)(Fig. 4.3). In contrast, MIG mRNA was not expressed in control nor LPS-stimulated cells at any of the time-points tested.

a)



b)

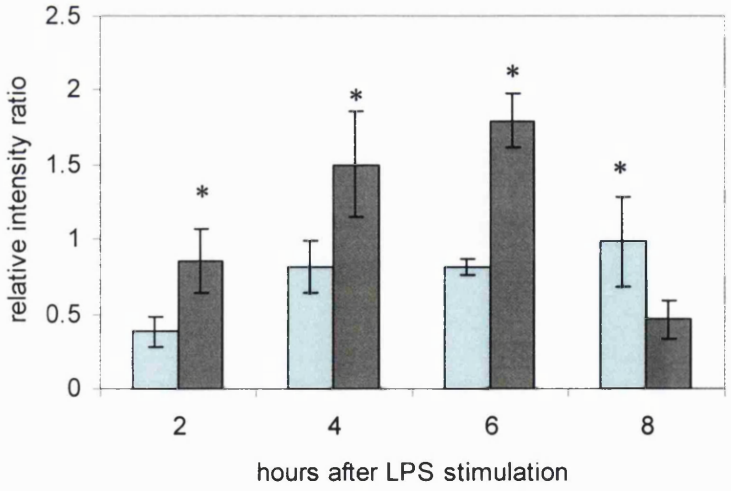


Figure 4.3: a) IP-10 and MIG mRNA expression by H357 cells with or without LPS stimulation. mRNA transcripts for 18S, IP-10 or MIG in H357 cells without (CON) or treated with LPS (LPS) for 2, 4, 6 or 8 hours. b) Densitometric analysis of IP-10 mRNA relative to 18S expression with (■) and without (■) LPS treatment. * indicates a significant difference between IP-10 production of untreated and LPS-treated cells.

4.3.2 Antimicrobial effect of MIG, IP-10, CTACK or MEC upon S.sanguis and E.coli

The clear zones of bacterial growth depletion that were produced after antimicrobial activity of the chemokines are indicated in Fig 4.4. All of the chemokines tested demonstrated a level of antimicrobial activity at the tested concentration (Table 4.1). MIG has the most effective of the tested chemokines against both *S.sangius* and *E.coli.* CTACK and IP-10 had a less antimicrobial action effective against *S.sangius* than *E.coli.* MEC had the greatest antimicrobial action against *E.coli.*

MIG IP-10 MEC CTACK -ve

a) *S.sanguis*

b) *E.coli*

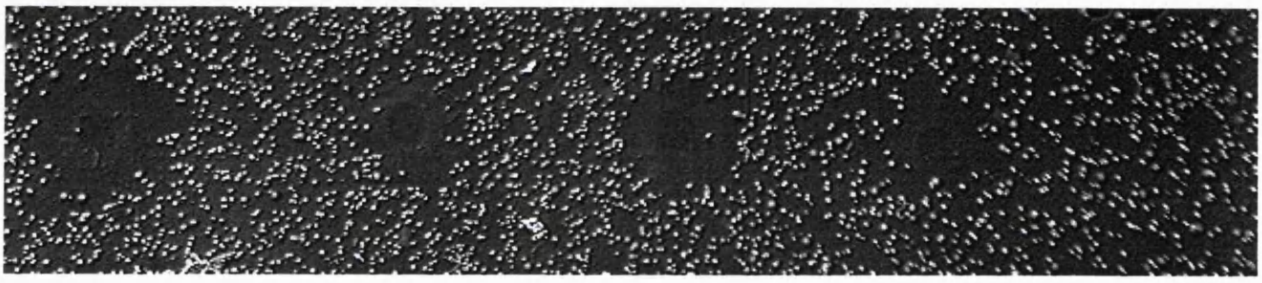


Figure 4.4: Radial diffusion gels demonstrating the antimicrobial activity of 100μg/ml human recombinant MIG, IP-10, MEC and CTACK upon *S.sanguis* and *E.coli* bacterium. The negative control (-ve) contains 0.01% of acetic acid only. The diameter of the cleared zone around the well containing chemokine represents the antimicrobial properties of the chemokine.

Table 4.1: Antimicrobial activity of 100μg/ml human recombinant MIG, IP-10, MEC and CTACK upon *S.sanguis* and *E.coli* bacterium. Figures represent the diameter of bacterial clearance (– the well diameter) and multiplied by 10 to convert to units. This represents the mean of 3 experiments (±standard deviation). The negative control had no effect on the microbial growth in any of the experiments tested.

	MIG	**IP-10**	**MEC**	**CTACK**
E.coli	34.6 (±6.80)	14.9 (±5.65)	15.4 (±6.04)	20.6 (±0.90)
S.sanguis	44.4 (±14.05)	8.4 (±3.52)	21.0 (±5.70)	10.0 (±5.45)

4.3 Discussion

Chemokines are known to have antimicrobial effects but little is known of the action of chemokines derived from the oral mucosal epithelium. The present study is the first to examine the expression of CXC ELR- chemokines by oral epithelial cells when stimulated by bacterially-derived products such as lipopolysaccharide (LPS). The present study has established that a cell line of oral origin is capable of expressing mRNA of CXC ELR- chemokines when stimulated with LPS. There was however some differential expression of the examined chemokines. In the present study, IP-10 mRNA expression was increased in an oral epithelial cell line after LPS stimulation, whereas MIG mRNA expression was not induced over the same time-period. As previously demonstrated IP-10 can act as a potent chemoattractant of lymphocytes (Chapter 3), hence this local production of IP-10 by oral epithelial cells in response to LPS could have important effects upon oral inflammation, perhaps crucially in the initial stages of inflammation.

Previous studies have also shown that LPS treatment alone can induce or enhance IP-10 mRNA expression in a number of different cell types of different origins (endometrial cells (Kai *et al,* 2002), breast carcinoma cell lines (Sun *et al,* 2001; Zaks-Zilberman *et al,* 2001), murine macrophages (Kopydlowski *et al,* 1999; Meyer *et al,* 1999), fibroblasts (Meyer *et al,* 1999) and osteoblasts (Gasper *et al,* 2002)). However, in contrast to the present findings LPS stimulation did not induce IP-10 from cultured cutaneously-derived keratinocytes (Boorsma *et al,* 1994), suggesting perhaps that oral keratinocytes are more responsive to LPS stimulation than cutaneous keratinocytes. This difference in expression may reflect the high bacterial load of the mouth,

necessitating the need for response to bacterial invasion. Alternatively, it may reflect differences in cell culture techniques between the two different studies.

The rapid expression of mRNA of IP-10 as observed in the present study accords with that of murine macrophages in which levels of IP-10 mRNA expression peaked at 4 (Widney *et al,* 2000) to 6 hours (Kopydlowski *et al,* 1999) after LPS stimulation. However, unlike the present study in which IP-10 levels fell after 6 hours, IP-10 mRNA levels remained high in murine macrophages. Furthermore, the peak of mRNA induction for IP-10 expression is considerably less than that of the same cell type following IFN-γ induction (see Section 3.3), suggesting that LPS stimulation is more transient than IFN-γ in IP-10 production. This short-term effect may of course be important, or essential, to avoid over-stimulation of IP-10 in response to resident bacteria in proximity to the epithelium, and the potential inflammatory effects of IP-10 production that might ensue. IL-10 is known to be able to down-regulate production of LPS-induced IP-10 in macrophages (Kopydlowski *et al,* 1999). This cytokine is present within OLP lesions (Simark-Mattson *et al,* 1999) and thus may act to down-regulate expression of IP-10 in oral inflammation. However, in an IL-10 producing cancer cell line both IP-10 and MIG were still produced (Sun *et al,* 2001) in response to LPS, suggesting that the influence of this cytokine may not always be paramount to regulate IP-10 expression and that other factors are probably involved in regulation of LPS-stimulated IP-10 production.

There are few reports of LPS-induced production of MIG. The present study revealed that MIG mRNA was not expressed by H357 cells when stimulated with LPS. In contrast to the present study, MIG mRNA was found to be expressed in LPS-

stimulated murine dendritic cells (Meyer *et al,* 1999), however, in another study, this chemokine was not induced in the same murine cell line by LPS, despite induction of MIG by IFN-γ (Widney *et al,* 2000). Furthermore, in common with the present findings, murine dermal adult fibroblasts demonstrated no constitutive or LPS-induced expression of MIG (Meyer *et al,* 1999), despite IP-10 production by the same cell type.

It is possible that MIG displays a delayed response in comparison to IP-10, as MIG mRNA induction in lung tissue of intravenously LPS-treated mice displays a later induction to IP-10 (Widney *et al,* 2000), and is never expressed to the same levels as IP-10. Correspondingly, whole blood cells stimulated with a variety of bacteria did not induce MIG production to similar levels of IP-10 production (Lauw *et al,* 2000). Indeed, this is similar to the present findings for MIG and IP-10 protein secretion in IFN-γ-stimulated H357 cells (see Section 3). However, in the lung tissue it was found that MIG was induced by 4 hours (Widney *et al,* 2000), a time point tested in our study thus suggesting that MIG is indeed not produced in oral epithelium following LPS-stimulation.

The present study did not examine the effects of I-TAC expression by oral epithelial cells in response to LPS, however, murine macrophage cell lines do express I-TAC after LPS stimulation (Widney *et al,* 2000) with a later dynamic to that of IP-10 and, therefore, may also be induced by LPS in epithelial cell types. If this was the case, it may also prove important for the initiation of oral inflammation.

Many studies report that LPS and IFN-γ act synergistically to induce the production of high levels of IP-10 mRNA, for example, in breast carcinoma cells (Sun *et al,* 2001), unlike some other chemokines where IFN-γ acts to down-regulate LPS-mediated production (Kopydlowski *et al,* 1999). It is then possible that IP-10 levels could be enhanced in oral inflammation where there is a presence of both LPS and IFN-γ. Furthermore, the prior incubation of oral epithelial cells with IFN-γ can induce responsiveness to LPS, perhaps through the enhancement of specific TLRs (Uehara *et al,* 2002).

IP-10 was expressed in unstimulated H357 cells, unlike the results found in Section 3.3, perhaps reflecting the addition of serum to the cell culture medium in this experiment. Serum was added in this experiment as LPS stimulation of IP-10 is dependant upon serum-derived factors in macrophages (Perera *et al,* 1998).

It has been shown that oral epithelial cells can express TLR-4 (Uehara *et al,* 2002) and thus may be functionally able to signal through this molecule. It is thought that CD14 and LPS binding protein are also required for LPS signalling through the TLR-4 molecule (Reviewed by Heumann & Roger, 2002). However, as these molecules are not expressed on oral epithelial cells (Uehara *et al,* 2002), the addition of serum would presumably provide these factors for epithelial cells (as LPS binding protein requirement is fulfilled by the addition of serum in CD14-expressing macrophages (Perera *et al,* 1998)). Therefore, *in vivo* the presence of soluble CD14 and LPS binding protein in body fluids would presumably allow LPS signalling in oral epithelial cells. Indeed, it has recently been shown that CD14 is produced constitutively in saliva (Uehara *et al,* 2003), suggesting that oral keratinocytes may

utilise these receptors and therefore, be more responsive than cutaneous cells, perhaps explaining the differing results in the responsiveness of LPS by oral and cutaneous epidermal cells.

However, as the oral epithelium does not express CD14, it possibly means that it is less susceptible to LPS stimulation than macrophages or dendritic cells (reviewed by Svanborg *et al,* 1999). However, binding of LPS in the presence of soluble CD14 would presumably stimulate IP-10 secretion from oral epithelium which may increase T cell migration to this area. Furthermore, in contrast to previous studies it has been found in some recent studies that keratinocytes may actually express CD14 constitutively (Song *et al,* 2002), suggesting that they may be able to signal in the absence of soluble CD14.

It is possible that the addition of serum in this experiment added low levels of IFN-γ which stimulated IP-10 from the H357 cells and if this was the case then the LPS-stimulated IP-10 expression may represent the synergistic effect of these 2 factors. However, as MIG was not upregulated in these conditions it suggests that IFN-γ is not having an effect on LPS-mediated stimulation, as it has been previously that IFN-γ stimulation can upregulate MIG mRNA expression (see Chapter 3).

LPS derived from different bacteria differ in their ability to stimulate monocyte chemoattractants from lung fibroblasts and epithelial cells (Koyama *et al,* 1999). This may also be the case for the stimulation of IP-10, however, LPS stimulation of IP-10 occurs through a different pathway than most cytokines / chemokines. IP-10 production by *E.coli* LPS is mediated through TLR-4 in macrophages in common

with other pro-inflammatory cytokines (Kawai *et al,* 2001). However, whereas the production of most inflammatory cytokines in response to LPS signalling is mediated through the adaptor molecule, myeloid differentiation protein-88 (MyD88) (Kawai *et al,* 2001), IP-10 production is independent of this molecule and instead signals by toll-IL-1 receptor domain-containing adapter protein (TIRAP) (Kawai *et al,* 2001). Instead it is thought that the production of this chemokine relies upon the early induction of IFN-β and subsequent activation of the STAT-1 pathway (Toshchakov *et al,* 2002). Therefore, IP-10 production could be stimulated in different circumstances to other LPS-induced cytokines.

Gram-positive bacteria, such as *S.sanguis* contain components other than LPS that are known to stimulate chemokine release from various cell types and it would be interestingly to determine whether these are also capable of stimulating the production of the CXC ELR- chemokines in oral epithelium. Many gram-positive bacterial components act on a different toll like receptor, TLR-2 (Reviewed by Heumann & Roger, 2002), which is functionally expressed upon keratinocytes (Kawai *et al,* 2002). However, TLR-2 agonists do not induce IP-10 production in macrophages (Toshchakov *et al,* 2002), dendritic cells (Re *et al,* 2001) nor osteoblasts (Gasper *et al,* 2002) *in vitro* as TLR-2 signalling appears to be MyD88-dependant. This suggests that TLR-2 agonistic bacterial products would also not induce IP-10 in epithelial cells. Therefore, only products bound by TLR-4 would be influential in upregulating IP-10 production in epithelial cells.

In addition, as LPS-mediated IP-10 production is presumably TLR-4 dependant, it strongly suggests that oral keratinocytes bear functional TLR-4, therefore, LPS-stimulation of epidermal cells is not due to TLR-2 agonist contaminants in LPS preparations (Hirschfeld *et al,* 2000), as previously suggested (Kawai *et al,* 2002).

Furthermore, IL-8 is powerfully induced by TLR-2 agonists and to a lesser extent by TLR-4 agonists (Re *et al,* 2001). As IL-8 chemokine is not produced by keratinocytes in OLP (Little *et al,* 2003), if OLP is induced by an innate response to bacterial flora, it may be due to a TLR-4 agonist as opposed to a TLR-2 agonist. Interestingly, IL-8 was enhanced in gingival fibroblasts by a *Porphyromonas gingivalis* protease, gingipain-R after T cell contact, whereas T cell contact increased IP-10 production in this cell type (Oido-Mori *et al,* 2001), but prior gingipain-R incubation decreased this production. This suggests that chemokine modulation in oral cells by bacterial products is complex and many different factors, including T cell contact, may play a factor in chemokine induction during an immune response.

A level of tolerance to LPS stimulation can be gained by repeated stimulation through either TLR-2 or TLR-4 in macrophages (Dobrovolskaia *et al,* 2003). However, the MyD88-independent pathway only appears to become tolerant if stimulated through TLR4 continuously (Dobrovolskaia *et al,* 2003). If this was the case for epithelial cells, IP-10 might be produced in response to TLR-4 stimulation, even after prolonged TLR-2 stimulation, whereas other inflammatory chemokines/ cytokines would not. This may induce an IP-10 based T cell inflammatory response in these circumstances. Therefore, perhaps in situations where the microbial flora becomes predominately

'TLR-2-based', IP-10 would be more readily produced by TLR-4 stimulation and therefore cause a Th1 based inflammation.

Furthermore, bacterial product stimulation of dendritic cells can upregulate CD40 and CD86 expression (Re *et al,* 2001), suggesting that bacterial product stimulation of oral epithelium could also enhance the expression of these molecules, and thus after ligation cause the upregulation of the inflammatory response, perhaps by upregulation of the CXC ELR- chemokines (see Chapter 3).

The present studies suggest that in certain circumstances, bacterial products could stimulate oral epithelial cells to produce an inflammatory response. Specifically if this response was stimulated through TLR-4 agonists (perhaps after continuous TLR-2 stimulation) this may induce epithelially-derived IP-10-mediated inflammation. This inflammation would presumably be characterised by activated memory T cell infiltration localised under the basal epithelium reminiscent of the pathology of OLP. If LPS stimulation was driving IP-10 production in OLP epithelium, this may also assist in explaining the undetectable levels of IFN-γ found by RT-PCR in OLP (Section 3.3), although small levels in the tissue may synergise with LPS.

In the second group of experiments of this section MIG was shown to be a potent antimicrobial agent against both *S.sanguis* and *E.coli* whilst IP-10 was also found to have similar antimicrobial activity but to a lesser degree from MIG. This confirms the study by Cole *et al,* 2001 that found the CXC ELR- chemokines act antimicrobially, and that MIG was more effective than IP-10 upon *E.coli.* Although only one oral bacterial species was tested in this study, the present data hints that antimicrobial

properties of these chemokines may assist in countering bacterial growth in the oral cavity. As these chemokines are upregulated during OLP pathogenesis (Chapter 3), they may function in an antimicrobial capacity during this condition and that subsequent T cell infiltration would be likely to occur after these chemokines are produced.

The low production of MIG by oral epithelial cells following IFN-γ or LPS stimulation could potentially be a means of avoiding an over-active anti-microbial response due to the potent antimicrobial activity of MIG. However, the fact that dermal macrophages appear to be expressing this chemokine in large quantities in lichen planus (Flier *et al,* 1999), suggest that these cells may utilise MIG as an antimicrobial agent in this condition. Furthermore, this may assist in explaining the lower binding affinity of MIG to CXCR3 (Cox *et al,* 2000) if it is predominately induced to respond antimicrobially.

IP-10 had a lower antimicrobial action towards *S.sanguis* than MIG. However, as IP-10 chemokine can be highly induced in oral epithelial cells, it may, therefore, have a localised influence upon flora where IP-10 is upregulated to high concentrations by LPS or IFN-γ (and perhaps a combination of other cytokines/ factors). The fact that oral epithelial cells (in the presence of serum) can be induced to express IP-10 by *E.coli* LPS and then act in an antimicrobial fashion against the same bacterium suggests that this could be an important mechanism in regulating bacterial colonisation of epithelial areas. It is interesting to note that IP-10 was considerably more effective against *E.coli*, whose LPS is a TLR-4 agonist, whereas *S.sanguis* as a gram positive bacteria is therefore more likely to contain TLR-2 agonists. The

preliminary results of these studies may suggest that the upregulation of IP-10 is specifically upregulated and acts mainly antimicrobially towards TLR-4 agonistic bacterial products.

The antimicrobial effect of IL-8 was not investigated in this study, however, IL-8 was not found to have significant antimicrobial properties (Cole *et al,* 2001) and this chemokine is not upregulated from oral keratinocytes during OLP (Little *et al,* 2003), suggesting there is a general upregulation of bacterial killing mechanisms in this condition.

The results of the present study indicate that both MEC and CTACK exert antimicrobial properties against *E.coli* and *S. sanguis*, albeit at the high concentrations tested. MEC has recently been shown to have a microbicidal activity against a range of oral bacteria (Heishami *et al,* 2003), and the present study confirms that this chemokine was also effective against the oral commensal *S.sanguis*. This is the first report of the antimicrobial effect of MEC upon the enterobacterial *E.coli*. The fact that it displays such an effect is perhaps not surprising as MEC is also expressed in the colon (Pan *et al,* 2000) and thus may also act as a secretory antimicrobial agent against resident colonic bacteria such as *E.coli*. CTACK is capable producing an antimicrobial effect upon *E.coli*, in fact, to a greater degree than against *S.sanguis*. This may be surprising as CTACK is expressed in the oral mucosa (Chapter 5), but there are no reports of expression of this chemokine within the gastrointestinal system. It would be interesting to assess the antimicrobial action of CTACK against commensal bacteria of the skin to assess whether there may be some antimicrobial

role of the homing chemokines in the sites of their expression against tissue-specific bacterial colonisations i.e. CTACK against *S.epidermis* and MEC against *E.coli.*

Interestingly, MEC and CTACK have been found to exert anti-Candidal activity (Heismema *et al,* 2003), indeed these molecules share structural similarities to histadine, an anti-Candidal protein. An association between *Candida* and pathogenesis of OLP has previously been suggested (Lundstrom *et al,* 1984), and it is now known that infection of human oral epithelial cells with *Candida* can cause an increase in pro-inflammatory cytokine production (Schaller *et al,* 2002; Steele *et al,* 2002) - even by relatively non-virulent strains (Schaller *et al,* 2002). Therefore, perhaps CTACK is not antimicrobial when expressed constitutively, but when production is enhanced, for example, after proinflammatory cytokine treatment (see Chapter 5), there is an antimicrobial effect against *Candida.* It would be interesting to establish whether *Candidal* infection of oral epithelium increases CTACK or MEC production from these cells.

As secretions of milk and saliva both contain high levels of MEC (Hieshima *et al,* 2003), it is interesting to note that treatment of OLP with products containing bovine colostrum correlated with an alleviation of symptoms (Pedersen *et al,* 2002). This may be partially due to the presence of antimicrobial MEC within this product. Furthermore, IgA is upregulated in saliva during OLP (Sistig *et al,* 2002), suggesting the saliva gland is activated during this condition and thus may also be producing large concentrations of MEC, both in order to recruit IgA producing cells, and for antimicrobial purposes.

Although not tested in the present section, studies have also shown MIP-3α is also antimicrobial against *E.coli* (Hoover *et al,* 2002) even at low concentrations. Therefore, perhaps the differential production of MIP-3α witnessed for the oral and cutaneous epithelial cells (see Chapter 6) might be due to the antimicrobial properties of this chemokine and the possible requirement of the mucosal environment to maintain homeostatic control of bacterial growth in this environment.

Therefore, the production of IP-10 in oral epithelial cells, in oral inflammatory conditions such as OLP maybe induced by resident bacteria that contain TLR-4 agonists (Fig 4.4). If this inflammation was caused after constant TLR-2 stimulation, this may cause an IP-10 based inflammation, presumably causing an influx of memory Th1 CD4+ cells (see Section 3) similar to the inflammation witnessed in OLP. Furthermore, IP-10 is antimicrobial at high concentrations suggesting that it may be upregulated by oral epithelial cells in order to respond to adherence or invasion by bacteria. The antimicrobial activity of the chemokines tested is likely to be an important mechanism in the homeostasis of oral bacterial colonisation. Any IFN-γ in the oral epithelial area (which could presumably be produced by the infiltrating Th1 cells) may synergise with TLR-4 agonists to cause an increased inflammatory state (Fig 4.5).

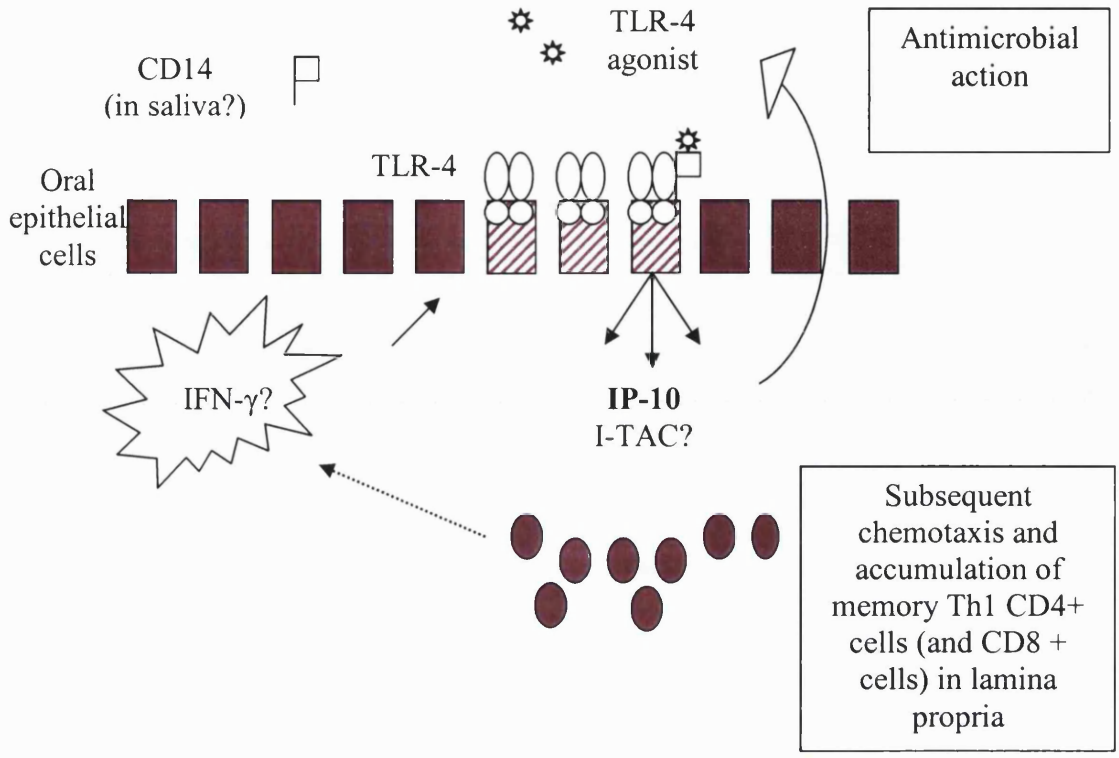


Figure 4.5: Potential role of bacteria in the pathogenesis of OLP. TLR-4 agonists of oral bacteria bind to TLR-4 expressed upon oral epithelial cells, perhaps through binding to CD14 in saliva. IP-10 (and I-TAC?) is produced in a MyD88-independent process (and therefore may be produced after constant TLR-2 stimulation leading to tolerance of other MyD88-dependant cytokines/ chemokines) and would probably act as an antimicrobial agent at high concentrations as well as a chemoattractant for memory Th1 CD4+ cells. These infiltrating cells may also produce IFN-γ which would possibly synergise with LPS to produce high concentrations of IP-10 in the lesions.

Chapter 5: CTACK in oral epithelium and oral lichen planus

5.1 Introduction

Cutaneous T-cell attractant chemokine (CTACK), or CCL27, is a chemokine noted for its properties to attract a subset of T cells bearing the receptor CCR10 (Homey *et al,* 2000). These cells have been characterised to include a subset of memory T-cells expressing the skin-homing molecule, cutaneous lymphocyte antigen (CLA).

It is known that CTACK is highly expressed in cutaneous keratinocytes, both during healthy and some inflammatory conditions, such as psoriasis (Morales *et al,* 1999). In healthy skin samples, there is expression of CTACK particularly in the basal layers of the epidermis, however, in psorasis and contact dermatitis, suprabasal keratinocytes also exhibit strong CTACK expression, with basal cells releasing large amounts of this chemokine into the dermis (Homey *et al,* 2002). Furthermore, CTACK expression can detected in basal and suprabasal keratinocytes after six hours of nickel exposure in related contact dermatitis (Homey *et al,* 2002).

CCR10 expression mostly occurs on CD4+ T cells and is restricted to CLA+ T cells. However, the receptor CCR10 is only expressed by 30-40% of CLA+ cells (Homey *et al,* 2002). Soler and co-workers (2002) found that the CCR10+ cells could be further characterised to a CD27-CCL7-CD45RO+CLA+ subset, which represent a subset known as 'memory effector' cells. These cells have lost the ability to home to the lymph node (through the absence of chemokine receptor CCL7) and, therefore, it was thought that they probably act in the surveillance of peripheral tissue in response to previously encountered antigens.

The degree of CCR10 involvement in cutaneous inflammation is unclear. In one study, there was a strong expression of CCR10 on skin-infiltrating dermal leukocytes and intra-epidermal lymphocytes. Indeed most of the infiltrating CD3+ cells were CCR10+ during inflammation (e.g. psoriasis, atopic or allergic contact dermatitis)(Homey *et al,* 2002), whereas in another study of candidal and bacterial extract-induced skin delayed hypersensitivity (DTH) inflammation there was no selective infiltration of CCR10+ cells within the T cell population (Soler *et al,* 2002). Thus the exact specialised function of these CCR10+ cells remains unclear.

The possible role of this chemokine as a target of anti-inflammatory treatment has been considered. Treatment with a neutralising CTACK antibody reduced earswelling after sensitation with dinitrofluorobenzene (DNFB) in a mouse model (Homey *et al,* 2002). The antibody reduced the recruitment of lymphocytes to the skin of ovalbumin-sensitised mice by more than 90% when compared with isotype-treated controls (Homey *et al,* 2002). In contrast, however, a study by Reiss *et al* (2001) suggested that CTACK neutralisation does not reduce ear-swelling, unless in combination with anti-thymus and activation-regulated chemokine (TARC) treatment also, as they appear to have over-lapping roles (Reiss *et al,* 2001). In addition, Soler *et al,* 2002 argued that anti-CTACK treatment must reduce ear-swelling not by reducing the T cell infiltration of CCR10+ cells, as this reaction is a T cell independent mechanism. However, the possible use of anti-CTACK strategies should not be entirely discounted at this time as such an approach would specifically only dampen immune responses in the areas where CTACK is expressed (at present only demonstrated in the skin), while presumably other areas would remain unaffected.

Thymus and activation-regulated chemokine (TARC) is another chemokine suggested to be associated with tissue-specific homing to the cutaneous environment. TARC is expressed by endothelial cells in skin and expression was found to be increased in cutaneous lichen planus (Campbell *et al,* 1999). This chemokine attracts cells bearing the CCR4 chemokine receptor which is expressed on memory T cells that bear CLA (Campbell *et al,* 1999), however, it was found that in an experimental delayed-type hypersensitivity mouse model, that CCR4- cells are still capable of homing to the skin, suggesting that CCR4 is not required for lymphocyte recruitment to inflamed skin (Reiss *et al,* 2001).

Mucosae-associated epithelial chemokine (MEC), or CCL28, is a chemokine with 40% homology to CTACK. It attracts a similar profile of memory CLA+ T cells bearing CCR10 as CTACK. MEC is also able to attract CCR3 transfectants, expressed on eosinophils (Pan *et al,* 2000). MEC is poorly expressed in skin, but, instead is expressed in large amounts in mucosal sites, such as the colon, trachea and particularly in the salivary glands (Pan *et al,* 2000) where MEC expression is mostly confined to the epithelial cells (Pan *et al,* 2000).

The oral cavity has many immunological features in common with the skin, including the presence of CLA+ T cells, Langerhans cells and a stratified epithelium. However, despite many disorders affecting the skin and oral mucosa (e.g. Bechet's disease (Bang, 2001); epidermolysis bullosa simplex (Horn & Tideman, 2000), pemphigus vulgaris (Scully *et al,* 1999) and lichen planus), it remains unknown which, if any, tissue specific chemokines are expressed in the oral mucosal environment. There are no reports documenting the expression of MEC or CTACK in the healthy or inflamed

oral mucosa. Yet these chemokines would seem to have a central role in oral mucosal immunosurveillence, and possibly be mediators of oral mucosal chronic inflammation. It is possible that CTACK could also be involved in the trafficking of memory T cells to the oral mucosa. For example, CTACK has a proven role in recruitment of a subset of CLA+ T cells (Morales *et al,* 1999), and is also implicated in inflammatory conditions involving memory T cells bearing CLA (Morales *et al,* 1999; Homey *et al,* 2002). Certainly the CLA+ cell subset has been reported within normal oral mucosa and lesions of OLP (Walton *et al,* 1997). In addition the ligand for CLA - E-selectin - is also expressed in oral mucosa and there is an increased intensity of expression during both skin and oral lichen planus (Walton *et al,* 1997). Although it is not thought that there is a selective recruitment of CLA+ T cells in oral or cutaneous lichen planus, there is a significant increase in the numbers of CLA+ cells in the epithelium during these conditions (Walton *et al,* 1997). It may be that an alteration in the expression of CTACK during these inflammatory conditions could be responsible for this modification in T cell homing, which could crucially lead to interactions between CLA+ T cells and oral epithelial cells (Brown *et al,* 1999). This interaction could be central in a disease currently thought to be mediated by auto-immune T cells acting upon the epithelium (Eisen, 2003; Agarwal & Saraswat, 2002; Thornhill, 2001)

Furthermore, anti-CTACK, has been shown to be more potent than tacrolimus (now suggested as a treatment for OLP conditions (Kaliakatsou *et al,* 2002) in reducing inflammation in patients with inflammatory skin conditions (Homey *et al,* 2002). Therefore, there are potential therapeutic uses of blocking CTACK in cases of oral inflammation if this chemokine was found to be expressed in the oral cavity,

especially in a condition such as lichen planus. Hence the aim of this section was to determine whether CTACK can be expressed by the oral epithelium, and if expression is increased in oral lichen planus, and thus establish if this chemokine has a central role in the pathogenesis of OLP.

Specifically, the aims of this section were to:

- Determine the expression of CTACK mRNA in unstimulated primary oral epithelial cells and with and without IFN-γ stimulation
- Determine the production of CTACK protein primary oral epithelial cells with and without stimulation with IFN-γ
- Determine the effects of pro-inflammatory cytokine treatment (IFN-γ or TNF-α with IL-1β) upon the production of CTACK by oral (H357) and cutaneous (UP) keratinocyte cell lines
- Determine the effects of CTACK blocking upon memory (CD45RO) and CLA+ T cells chemotaxis to supernatants derived from oral epithelial cell cultures stimulated with or without TNF-α and IL-1β
- Determine the expression of CTACK mRNA in normal oral mucosal and oral lichen planus tissue

5.2 Materials & Methods

5.2.1 Expression of CTACK mRNA by IFN-γ-stimulated and non-stimulated primary oral epithelial cells with and without IFN-γ stimulation

The procedure for cytokine treatment assay (Section 2.3.6) was utilised under the following conditions: primary cell lines (2.3.1) were cultured in the presence or absence of 1000U/ml IFN-γ (Sigma-Aldrich, Poole, Dorset) or no treatment for 24 hours.

RNA isolation and single strand synthesis of the primary cells was performed as described previously (Section 2.9.2). RT-PCR of isolated CTACK RNA was undertaken also as described previously (Section 2.9.4). The following primers were generated:-

- CTACK: (forward 5'- CTGTACTCAGCTCTACCGAAAGCC -3', reverse 5'- GCCCCATTTTTCCTTAGCATCCC -3)

A magnesium concentration of 4.0mM was used for the CTACK RT-PCR reaction.

As a positive control for CTACK RT-PCR, human skin total RNA (Stratagene, Amsterdam, Netherlands) was obtained, single strand synthesis carried out (see Section 2.9.3) and was utilised.

5.2.2 CTACK production by primary oral epithelial cells with or without IFN-γ stimulation

Primary cells were incubated with or without 1000U/ml IFN-γ (Sigma-Aldrich, Poole, Dorset) for 24 hours as described previously (Section 2.3.6).

Levels of CTACK in cell culture supernatants were established by using a Duoset ELISA development system for human CTACK/CCL27 (R&DSystems, Oxon, UK). The capture antibody (mouse anti-human CTACK), was used at a concentration of 2μg/ml, diluted in PBS. After washing the coating antibody, 100μl of blocking buffer (1% BSA, FractionV (Sigma, Poole, UK), 5% sucrose (BDH, Poole, UK) in PBS (Sigma, Poole, UK), pH7.2-7.4, 0.2μm filtered) was added to each well and incubated for 2 hours at room temperature. Biotinylated goat anti-human CTACK was employed as the secondary antibody. All antibodies (except capture) and standards were diluted in 1% BSA in PBS, pH 7.2-7.4, 0.2μm filtered. The ELISA protocol as detailed in Section 2.4 was followed from this point, although OPD was incubated for 20mins.

5.2.3 Effects of IFN-γ or TNF-α with IL-1β upon the production of CTACK by oral (H357) and cutaneous (UP) keratinocyte cell lines

H357 (2.3.2) and UP (2.3.3) cells were incubated with 10ng/ml recombinant human TNF-α (R&D systems, Oxon, UK) with 5ng/ml IL-1β (Peprotech, London, UK) or 1000U/ml IFN-γ (Sigma-Aldrich, Poole, Dorset) for 3, 6 or 24 hours. Cells were also incubated without IFN-γ or TNF-α with IL-1β.

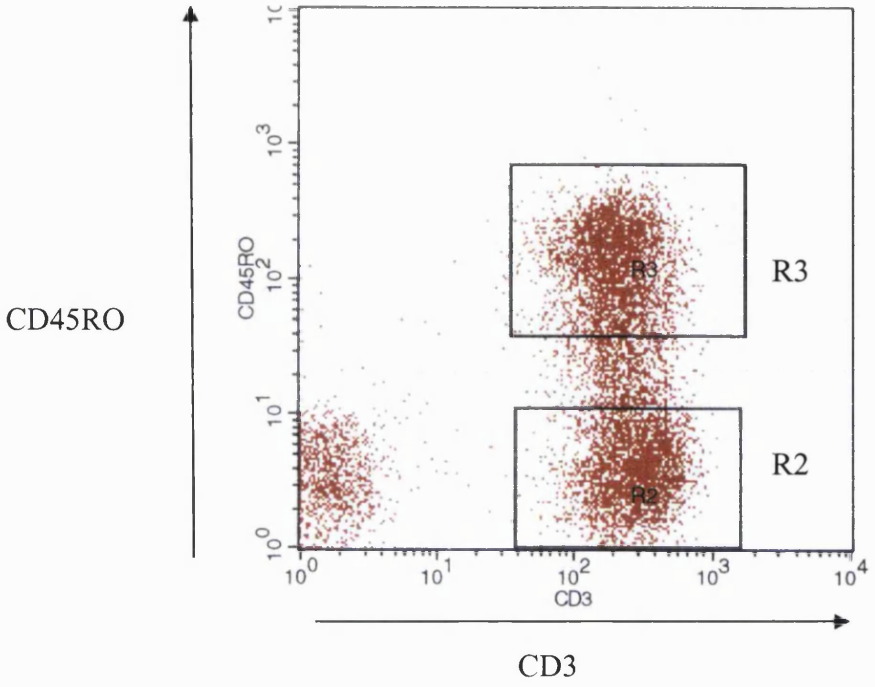
The levels of CTACK produced in the extracted supernatants were then determined by CTACK ELISA as described for the primary cell supernatants.

5.2.4 Effects of CTACK blocking upon the memory and CLA+ T cells chemotaxis to supernatants of H357 or UP cells following stimulation with IFN-γ or TNF-α with IL-1-1β

Supernatants derived from the above cytokine treatment assays for H357 and UP cells were incubated with no antibody, with monoclonal anti-human CTACK antibody (R+D systems, Oxon, UK) or with mouse anti-human IgG_{2a} isotype control (Clone 11711.11) (R+D systems, Oxon, UK) for 30 minutes before placing in a transwell. Chemotaxis of peripheral blood lymphocytes was undertaken as detailed previously (Section 2.6).

The antibodies utilised to label the migrated PBMC were monoclonal mouse anti-human CD45RO-R-Phycoerythrin (Clone UCHL1, IgG_{2a}), monoclonal rat anti-human CLA-FITC (HECA-452, IgM) and monoclonal mouse anti-human CD3 Cy-chrome (Clone UCHT1, IgG_1), all were utilised at a concentration of $6\mu l/10^6$ cells (all antibodies were from BD Pharmingen, Cowley, Oxford). Flow cytometry acquisition and analysis of the migratory cells was carried out as described in Section 2.7. The cell populations that were analysed after migration in this experiment are indicated in Fig 5.4. Populations were analysed from within the peripheral blood lymphocyte population (Fig 2.3) and gated upon the level of fluorescence to CD3 and CD45RO or CD3 and CLA into CD3+CD45RO+ cells and CD3+CLA+ cells (Fig 5.1).

1)



2)

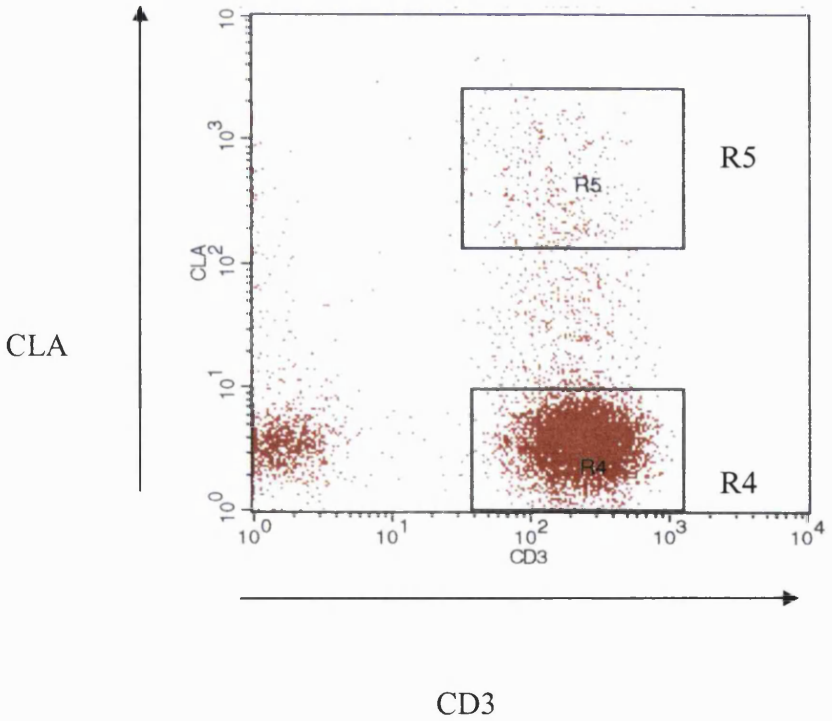


Figure 5.1: The cell populations analysed by 3 colour-FACS. The cytometry plots for the PBL population labelled with 1) anti-CD3 and CD45RO with populations divided into CD3+CD45ROlo (R2) and CD3+CD45ROhi (R3) and 2) anti-CD3 and CLA with gating corresponding to CD3+CLA+ (R4) or CD3+CLA- (R5).

5.2.5 CTACK expression of CTACK in normal oral mucosa and oral lichen planus tissue

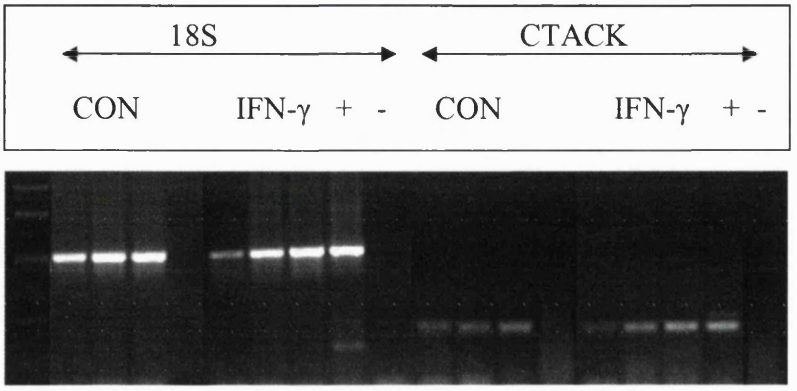
RNA isolation and single strand synthesis of tissue derived from OLP lesions or NOM was performed as described in Section 2.9.2. RT-PCR (as described in Section 2.9.4) determined the presence of mRNA to CTACK, utilising conditions as stated for the primary cells.

5.3 Results

5.3.1 Expression of CTACK mRNA by IFN-γ stimulated and non-stimulated primary oral epithelial cells

CTACK mRNA was expressed in oral epithelial cells in the presence or absence of IFN-γ stimulation (Fig 5.2). IFN-γ stimulation did not significantly increase ($p>0.05$) expression of mRNA in examined specimens as detected by densitometry analysis of RT-PCR products.

i)



ii)

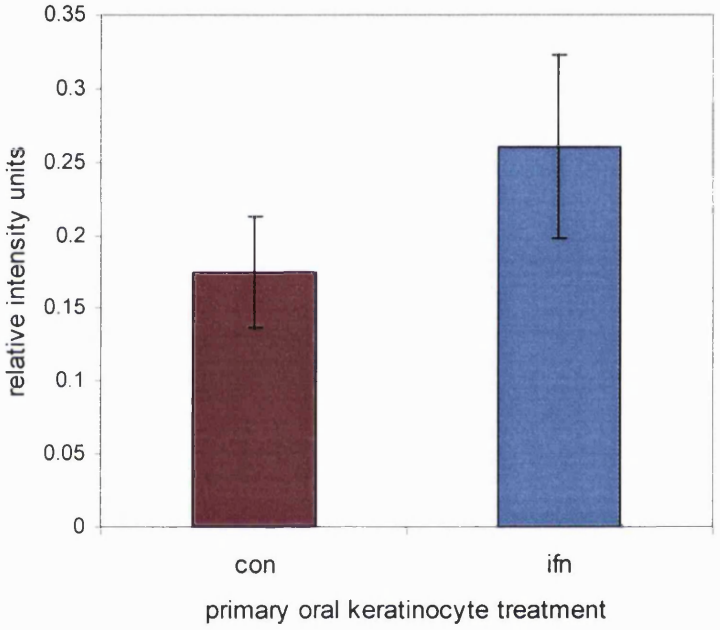


Figure 5.2: RT-PCR of CTACK mRNA in primary epithelial cell lines. i) Expression of 18S and CTACK expression in the 3 keratinocyte cell cultures non-stimulated (CON), and treated with IFN-γ for 24hours (IFN-γ). + represents the positive control for 18S and CTACK expression, skin cDNA. Lanes marked – are the negative controls for these samples. ii) Densitometric analysis of the average CTACK mRNA expression (normalised to 18S expression) in the 3 different primary oral keratinocytes treated with IFN-γ (ifn) or non-treated (con).

5.3.2 Production of CTACK by IFN-γ-stimulated and non-stimulated primary oral epithelial cells

CTACK protein was detected in the supernatants of both IFN-γ-stimulated and non-treated primary oral epithelial cell cultures. IFN-γ increased the concentration of CTACK produced by each tested primary oral epithelial cell culture tested, and in 1 of the 3 cultures was this significantly raised compared to unstimulated cells ($p<0.05$) (see Fig 5.3). Overall, levels of CTACK were increased significantly ($p<0.05$) by IFN-γ stimulation.

Of note CTACK was released by unstimulated oral keratinocytes in high concentrations, particularly when compared to findings for the production of IP-10 or MIG from the same cell type (see Fig 3.3).

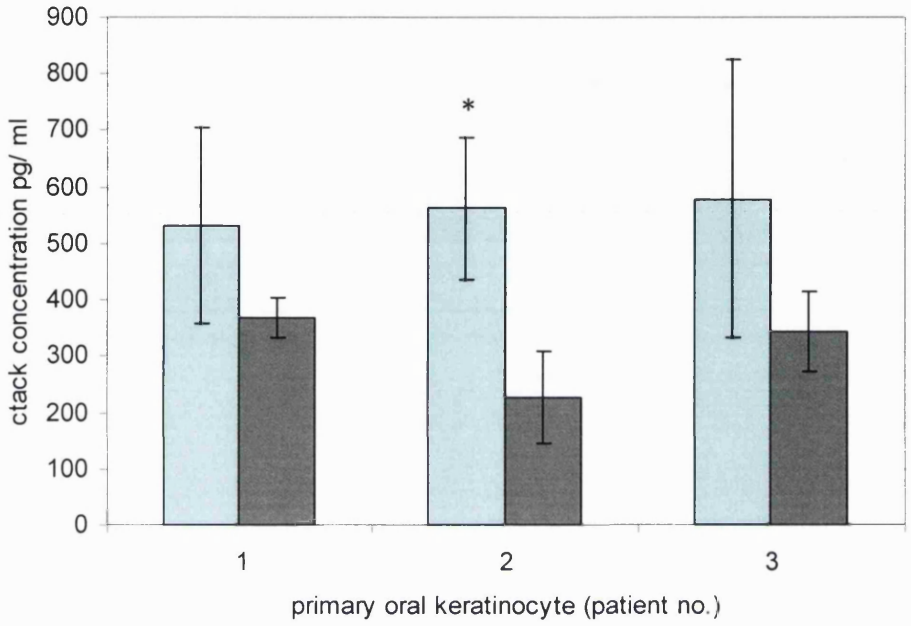


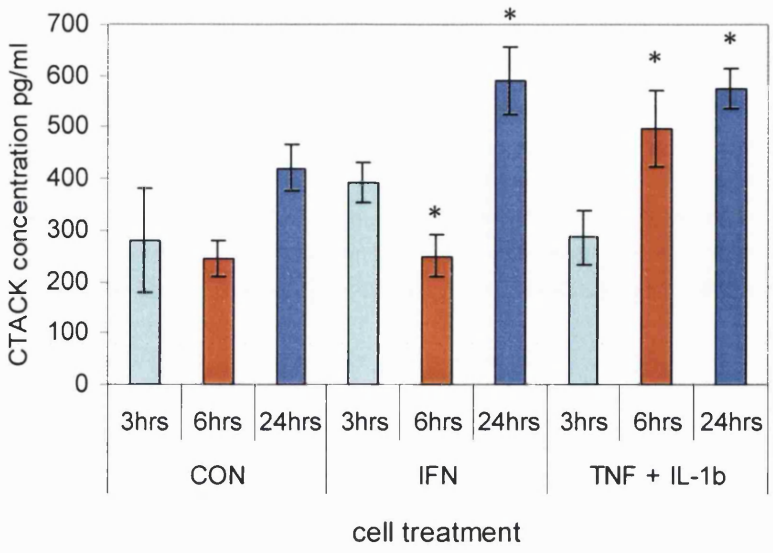
Figure 5.3: The concentration of CTACK produced in 3 different primary oral keratinocyte cell lines stimulated with 1000U/ml interferon-γ ( ) or left untreated ( ) for 24 hours. (* represents a p value of <0.05).

5.3.3 Effects of pro-inflammatory cytokine treatment (IFN-γ or TNF-α with IL-1β) upon the production of CTACK by oral (H357) and cutaneous (UP) keratinocyte cell lines

CTACK was produced by unstimulated H357 oral carcinoma cells, and without stimulation the levels of production increased with time (Fig 5.4). TNF-α and IL-1β stimulation initially had no effect upon the release of CTACK, however, the amount of release increased over each time point to become significantly greater than that of the unstimulated cells after 6 and 24hours. IFN-γ-stimulation also significantly increased the production of CTACK, but had a slower effect compared to TNF-α with IL-1β, as it only gives rise to significantly increased production after 24hours.

CTACK was produced by both the unstimulated and stimulated skin keratinocyte cell line (UP) (Fig 5.4). Levels of CTACK production were highest at 24hours. IFN-γ treatment slightly increased CTACK production by the cutaneous cells after 3 and 24hours compared with the unstimulated cells, but these increases were not significant. TNF-α with IL-1β stimulation likewise increased the production of CTACK at 24hours to a significant degree ($p<0.05$). Of note the levels of production of CTACK by non-stimulated cutaneous cells were equivilant to levels produced by unstimulated oral cells at 3hours.

i) H357 cells



ii) UP cells

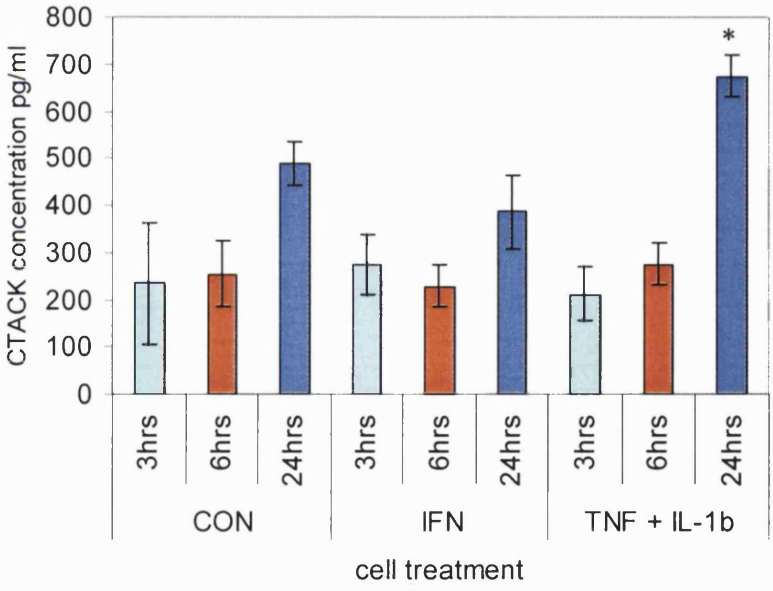


Figure 5.4: The concentration of CTACK produced (pg/ml) by i) H357 or ii) UP cells treated either with interferon-γ (IFN) or TNF-α with IL-1β (TNF+IL-1b) for 3, 6 or 24 hours as determined by ELISA. CON represents control cells without stimulation. Each result is the mean of triplicate samples ±SD. * represents a significant difference $p<0.05$ between the stimulated and unstimulated cells at the same time point.

5.3.4 Effects of CTACK blocking upon the memory and CLA+ T cells chemotaxis to oral supernatant

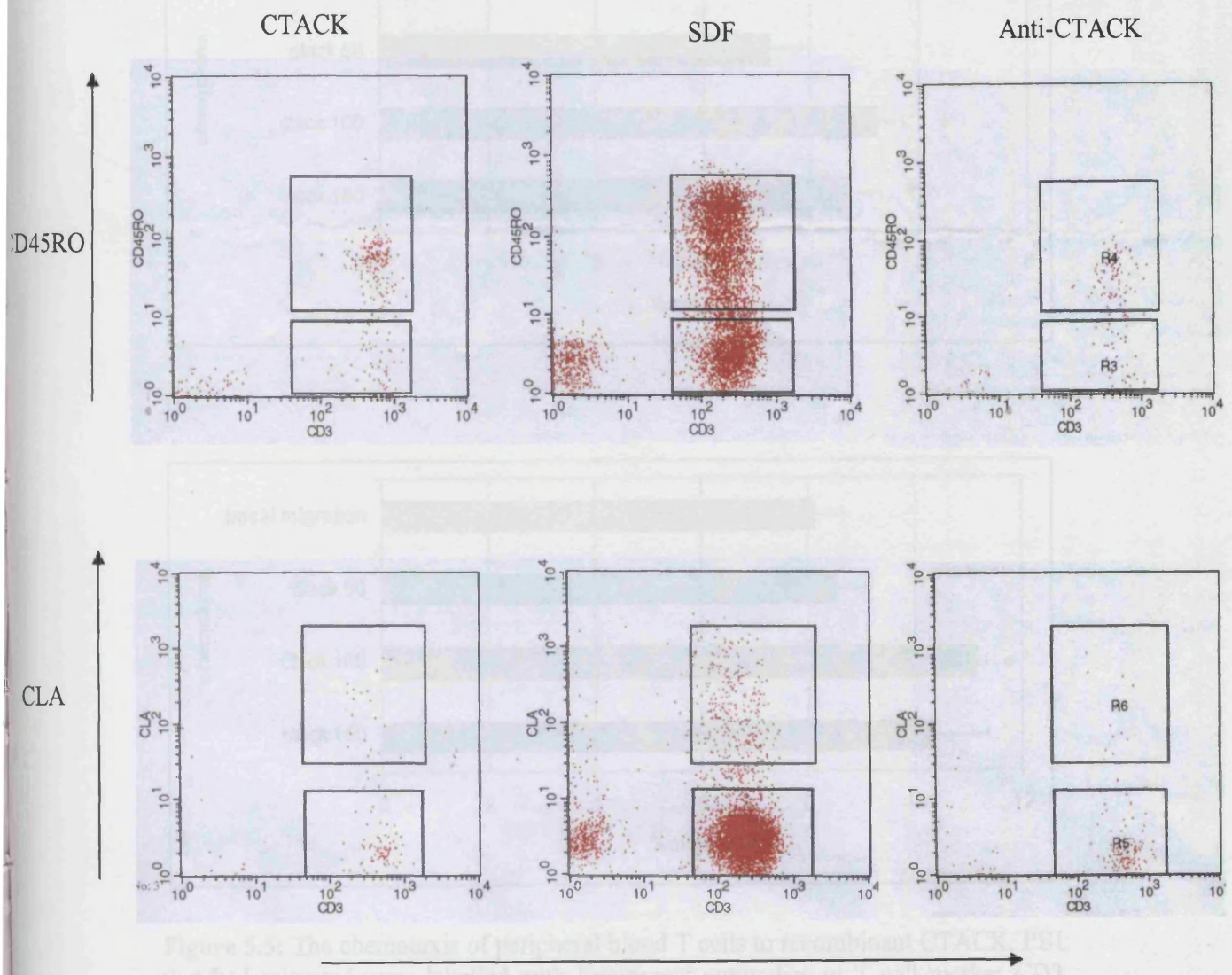
Recombinant human CTACK selectively attracted a memory and CLA+ T cell population as shown by the dot plots (Fig 5.5.1). The chemotactic action of recombinant CTACK at concentrations of 100 and 150nM was significantly greater than control buffer (Fig 5.5.2). In addition, CTACK increased the migration of memory T cells (CD45RO+) expressing CLA from peripheral blood above the level of basal migration, to a significant degree at 100mM as shown in Fig 5.5. The pre-incubation of CTACK with anti-CTACK antibody reduced the migration of memory and CLA+ T cells selectively without a reduction of naïve and CLA- cells (Fig 5.5.1).

In this experiment the supernatants of H357 and UP cells stimulated with TNF-α with IL-1β for 24hours were employed as potential chemoattractants as this incubation had resulted in the greatest production of CTACK in a previous experiment.

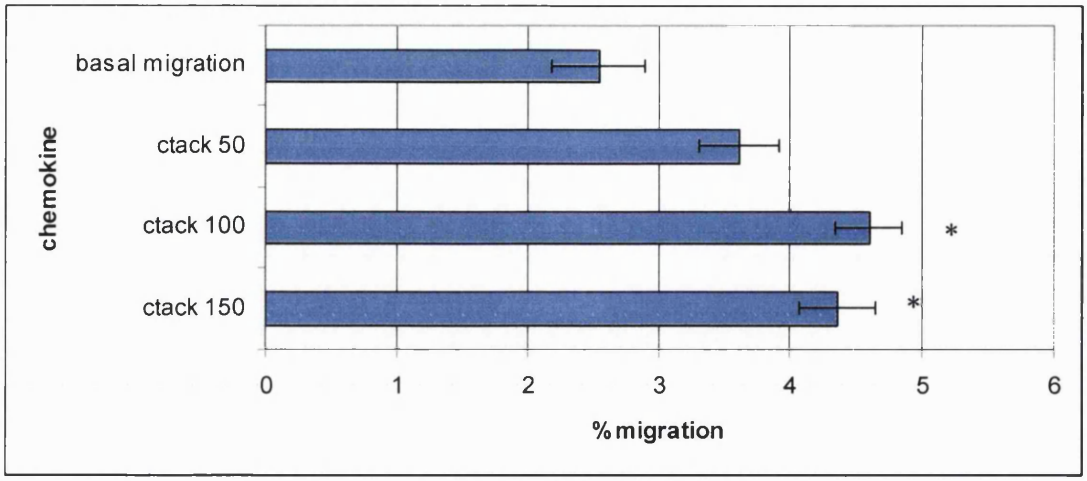
Supernatants of stimulated and unstimulated H357 and UP cells induced the migration of memory (CD45RO+) and CLA-positive T cells above the level of basal migration. However, they did not induce a significant migration of naïve T cells (Fig 5.6). Supernatants of TNF-α and IL-1β-stimulated UP and H357 cells significantly increased the migration of memory (CD45RO+) and CLA+ T cells compared to the control unstimulated cell supernatants. The supernatant of H357 cells induced a higher percentage migration of memory T cells than the UP cells regardless of the previous stimulation or non-stimulation with TNF-α and IL-1β.

Pre-incubation of oral and cutaneous keratinocyte cell line supernatants with anti-CTACK antibody reduced the migration of memory T cells (CD45RO+). This reduction of migration was significant ($p>0.05$) with supernatants of TNF-α/IL-1β-treated H357 and UP cells. However, anti-CTACK treatment did not reduce the migration of CLA+ T cells towards the supernatant of unstimulated H357 cells. Interestingly, by blocking CTACK activity there was a significant increase in the migration of naïve T cells to both cutaneous and oral supernatants compared to the supernatants that had not been pre-treated.

1)



2)



3)

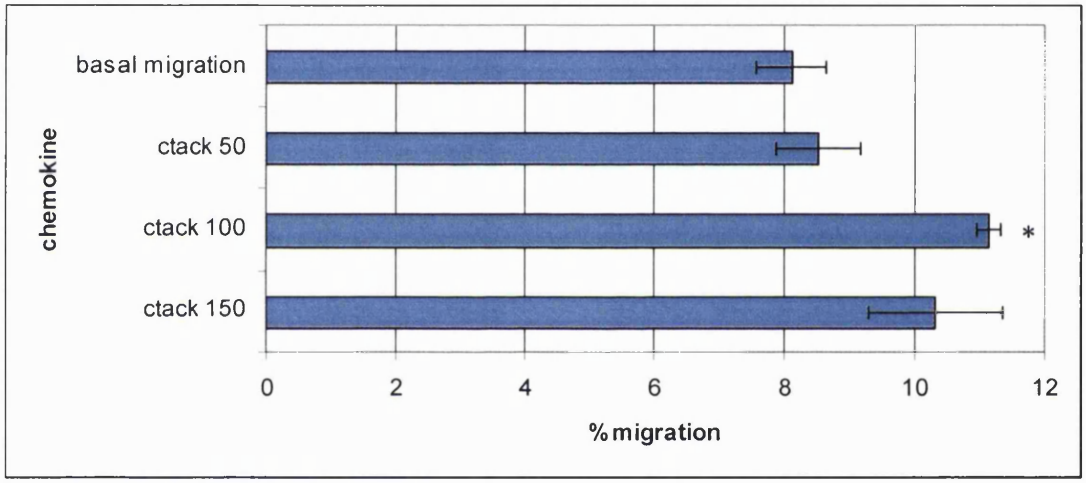
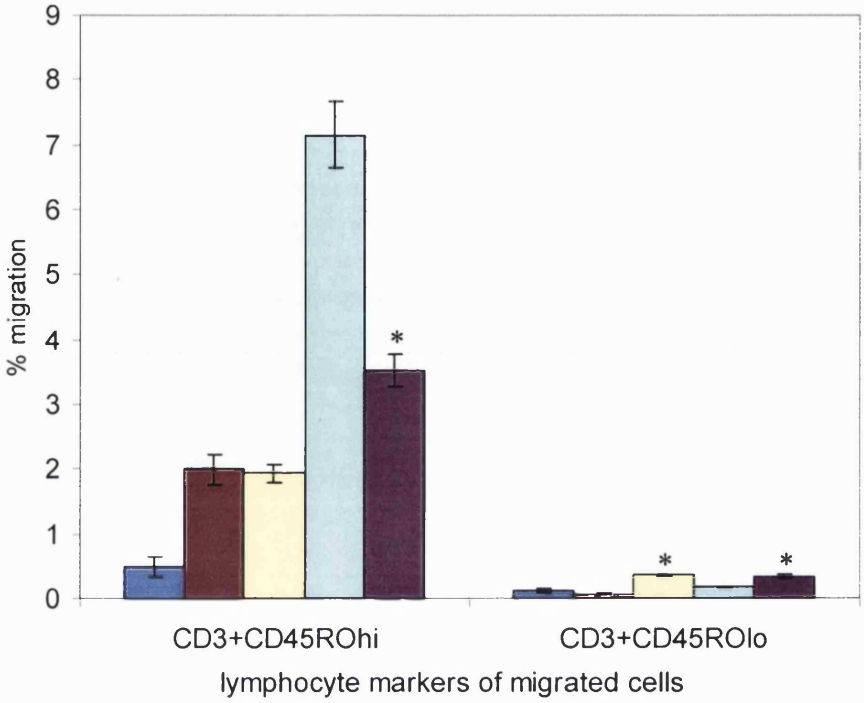
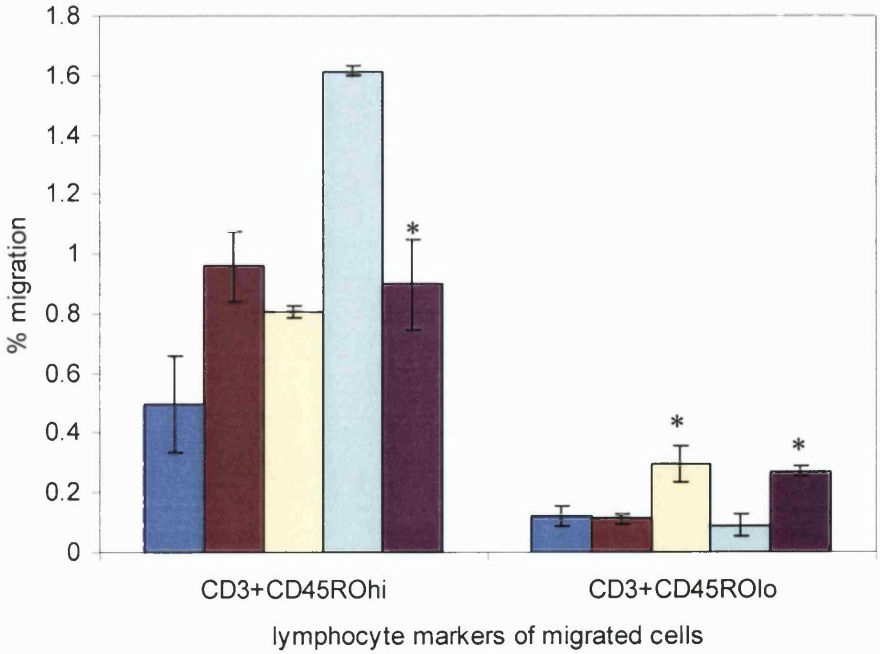


Figure 5.5: The chemotaxis of peripheral blood T cells to recombinant CTACK. PBL that had migrated were labelled with fluorescent antibodies to T cell marker CD3, memory cell marker CD45RO and 'skin-homing' marker CLA.
1) Dot plots showing migration to 100mM CTACK, SDF-1α or 100mM CTACK incubated with anti-CTACK: cell populations migrated to CTACK are enriched in CD3+CD45RO+ and CD3+CLA+ cells which is blocked by anti-CTACK treatment. An isotype control antibody showed no significant difference in the population migration to CTACK (data not shown). % migration is the normalised chemotaxis of CD3+CD45RO+ (2) or CD3+CLA+ (3) cells compared to the number of specific input cells to 50, 100 or 150mM of CTACK (ctack 50, ctack 100 and ctack 150 respectively). Basal migration is the migration of cells to cell culture medium only. * represents a significant increase in the % migration ($p<0.05$) compared to basal migration.

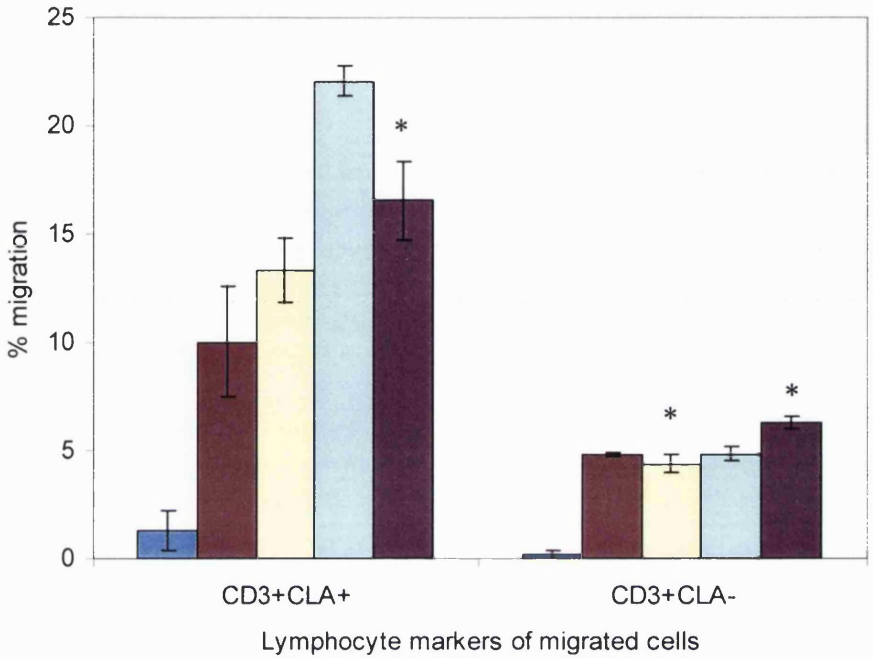
1) Chemotaxis of CD3+CD45ROhi and CD3+CD45ROlo peripheral blood cells towards unstimulated or TNF-α with IL-1β-stimulated H357 cells



2) Chemotaxis of CD3+CD45ROhi and CD3+CD45ROlo peripheral blood cells towards unstimulated or TNF-α with IL-1β-stimulated UP cells



3) Chemotaxis of CD3+CLAhi and CD3+CLAlo peripheral blood cells towards unstimulated or TNF-α with IL-1β-stimulated H357 cells



4) Chemotaxis of CD3+CLAhi and CD3+CLAlo peripheral blood cells towards unstimulated or TNF-α with IL-1β-stimulated UP cells

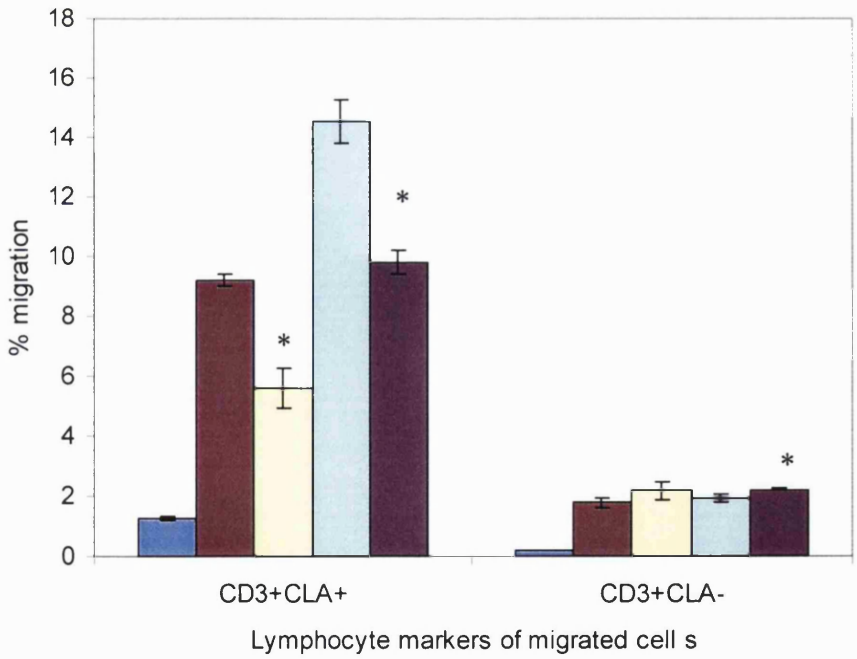


Figure 5.6: The normalised migration of T cell subsets to supernatants of non-treated or TNF-α and IL-1β treated keratinocyte cell lines.
The migration of CD3+CD45ROhi ('memory' T cells) or CD3+CD45ROlo ('naïve' T cells) to oral (H357) (1) or cutaneous (UP) 2) cell line supernatants untreated with (■) or without (□) pre-incubation with an anti- CTACK antibody, or with TNF-α and IL-1β incubation with (▢) or without, (■) pre-incubation with an anti-CTACK antibody. The migration of CLA+CD3+ or CLA-CD3+ cells to oral (H357) (3) or cutaneous (UP) (4) cell line supernatants incubated as described above. Basal migration is the migration of these cell types to cell culture medium alone (■). * represents a significant difference ($p<0.05$) in the migration induced by supernatants pre-treated with anti-CTACK or untreated.

5.3.5 Expression of CTACK in OLP and NOM tissue

CTACK mRNA is expressed in the oral inflammatory disorder OLP and in NOM, however, to low levels and to differing degrees in different samples (Fig. 5.7).

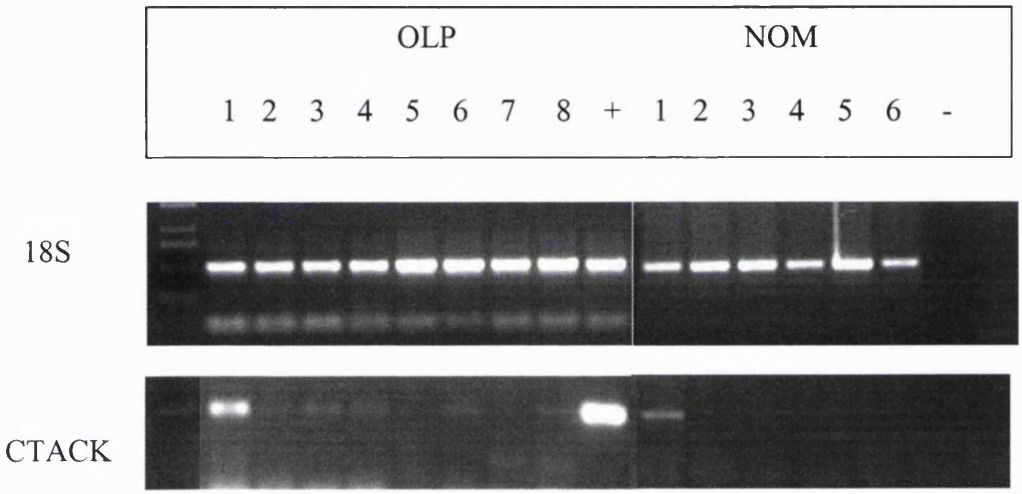


Figure 5.7: Expression of 18S and CTACK mRNA in OLP samples (1-8), NOM tissue (1-6) and positive control (+) skin mRNA.

5.4 Discussion

This is the first study to examine the ability of oral mucosal cells to express and produce immunologically-active CTACK. The results of the study indicate that CTACK can be expressed and secreted by oral epithelial cells and that this production of CTACK actively promotes chemotaxis of memory CLA+ T cells. Hence CTACK has the potential to play a role in attracting lymphocytes to the oral epithelium - as occurs in oral lichen planus - and thus indicating that this chemokine may have a role in the pathogenesis of immunologically-mediated oral mucosal disorders such as lichen planus.

In the present study CTACK mRNA was found to be expressed constitutively in primary oral epithelium. This is the first report of a tissue-specific chemokine expressed in oral epithelial cells. These present results are in agreement with studies of skin-derived keratinocytes in which CTACK mRNA was found to be expressed in the absence of cytokines and that the transcript could also be upregulated in cutaneous keratinocytes by pro-inflammatory cytokine treatment (TNF-α and IL-1β) (Morales *et al,* 1999). However, another study found that although treatment of cultured primary and transformed keratinocytes showed increased CTACK mRNA after stimulation with TNF-α and interleukin-1β, there was no increase after stimulation with IL-4 or IFN-γ (Homey *et al,* 2002). In the present study there was an increase after IFN-γ treatment, but this increase was not significant.

The results of the CTACK ELISA indicated that there is also a constitutive production of CTACK protein by oral primary cells and this level increases when treated with interferon-γ, significantly in 1 of the 3 cell lines. This accords well to the findings for

RT-PCR in the same cell type. There are no reported ELISA studies for CTACK production in other cell types, however, immunohistochemistry in skin samples showed that CTACK corresponded predominately to the epidermal cells, suggesting that these cell types produce CTACK. The present study has shown that there is a similar pattern to cutaneous tissue where there is a constitutive expression, which can be upregulated during inflammatory conditions (Homey *et al,* 2002). The present findings of high levels of constitutive CTACK release support the theory that CTACK acts as a homing molecule in healthy tissue, and can be up-regulated during inflammatory conditions. However, it then appears unusual that Homey *et al* (2002) reported that there were no CCR10+ cells found in healthy skin tissue. The presence of CCR10+ cells remains to be investigated in the oral mucosa, but as yet there is no commercial antibody available for such a study.

In the investigation of the production of CTACK from both oral and cutaneous keratinocyte cell lines there was a level of production in untreated cell types, suggesting that this chemokine is constitutively produced in oral, as well as cutaneous cell types. However, TNF-α and IL-1β treatment of these cells increased the production of this chemokine after 24hours of treatment, in the case of the H357 cells to a significant degree. As previously discussed, this is the first study reported to investigate CTACK release using ELISA however, these findings parallel those of RT-PCR of cutaneous keratinocyte mRNA that underwent an increase after TNF-α and IL-1β treatment, to increase transcripts 8 to 30 fold (Morales *et al,* 1999).

Although cytokine treatment of the cutaneous cell line did induce an increase in CTACK production, the oral cell line was more sensitive to cytokine stimulation,

even producing a significant different production after interferon-γ treatment at 24hrs. This may reflect the origin of the oral cell line (from a tongue squamous cell carcinoma), whereas the skin cell line is derived from healthy transformed skin keratinocytes (see Section 2.3). However, the production in the H357 cells appears similar to the dynamics of the oral primary epithelial cells which overall showed a significant increase in the production of CTACK protein and relative level of mRNA after IFN-γ stimulation. This suggests that CTACK production from oral epithelial cells is more sensitive to pro-inflammatory cytokine stimulation than the cutaneous keratinocytes. The cutaneous and oral mucosal environments have to confront different immunological stimuli (see below) and it may be due to these different influences that a differential production of this chemokine occurs.

It was observed that CTACK attracts memory T cells (CD45RO+) and those T cells that express CLA above the level of basal migration. This corresponds with other studies showing that 100nM CTACK attracted an increased migration of CLA+ memory T cells, but not CLA- memory cells (Morales *et al,* 1999). A range of 2-24% of the input CD4+CLA+ memory T cell population migrated at this concentration, suggesting that CTACK attracts a subset within the CLA+ cell population whose size may vary from donor to donor (Morales *et al,* 1999). Indeed the result of this study fits into this range by attracting over 11% of the CD3+CLA+ population at the same concentration of CTACK.

As previously mentioned CLA+ T cells can be detected in the oral mucosa (Walton et al, 1997), and as CTACK can be produced by oral keratinocytes, this chemokine may be, at least partially responsible for the migration of this cell type to the oral mucosa.

Untreated oral and cutaneous supernatants are capable of attracting a large population of cells bearing characteristic markers for memory (CD45RO+) and CLA+ T cells, but these numbers increase significantly following TNF-α and IL-1β treatment. This suggests that oral keratinocytes produce chemotactic elements for these cell types, which are increased after pro-inflammatory signals. Furthermore, the treatment of supernatants with anti-CTACK reduces the migration of memory T cells. This suggests that this chemokine is involved in the migration of these cell types to both oral and cutaneous keratinocytes. Although anti-CTACK treatment did reduce the migration of memory cells to untreated keratinocyte supernatants, this was not to a significant degree. This suggests that although CTACK can be produced constitutively in keratinocytes, the effect that this chemokine has on migration during non-inflamed periods is possibly minimal.

Interestingly, although supernatants derived from TNF-α and IL-1β treated cutaneous and oral keratinocytes both demonstrate a significantly reduced ability to attract memory (CD45RO+) and CLA+ T cells when CTACK is blocked, in cutaneous cells the block reduced the migration to the level of non-treated cells, suggesting a large component of TNF-α and IL-1β-influenced migration in cutaneous cells is caused by this chemokine. The reduction of migration after blocking CTACK in TNF-α and IL-1β treated oral cell supernatants to levels above non-treated supernatants suggests that there are other influences in the supernatants, besides CTACK (presumably other chemokines) also affecting migration of this cell type. There are no reported chemotactic effects of TNF-α and IL-1β, but TNF-α is a crucial cytokine in directing migration (Reviewed by Sedgwick *et al,* 2000) and therefore may be influencing the

induction of other chemokines from epithelial cells. It should also be noted in the present study the migration of 'resting' peripheral blood cells was examined, thus activated cells, which would be likely to express other chemokine receptors, for example, CXCR3, may not be blocked to such a large extent by the blocking of CTACK activity. CTACK would thus appear to have a role in the early migration of non-activated T cells to inflamed epithelial sites in the skin and oral mucosa.

The subsequent increase in naïve cells migrating after blockade of CTACK appears to be a reflection of the reduced memory cell population migration, thus allowing more 'random' migration to occur, including CD3+CD45ROlo cells.

In OLP and NOM tissue CTACK mRNA was expressed, but only at low levels in most of the samples tested. This is perhaps surprising as the concentration of CTACK released from oral keratinocytes was found to be similar than the amount from skin keratinocytes. However, the presence of the tissue-homing chemokine TARC in skin contact allergenic diseases is expressed early in inflammation (Sebastini *et al,* 2001; Martin *et al,* 2002) opposed to the CXC ELR- chemokines (Sebastini *et al,* 2001) which are expressed later. Therefore, the homing chemokines may still play a role in progressing OLP lesions and not to the same extent in the chronic lesions tested in this study. Furthermore, CTACK could be involved in the adjustment of CLA+ cells within the epithelial microenvironment, as appears to be the case in lichen planus lesions (Walton *et al,* 1995).

Any migratory CCR10+ cells may play a role in the exacerbation of OLP lesions as they are capable of producing IFN-γ and TNF-α (Hudak *et al,* 2002) which may

induce further chemokine production from the epithelium. It is also interesting to note that TNF-α expression is found in most OLP lesions (Sklavounou *et al*, 2000) and TNF-α is produced in higher amounts in OLP-derived keratinocytes compared to normal tissue *in vitro* (Yamamoto *et al,* 1994). Furthermore, TNF-α and IL-1β can be produced by keratinocytes and Langerhan's cells after only 4-24 hours of contact allergens (Rambukkana *et al,* 1996). As some lichenoid reactions, for example, amalgam-associated, appear to be caused by the presence of contact allergens, it can be speculated that these allergens upregulate the expression of these pro-inflammatory cytokines in keratinocytes and Langerhans cells. The expression of these cytokines would appear to be sufficient (e.g. from the present *in vitro* studies) to cause the increase in chemokines such as CTACK (and MIP3-alpha) from the oral epithelium, which may be a causative factor in the pathogenic T cell infiltration witnessed in lichen planus. Furthermore, the fact that CTACK attracts effector cells is interesting as these cells can undergo expansion with a lesser requirement of antigen (Busch *et al,* 2000).

The influence that CTACK has during skin inflammation has been debated. Whereas Homey *et al* (2002) suggested that experimentally-induced skin inflammation could be blocked to a large extent by anti-CTACK treatment, Reiss *et al* (2002) and Soler *et al* (2002) suggest that TARC, the other known skin-homing chemokine, is perhaps more important in the migration of a large proportion of CLA+ cells to the skin (as the receptor for TARC, CCR4, is expressed on most CLA+ cells (Campbell *et al*, 1999), whereas, CTACK attracts a subset of CLA+ cells from within the memory effector phenotype. Soler *et al* (2002) suggest that CTACK may be more important in the migration of cells from within the cutaneous (thus, presumably also the oral) micro-

environment. As CTACK is produced predominately by the epithelium, it seems likely that this area would be the target within the oral mucosa for migratory CCR10+ cells. Alternatively, it has been suggested that as it attracts effector cells, this chemokine is responsible for the future recruitment of the larger subpopulation of CLA+ T cells, perhaps through the regulation of TARC expression. This perhaps explains why TARC is expressed predominately by the endothelium, whereas CTACK is expressed by the epithelium. However, CTACK can also be found upon the surface of endothelial cells (Homey *et al,* 2002), and thus may also have a role in the arrest of CCR10+ cells on vascular endothelium.

As CTACK is involved in the preferential migration of skin-homing T cells that have previously encountered antigens in the skin, this suggests that memory T cells re-circulating within the oral mucosa have immunological 'memory' for skin-derived antigens and *vice versa*. This may assist in explaining the fact that there is some cross-over in the occurrence of oral lichen planus and cutaneous lichen planus. The reaction in the secondary lymph nodes appears to determine the tissue homing specificity of T cells (Campbell and Butcher, 2002), thus it would appear to make sense that skin and oral homing cells feature in the same reaction, especially as it may be linked to the type of antigen-presenting cells in these tissues (as Langerhans cells are located in both skin and mucosa).

In fact, if an antigen sampling process exists in the oral mucosa, it is interesting to speculate that the T cells that respond to the homing tissue-specific chemokines, such as CTACK, which are thought to traffic during non-inflamed and periods of early inflammation and have lost the ability to home to the lymph nodes may be involved in

this antigen-sampling process. This would assist in explaining the fact that the chemokines are expressed in a tissue-specific manner (as it could be that tissue-specific memory T cells are more likely to encounter potential pathogens in the same area of the body and less likely to have a cross-reaction towards tolergenic self antigen of other areas of the body than systemic T cells). Furthermore, these chemokines are characteristically produced by the epithelium, which may act in order to bring these homing memory cells in contact with the epithelium. This would also correlate with present theories that CCR10+ cells are involved in immune surveillance of tissues (Hudak *et al,* 2002). Furthermore, the characterisation of these cells as effector memory cells (i.e. CCR7-) (Soler *et al,* 2003) suggests that they would more likely to produce a wide range of cytokines, including IFN-γ, upon antigen stimulation, unlike CCR7+ memory cells that only produce IL-2 (Sallusto *et al,* 1999).

Whether there is another adhesion molecule-ligand or chemokine-ligand that specifies the oral mucosa in the compartmentalism of the immune system is unknown, however, it is interesting to note that αeβ7-positive (CD103) T cells are upregulated in OLP, but not in the cutaneous condition (Walton *et al,* 1997), suggesting that this ligand may influence migration in inflammation of the oral cavity to a greater degree than the cutaneous environment. However, this upregulation may be in order for T cell adherence to epithelial cells (Reviewed in Agace *et al,* 2000) rather than a specific role in migration. In fact, CD103+ CTL demonstrate a specific lysis of epithelial cell targets (Rostapshova *et al,* 1998), suggesting that these cells could play a role in OLP pathogenesis. There may also be a chemoattractant specific for this cell type to the epithelial area.

It is also interestingly to speculate whether any other tissue-specific chemokines are expressed in the oral mucosa, especially as it shares features with other mucosal sites such as the bronchial tissue. The fact that almost all CLA+CCR10+ cells co-express the ligand for TARC (CCR4) (Hudak *et al,* 2002) and that TARC is also expressed in nasal tissue epithelium (Terada *et al,* 2001), cutaneous keratinocytes *in vivo* (Martin *et al,* 2002) and *in vitro* (Yu *et al,* 2002) and in the endothelium of cutaneous lichen planus patients (Campbell *et al,* 1999) would suggest that this chemokine can be expressed in the oral cavity. However, despite the relatively large distribution of TARC expression, CCR4+ expression on skin-homing cells is expressed to a higher degree than those homing to other tissues, such as bronchial tissue (Kunkel *et al,* 2002) and this level of expression correlated with a lesser ability in adhesion and chemotaxis assays. However, it remains to be seen if oral mucosa-homing cells are also CCR4hi and thus would have a high responsiveness to TARC. Interestingly, chemokine receptor expression on T cells has been shown to change in aged mice, including upregulation of CCR4+ cells (Mo *et al,* 2003). This may produce an increased responsiveness to TARC in older patients, the profile witnessed in OLP patients, which may in turn cause an increase in T cell infiltration to the cutaneous (and possibly the oral environment).

Although many studies utilise TARC as a classical Th2 marker (Reviewed in Romagnani, 2001) it was found that CCR4+ cells are also associated with contact hypersensitivity and DTH reactions (Kunkel *et al,* 2002), both considered Th1 reactions, and akin to the proposed reaction in OLP. Thus the expression of this chemokine in cutaneous lichen planus is not surprising. Furthermore, other studies

have suggested that TARC is produced during autoimmune conditions (Kim *et al,* 2002) and therefore may also play a role in this type of inflammation.

The role of TARC in the epithelium remains elusive, Th1 or Th2 chemokines did not upregulate TARC expression in keratinocytes (Sebastini *et al,* 2002) and although TARC mRNA was upregulated in stimulated keratinocytes, there was no effective secretion of TARC protein in some studies (Horikawa *et al,* 2002).

MEC, the chemokine which shares CCR10 binding with CTACK may also be expressed in the oral mucosa. As Kunkel & Butcher, 2002 discuss, although they are disparate areas of the body, the colon (where MEC is expressed) has a similar microflora to the oral cavity (Kroes *et al,* 1999) and the production of MEC may attract cells that are protective against this environment. If MEC was also found to be produced by the oral mucosa, this would suggest that the oral mucosa is a unique cross-over between the cutaneous and mucosal immunological systems, presumably able to provide protection to mucosal and cutaneous-associated pathogens.

Furthermore, MEC is highly expressed in the salivary gland, and the proximity of the oral mucosa to this tissue makes it a candidate for expression in this area also. In fact, it has recently been shown that MEC is secreted in saliva and functions as an antimicrobial molecule against oral bacterium (Hieshima *et al,* 2003). However, the expression in the salivary gland is perhaps a reflection of the glandular nature of this tissue (as this chemokine is also expressed in the mammary glands) rather than its mucosal nature. In fact, this chemokine has recently been shown to be important in influencing the migration of cells that produce IgA (Lazarus *et al,* 2003; Kunkel *et al,*

2003), in a similar role to the related tissue-specific chemokine TECK (Bowman *et al,* 2002) which is expressed mainly in the small intestine (Kunkel *et al,* 2000). However, MEC appears to have no role in the migration of CCR10+ T cells. Intriguingly, IgA is upregulated in saliva of OLP patients (Sistig *et al,* 2002), presumably derived from the salivary glands, which express high levels of MEC (Hieshima *et al,* 2003). However, the production of secretory IgA is not a property of either the cutaneous or oral cavity, thus MEC may not expressed in these tissues.

Thus, the expression of CTACK in the oral epithelium could perhaps be of influence in causing the migration of 'effector-memory' CLA+ cells. The production of this chemokine in the oral epithelium at levels above cutaneous cells and the block of memory CLA+ T cell migration by using anti-CTACK, suggests that this chemokine may also play a large role in the migration of cells bearing CCR10 to the area of the oral epithelium, as well as the cutaneous epithelium. Its potential role as a 'homing' chemokine produced constitutively in the oral epithelium suggests that this chemokine may also play a role in the early recruitment of T cells that may influence the progression of oral inflammatory diseases, such as OLP (Fig 5.8).

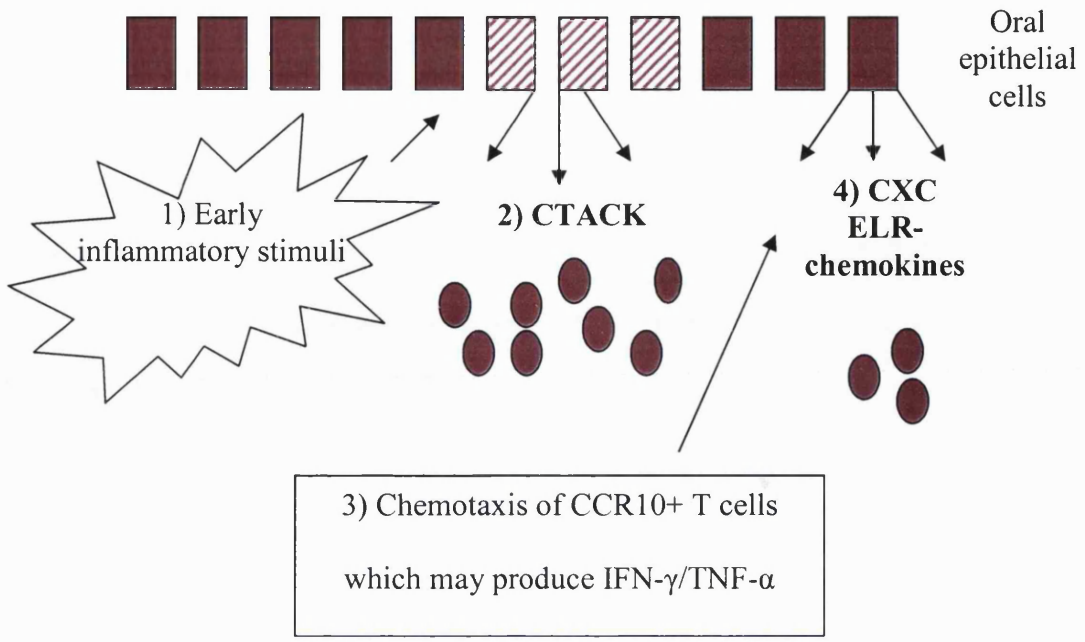


Figure 5.8: The potential role of CTACK in oral lichen planus. 1) An early inflammatory stimuli may enhance CTACK production from the oral epithelium (2), thereby inducing migration of 'effector' CCR10+ cells, which can produce IFN-γ rapidly after contact with antigen (3). This cytokine production in the vicinity of the epithelium may induce the production of the CXC ELR- chemokines from the oral epithelium (4) causing the Th1-based inflammatory state.

Chapter 6: MIP-3alpha production in oral epithelium and inflammation

6.1 Introduction

Macrophage inflammatory protein-3 alpha (MIP-3α), or CCL20, is a chemokine which belongs to the CC chemokine family, specifically attracting cells bearing CCR6 (Baba *et al,* 1997).

MIP3-α was first identified in liver and kidney tissue, but has since been discovered in many different cell types. Neutrophils, PBMC (Scapini *et al,* 2001), dermal microvascular endothelial cells (Charbonnier *et al,* 1999), epithelial cells [including intestinal (Fujiie *et al,* 2001; Izadpanah *et al,* 2001) and cutaneous keratinocytes (Schmuth *et al,* 2002; Charbonnier *et al,* 1999; Dieu-Nosjean *et al,* 2000)] can all produce MIP-3α. There is debate as to whether there is a constitutive or induced expression of MIP-3α in epithelial cells, but all studies agree that TNF-α and IL-1β stimulation of epithelial cells enhances MIP-3α production. There may be an overlap in the role of MIP-3α as a homing chemokine, produced constitutively in some cases, but also actively upregulated in some types of inflammation. The *in vitro* production of MIP-3α in the oral mucosa has never been investigated, although a study suggested that this protein was upregulated in the gingival epithelium in periodontitis (Hosokawa *et al,* 2002).

MIP-3α attracts cells specifically bearing the receptor CCR6 (Charbonnier *et al,* 1999; Dieu-Nosjean *et al,* 2000). This molecule is expressed upon CD1a+ dendritic cells (CD34+ve derived), and furthermore the recruitment of these Langerhans cells to TNF-α/IL-1β-stimulated keratinocyte supernatant can be blocked by MIP3-α antibody

(Dieu-Nosjean *et al,* 2000). CCR6 has also found to be preferentially expressed on memory T-cells (CD45RO+) (Ebert & McColl, 2002; Liao *et al,* 1999), both on CD4+ and CD8+ subsets, although it is thought that these cells represent a resting memory T cell phenotype and, therefore, may be part of homeostatic homing (Liao *et al,* 1999). CCR6 is expressed on cells bearing skin homing molecule and mucosal homing molecules (Liao *et al,* 1999), suggesting that these cells will migrate to both skin and mucosal sites. However, CCR6 appears not to be induced on all memory T cells and therefore may represent a distinct subpopulation of memory T cells (Ebert & McColl, 2002).

MIP-3α and the receptor CCR6 have been found to be upregulated in inflamed tonsils (Dieu-Nosjean *et al,* 2000), psoriasis (Homey *et al,* 2000; Schmuth *et al,* 2002) and other skin inflammatory conditions (Schmuth *et al,* 2002), as well as periodontitis (Hosokawa *et al,* 2002). In psoriasis, the suprabasal areas of the epithelium showed strong levels of MIP-3α staining (Homey *et al,* 2000).

As previously outlined, oral lichen planus is characterised by a large inflammatory infiltrate that consists mainly of memory T cells (Walton *et al,* 1998). Furthermore, although there is debate on whether Langerhans cell numbers are increased in OLP, there may be an alteration in the location of these cells to the epithelial area (Chou *et al,* 1993; Rich *et al,* 1989). It may be that the large influx of memory T cells and the change of location of Langerhans cells in lichen planus may be at least partially due to MIP-3α expression in the epithelium of these patients (Fig 6.1).

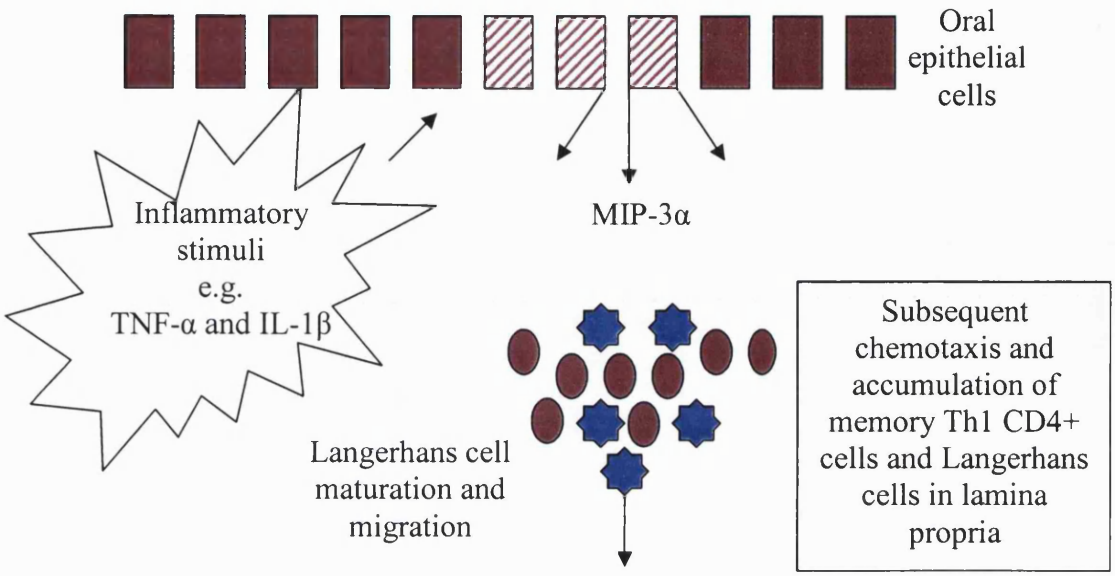


Figure 6.1: The potential role of epithelial cell-produced MIP-3α in OLP. The oral disorder is perhaps initially triggered by inflammatory stimuli. This, in turn may induce MIP-3α production from nearby oral epithelial cells which would presumably recruit large numbers of memory T cells and Langerhans cells bearing CCR6 in a band-like pattern in the oral lamina propria. Langerhans cells may then mature and travel to the lymph nodes to activate specific T cells, which would then presumably migrate and infiltrate OLP lesions.

Hence the aim of this section was to establish if MIP-3α can be expressed by oral mucosal cells and if the molecule and its receptor is upregulated in oral lichen planus. Specifically, the aims of this study were to:

- To determine the effects of pro-inflammatory cytokine (TNF-α with IL-1β or IFN-γ) treatment on the production of MIP-3α by oral and cutaneous keratinocyte cell lines
- To determine MIP-3α mRNA expression in oral and cutaneous keratinocyte cell lines following stimulation with TNF-α with IL-1β or IFN-γ)
- To determine MIP-3α and CCR6 mRNA expression levels in normal oral mucosa and oral lichen planus

6.2 Materials & Methods

6.2.1 Production of MIP-3α by oral and cutaneous keratinocyte cell lines stimulated with TNF-α with IL-1β or IFN-γ

The cytokine treatment assay as previously described (Section 2.3.6) was utilised under the following conditions: H357 (2.3.2) and UP (2.3.3) cells were incubated with 10ng/ml recombinant human TNF-α (R&D systems, Oxon, UK) with 5ng/ml IL-1β (Peprotech, London, UK) or 1000U/ml IFN-γ (Sigma-Aldrich, Poole, Dorset) for 3, 6 or 24 hours.

To determine the levels of MIP-3α in culture supernatants, ELISA was performed as described previously (section 2.4) utilising a MIP-3α (CCL20) ELISA development system (R&Dsystems, Oxon, UK). The capture antibody - mouse anti-human MIP-3α - as used at a concentration of 2μg/ml diluted in PBS. After washing the coating antibody, 100μl of blocking buffer [1%BSA, FractionV (Sigma, Poole, UK), 5% sucrose (BDH, Poole, UK) in PBS (Sigma, Poole, UK), pH7.2-7.4, 0.2μm filtered] was added to each well and incubated for 2 hours at room temperature. The secondary antibody used was biotinylated goat anti-human MIP3-α (R&D systems, Oxon, UK). All antibodies (except capture) and standards were diluted with 1% BSA in PBS (pH7.2-7.4, 0.2μm filtered). The general ELISA protocol (Section 2.4) was followed thereafter, except that OPD was incubated for 20mins.

6.2.2 MIP-3α mRNA expression in oral and cutaneous keratinocyte cell lines stimulated with TNF-α with IL-1β or IFN-γ

H357 and UP cells were treated were with the pro-inflammatory cytokines, TNF-α with IL-1β or IFN-γ, as described previously (7.2a). RNA isolation (2.9.2), cDNA synthesis (2.9.3) and RT-PCR of H357 and UP cells were performed as described previously. The primers generated and utilised in this study were:

MIP-3α (5'-CTGTACCAAGAGTTTGCTCC-3'; 5'-GCACAATATATTTCACCAAG-3') (Genosys-Sigma, Poole, UK)

These were utilised at an annealing temperature of 57°C and the general RT-PCR protocol was followed thereafter.

6.2.3 MIP-3α and CCR6 mRNA expression in normal oral mucosa and oral lichen planus

RNA isolation (2.9.2) and single strand cDNA synthesis (2.9.3) of NOM and OLP tissue was performed as described. RT-PCR was executed as described (2.9.4) for MIP-3α as described above for the H357 and UP cells. In addition, RT-PCR for CCR6 was carried out with the following primers (Genosys-Sigma, Poole, UK):

CCR6: (5'-TTTTTCTGCCCACAATCAGCGG-3'; 5'-GCATACCTGGCCATAGACTTTTT-3')

The same parameters as described for MIP-3α RT-PCR were utilised for this reaction.

6.3 Results

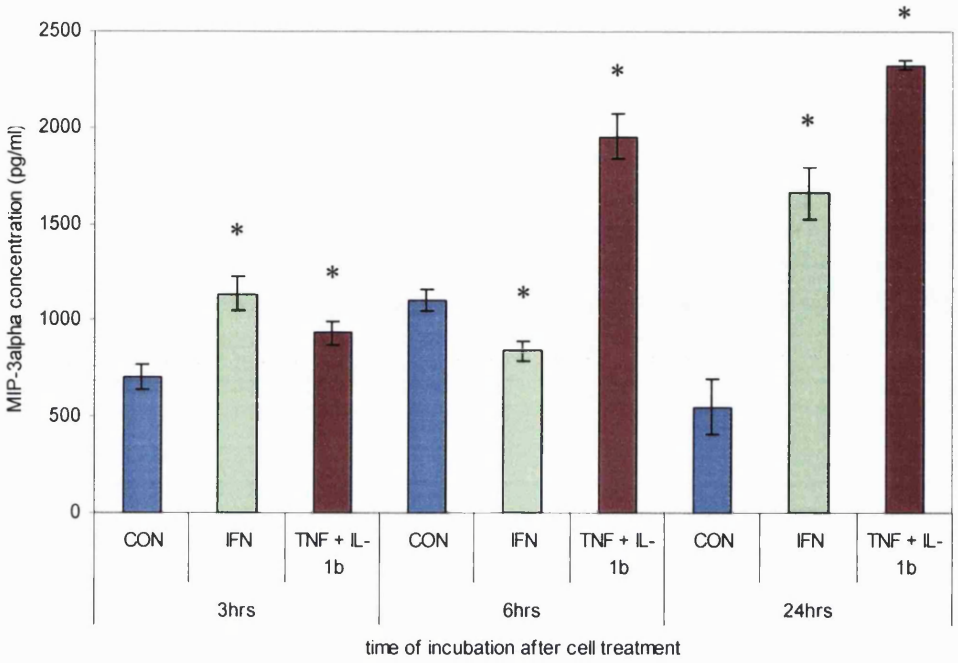
6.3.1 Production of MIP-3α by oral (H357) and cutaneous (UP) keratinocyte cell lines

MIP-3α was produced by unstimulated H357 (oral) cells but not UP (cutaneous) cells (Fig 6.2). Indeed, over 500pg/ml of MIP-3α was produced by the H357 cells only 3 hours of incubation time. In contrast, unstimulated UP cells only produced MIP3-α after 24 hours of incubation without cytokine stimulation.

The concentration of MIP-3α produced by H357 cells following stimulation with TNF-α and IL-1β was significantly higher than unstimulated cells at every time point investigated. The concentration produced by TNF-α and IL-1β treated H357 cells increased over time, with the greatest amounts being produced after 24 hours. IFN-γ stimulation induced significantly increased amounts of MIP-3α than non-stimulated cells at 3 and 24 hours, however, significantly less MIP-3α was produced after 6 hours of IFN-γ stimulation than the control cells.

The pattern of MIP-3α production with or without cytokine expression of cutaneous (UP) cells was different to that of the oral (H357) cells. Unstimulated UP cells did not produce any MIP-3α until 24 hours of incubation, levels never being as high as those of unstimulated H357 cells at any recorded time point. MIP-3α production was induced by TNF-α and IL-1β stimulation only after 6 hours of stimulation ($p<0.05$) and IFN-γ stimulation only induced production at the 24 time point but this was not a significant increase. The concentration of MIP3-α by the UP cells at 24 hours with or without cytokine stimulation was lower than the corresponding stimulation of the H357 cells.

i)



ii)

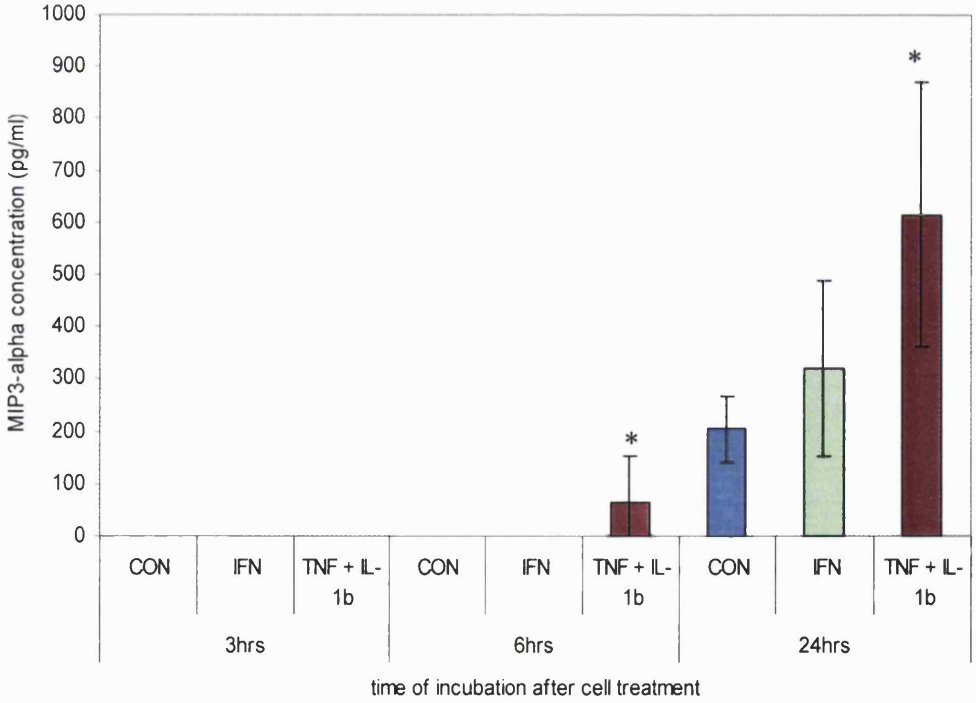


Figure 6.2: The concentration of MIP-3α produced in a) H357 cells or b) UP cells treated with IFN-γ (IFN), TNF-α and IL-1β (TNF + IL-1b) or untreated in culture (CON) for 3, 6 or 24 hours. The results are the mean results of triplicate experiments ±SD. (* represents a p value of <0.05 compared to unstimulated (control) cells at the same time point).

6.3.2 MIP-3α mRNA expression in oral and cutaneous keratinocyte cell lines with stimulation by TNF-α with IL-β or IFN-γ

Unstimulated H357 cells expressed MIP-3α mRNA, stimulation with TNF-α and IL-1β increased MIP-3α mRNA expression at 24hours ($p<0.05$) (Fig 6.3a & bi). IFN-γ stimulation did not significantly increase mRNA transcript expression at any time-point.

Unstimulated UP cells only expressed MIP-3α mRNA after 6 or 24 hours of incubation time (Fig 6.3a & bii). Stimulation with TNF-α and IL-1β did increase the expression of MIP-3α mRNA after 24hours of stimulation ($p<0.05$). Stimulation with IFN-γ only caused expression of MIP-3α mRNA after 6 and 24hours of incubation time ($p>0.05$). The ratios of MIP-3α mRNA to 18S mRNA by UP cells were lower in all instances compared to the respective treated H357 cells.

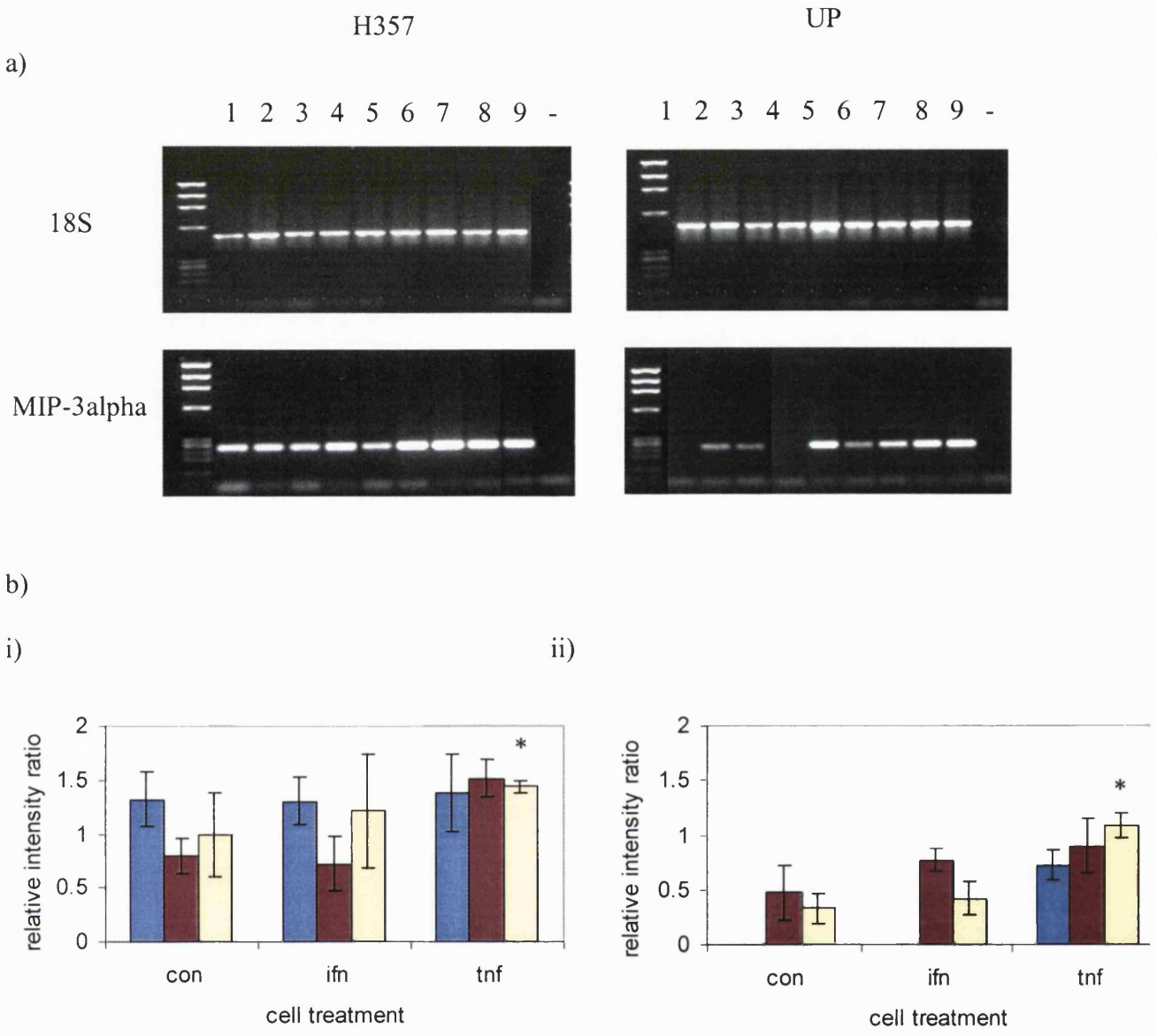


Figure 6.3: a) The expression of 18S and MIP3-α in H357 or UP cells 3, 6, 24 hours after no treatment (1-3 respectively), after treatment with 1000U/ml IFN-γ (4-6) or with TNF-α and IL-1β (7-9), – represents the negative control for this experiment, b) Densitometric analysis of the MIP-3α to 18S mRNA expression in the i) H357 and ii) UP cells treated as described above for 3 (■), 6 (■) or 24 (■) hours. * represents a significant difference ($p<0.05$) between treated and control cells at the same-time point.

6.3.3 Determine MIP-3α and CCR6 mRNA expression levels in normal oral mucosa and oral lichen planus

MIP-3α mRNA was expressed in all examined OLP and normal oral mucosa samples (Fig 6.3), but there was no significant between the two groups on average ($p>0.05$) (Table 6.1). In addition, expression of CCR6 mRNA was detected in 5/6 of both the OLP and NOM samples but again was not found to be significantly different ($p>0.05$). The mRNA levels for MIP-3alpha and CCR6 in both tissue types varied between samples, as represented by the relatively high standard deviation values.

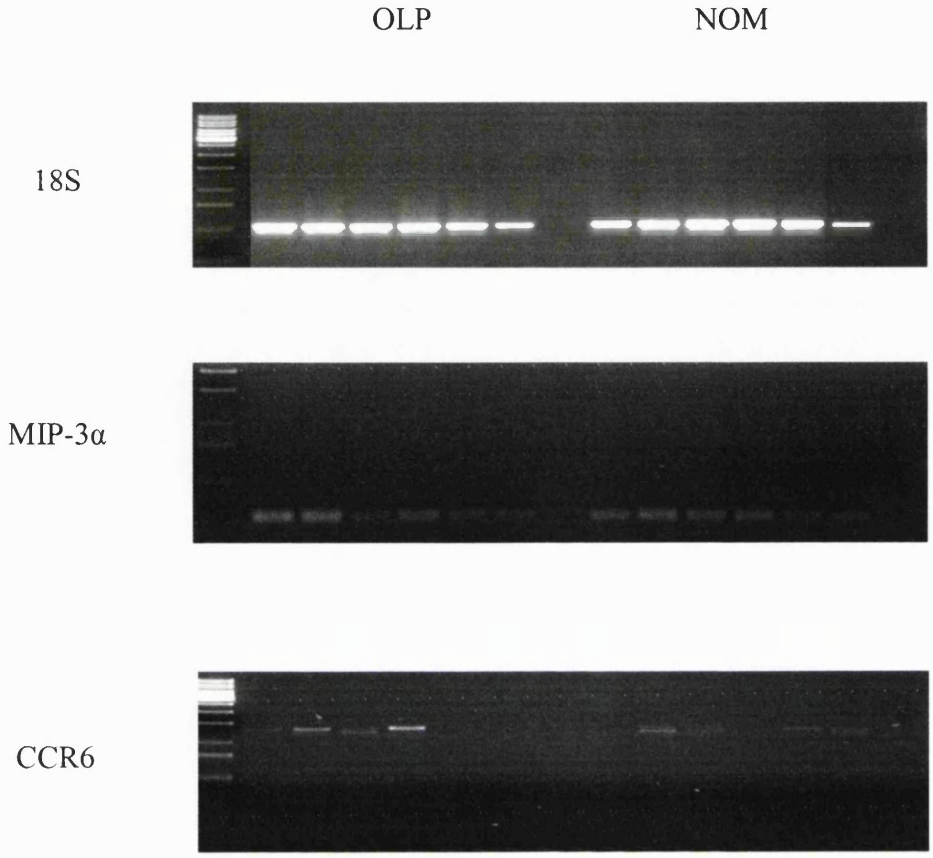


Figure 6.4: The expression of 18S, MIP-3α and CCR6 mRNA in six different oral lichen planus lesional tissue samples (OLP 1-6) and normal oral mucosa (NOM 1-6).

Table 6.1: Densitometric analysis of the MIP-3α and CCR6 mRNA expression in oral lichen planus (OLP) and normal oral mucosal (NOM) tissue. The values shown in the table are the mean ratios of six different samples of each tissue type. The standard deviation between the samples are indicated in brackets.

Tissue type	Ratio 18S : MIP-3alpha mRNA	Ratio 18S: CCR6 mRNA
OLP	0.132 (± 0.117)	0.178 (±0.112)
NOM	0.129 (± 0.087)	0.159 (± 0.132)

6.4 Discussion

The results of this study demonstrate that oral mucosal keratinocytes can constitutively produce MIP-3α in high concentrations. This production of MIP-3α is enhanced by inflammatory stimuli, such as TNF-α with IL-1β, and in some cases by IFN-γ. In contrast, cutaneous keratinocytes, such as the UP cell line, express only low levels of MIP-3α when unstimulated. MIP-3α production of cutaneous cells is not greatly enhanced by TNF-α and IL-1β nor IFN-γ stimulation until after 24hrs of stimulation. This is the first report of *in vitro* production of MIP3-α by oral epithelial cells thus demonstrating that this production could be of importance for memory T cell and Langerhans cell trafficking in the oral cavity.

The present observation of constitutive production of MIP-3α by resting oral epithelial cells is in agreement with findings in cutaneous keratinocytes by Chabonnier, 1999 however other studies (Homey *et al,* 2000; Dieu-Nosjean *et al,* 1999; Tohyama *et al,* 2001) did not observe MIP-3α protein production nor mRNA expression in unstimulated keratinocytes. Some, but not all studies (Izadpanah *et al,* 2001) of intestinal epithelial cells observed no constitutive expression of MIP-3α (Fujiie *et al,* 2001). It would presently seem that the detection of MIP-3α in resting cells may be dependant upon the specific cell type being examined and the method of cell culture.

The cells utilised for the study of oral keratinocytes was an oral carcinoma cell line and therefore may not be wholly representative of keratinocytes of the normal oral mucosa, However, in Chapters 3 and 5 the production of chemokines by the H357 cells has corresponded well to the findings of primary oral keratinocytes (refer to

Sections 3.3 and 5.3) and thus there would seem to be no reason why present observations would not correspond with findings of oral epithelial cells. There were striking differences in the pattern of production of MIP-3α production by the oral (H357) and cutaneous (UP) cell lines. Different cell lines from the same tissue origin can differ in MIP3-α expression patterns (Fujiie *et al,* 2001) and therefore may also differ between individual cell lines. However, as MIP-3α is considered to be important during mucosal immune responses (Tanaka *et al,* 1999; Fujiie *et al,* 2001; Izadpanah *et al,* 2001) (as opposed to cutaneous surfaces) a higher expression by the oral cells may reflect the mucosal nature of the oral epithelium. If this was the case then trafficking of CCR6-bearing cells would be expected to be higher to the oral mucosa than to cutaneous environment. However, recent findings have suggested that MIP-3α is also important during cutaneous inflammation (Schmuth *et al,* 2002; Homey *et al,* 2000). Differentiated keratinocytes are the main producers of TNF-α-stimulated MIP-3α (Tohyama *et al,* 2001) and therefore UP cells may represent a cell line derived from basal epithelial cells. Alternatively cutaneous keratinocytes perhaps demonstrate delayed induction in comparison to mucosal sites. For example, previous studies have only investigated MIP3-α protein production in cutaneous keratinocytes at time points greater than 20hours (Nakayama *et al,* 2001; Dieu-Nosjean *et al,* 2000; Homey *et al,* 2000), thus earlier expression is unknown and may be delayed in comparison to mucosally-derived keratinocytes, such as oral keratinocytes in the present study or intestinal epithelial cells (Fujiie *et al,* 2001).

The present results indicate that stimulation with TNF-α and IL-1β rapidly leads to production of MIP-3α by orally derived cells. In contrast, there was a delayed response to TNF-α and IL-1β, or IFN-γ stimulation by cutaneous cells. The present

findings for the H357 cells accord with those of intestinal epithelium (Izapadpanah *et al,* 2001) and neutrophils (Scapini *et al,* 2001) in that there is an up-regulation of MIP-3alpha after TNF-α and IL-1β stimulation in these cell types.

In the present study, IFN-γ enhanced the production of MIP-3α from oral keratinocytes after 3 and 24 hours, but did not significantly enhance production at any time-point in cutaneous keratinocytes. These results likewise accord with those of IFN-γ stimulation on intestinal epithelium in which no increased levels of MIP-3α production arose (Izadpanah *et al,* 2001). Our findings may also be linked to the finding that oral keratinocytes are more responsive to IFN-γ stimulation than cutaneous keratinocytes in upregulating ICAM-1 expression (Li *et al,* 1996) and IL-6 production (Li *et al,* 1999). Thus, IFN-γ stimulation may also cause an increased production of MIP-3α in comparison to cutaneous cells, as was the case in the present study.

The present results of MIP-3α mRNA expression with or without cytokine stimulation generally accorded with those of MIP-3α protein production. MIP3-α mRNA was expressed constitutively and was enhanced by TNF-α and IL-1β stimulation of the investigated oral and cutaneous epithelial cell lines. These results correspond to previous findings in keratinocytes (Dieu-Nosjean *et al,* Homey *et al,* 2000; Toyhama *et al,* 2001) and intestinal epithelial cells (Fujiie *et al,* 2001, Izhadpanah *et al,* 2001), where the combination of TNF-α and IL-1β was a strong stimulant for the production of MIP-3α.

MIP-3α mRNA was expressed in TNF-α and IL-1β stimulated keratinocytes after only 3 hours in the cutaneous epithelial cells (but not in unstimulated or IFN-gamma stimulated cells), whereas at the same time point there was expression in all treated and non-treated H357 cells, slightly increased by TNF-α and IL-1β treatment. There is a rapid induction of MIP-3α mRNA following stimulation with TNF-α (and IL-1β treatment) by neutrophils (Scapini *et al,* 1999) or intestinal epithelial cell lines (Izapadpanah *et al,* 2001). Neutrophil and intestinal epithelial cell mRNA decreases after 3 and 12hours stimulation respectively, whereas in this study mRNA levels remain static at all time-points following TNF-α and IL-1β treatment for H357 cells but continues to increase to 24hours in the UP cells. This later peak in UP cells is perhaps is due to the apparent slower induction for MIP-3α in this cell type.

Reports disagree on whether IFN-γ can increase MIP3-α mRNA expression. Some, but not all (Homey *et al,* 2000), reported studies suggest that IFN-γ does not enhance mRNA expression in keratinocytes (Tohyama *et al,* 2001). Even when there is increased expression the amount of MIP-3α mRNA produced with IFN-γ stimulation is less than that of TNF-α stimulation (Homey *et al,* 2000). In neutrophils, there are biphasic stimulatory effects of IFN-γ upon MIP3-α release, it significantly diminished the production of MIP-3α in neutrophils incubated with LPS for up to 6 hours, whereas it elicited a greater MIP-3α production after 21 hours of stimulation (Scalpini *et al,* 2002). As we found similar results for the H357 cells treated with IFN-γ alone in this study this may also be true for oral keratinocytes.

The production of MIP3-α by oral keratinocytes constitutively and during inflammatory conditions is likely to have significant consequences upon dendritic cell

and CCR6+ T cell migration to the oral cavity and hence could have a potential role in the pathogenesis of oral lichen planus. However, despite the high production of MIP-3α mRNA and protein in H357 cells, there was only a small upregulation of MIP-3α expression in the examined oral lichen planus. Although the upregulation of this chemokine has been reported in periodontitis (Hosokawa *et al,* 2002) and some other skin pathologies (Homey *et al,* 2000; Schmuth *et al,* 2002), there appears to be a differential upregulation. Of note, in diseases previously compared to OLP; chronic graft-versus-host disease and allergic contact dermatitis, MIP3-α is only slightly upregulated (Schmuth *et al,* 2002). The small upregulation of MIP3-α in OLP may be surprising considering that there are TNF-α and IL-1β-expressing cells located in OLP (Simark-Mattson *et al,* 1999) and that large amounts of this chemokine can be produced from oral epithelial cell line even compared to a cutaneous epithelial cell line.

It is thus now important to try to explain if indeed MIP3-α does have a role in the pathogenesis of OLP. There are perhaps conflicting results; *in vitro* oral epithelial cells seem capable of expressing and producing MIP3-α, but in oral lichen planus MIP-3α expression is not greatly increased. The chronicity of OLP may mean that by the time the disease has manifested clinically and the biopsy tissue obtained the expression of this chemokine may have already peaked and its role in early development of OLP missed. Indeed, CCR6 is only expressed on immature dendritic cells (Vanbervliet *et al,* 2002), whereas, in mature dendritic cells, CCR7 is upregulated (Sallusto *et al,* 1999), therefore allowing the migration of these cells to the lymph nodes. The chronic nature of OLP may suggest that Langerhans cells in this condition are of a mature status and thus would not express CCR6 nor respond to

MIP-3α, and indeed studies have shown that Langerhans cells of OLP appear dendritic, thus suggesting that they are already activated (Farthing *et al,* 1990). In addition, Langerhans cells in inflamed peripheral tissue express other chemokine receptors, such as CCR5 (Reviewed in McColl, 2002) and thus may respond to RANTES expressed in OLP (Little *et al,* 2003) as opposed to MIP-3α which may have more influence at an earlier stage of inflammation. Indeed, recent evidence suggests that during *in vivo* contact hypersensitivity reactions MIP-3α is one of the first chemokines expressed and decreases after only 3hours (Mitsui *et al,* 2003).

The expression of MIP-3α is localised to epithelial cells bordering the external environment (Vanbervliet *et al,* 2002) and not the cutaneous basal epithelium (Tohyama *et al,* 2001). In contrast, the immune reaction of OLP appears to be centred upon the basal epithelium and therefore, the expression of this chemokine may not be activated during this condition. Neutrophils can produce MIP-3α (Scapini *et al,* 2001), which may explain the higher findings of this chemokine in periodontitis compared to OLP and normal gingiva (Hosowaka *et al,* 2002) as periodontitis is characterised by large numbers of neutrophils (Kantarci & Van Dyke, 2002). Hence, although MIP-3α can be expressed by oral epithelial cells, it may not actually play a great role in maintaining OLP.

In the present study it was found that there was not a large increase in the expression of CCR6 mRNA in comparison to normal oral mucosa. CCR6+CLA+ cells are upregulated in the blood of psoriasis patients opposed to atopic dermatitis patients (Ong & Leung, 2003), furthermore, these cells produce high levels of IFN-γ. This suggests that the expression of CCR6 cells is closely linked to Th1 skin inflammation.

The fact that there was no upregulation of CCR6+ mRNA levels in OLP inflammation is perhaps related to the fact that there is low-undetectable levels of IFN-γ in OLP tissue (Chapter 3). Furthermore, CCR6 can be stored intracellularly, probably reflecting complex regulatory mechanisms (Egbert & McColl, 2002). Therefore RT-PCR may detect the expression of stored mRNA that is not expressed, perhaps also the case for normal tissue studied. Interestingly a study also found that increased CCR6 production did not necessarily correspond to an increased chemotactic response to MIP-3α *in vitro* (Ebert & McColl, 2002). However, gingival lymphocytes isolated from periodontitis patients were capable of migrating to recombinant MIP-3α suggesting that these cells are relevant in oral inflammation (Hosokawa *et al,* 2002).

Therefore, the present evidence suggests that MIP-3α is not playing a role in established OLP lesions, however, the presence of MIP3-α in oral tissue may play a number of roles. For example, beta-defensins can also bind CCR6 acting chemotactically (Yang *et al,* 1999). Any upregulation in CCR6 in oral tissue could therefore possibly be cells responding to this defensin and not in fact to any upregulation in MIP-3alpha, perhaps also the case for psoriasis patients. In fact, beta-defensin-2 is also expressed in OLP (Abiko *et al,* 2002). This again suggests that there is an overlap between chemotactic and antimicrobial functions in these molecules, which may have important implications for oral diseases.

In addition, MIP3-α production can also influence the adherence of memory T cells to ICAM-1 in endothelial cells (Campbell *et al,* 1998), and the production of MIP-3α in oral epithelium could possibly involved in this function. Keratinocytes in areas overlying the infiltrate in OLP were also detected as ICAM-1 positive in a majority of

lesions (Walton *et al,* 1994) and T cells can bind to the oral epithelium in oral inflammatory conditions, such as oral lichen planus. It may be that MIP-3α assists in mediating the binding of T cells to ICAM-1 and therefore possibly contributes to the pathology of inflammation involving epithelial surfaces.

Furthermore, CD40 ligation can enhance the level of MIP-3α production in dendritic cells (Homey *et al*, 2000), and therefore it is interesting to speculate whether MIP-3α production could be enhanced by ligation of this molecule in oral epithelial cells, which we have shown expresses this molecule in OLP (Chapter 3). The increased production of this chemokine in the epithelial area would presumably induce further migration of immature dendritic cells and memory T cells.

Chapter 7: Conclusions

This study aimed to characterise the expression and/or production of five chemokines (known to induce memory T cell migration) in oral epithelial cells and oral (lichen planus) tissue. This study demonstrated that oral epithelial cells are capable of producing different types of chemokines, for example, inflammatory chemokines (the CXC ELR-chemokines), tissue-specific chemokines (CTACK), as well the LC-attracting chemokine MIP-3α. In addition, it was shown that oral epithelial cells can respond to stimulation with inflammatory cytokines by inducing or increasing the production of all studied chemokines. These studies support previous evidence that epithelial cells are actively involved in immune responses (Reviewed by Grone, 2002) and that part of this role includes the production of chemokines either produced under resting conditions or in response to inflammatory stimuli. Thus it is possible that epithelial cells are involved in the initiation and persistence of inflammatory conditions.

In addition, this study confirms previous findings that chemokines can be produced by oral epithelial cells, and often to a greater level than the production by skin epithelial cells (Li *et al,* 1996; Li *et al* 2000). This suggests that oral epithelial cells are more responsive to inflammatory stimuli than skin epithelial cells, and that stimulation of oral mucosal membranes may result in a larger inflammatory reaction compared to skin. In relation to these findings it is interesting to note that oral lichen planus displays a delayed resolution in comparison to the cutaneous variant.

It was also confirmed in this study that the production of chemokine/s by oral epithelial cells can attract memory T cells, and that IP-10 and CTACK induce memory T cell migration. As OLP lesions feature large numbers of memory T cells, these epithelial cell-derived chemokines may play a role in the formation and persistence of OLP lesions. A study by Yamamoto *et al,* 1994 demonstrated that supernatants derived from epithelial cell culture from OLP patients were capable of attracting mononuclear cell populations (but did not study specific T cell subsets that undertook migration). Thus chemokines from epithelial cells could be playing a role in generating the inflammatory infiltrate witnessed in OLP lesions.

As detailed in this study, CTACK and MIP-3α are produced constitutively by oral epithelial cells, whereas the CXC ELR- chemokines are produced in large amounts only after inflammatory stimuli. Therefore, the former chemokines may have a greater role in initiating inflammation, whereas the CXC ELR- chemokines may play a role in the maintenance of inflammation in OLP lesions. Indeed in cutaneous lichen planus, the CXC ELR- chemokines are produced in abundance by epithelial cells (Flier *et al,* 2001; Spandau *et al,* 1998). Given that the evidence in this thesis (and other studies) suggests that oral epithelium is more responsive to inflammatory stimuli than skin epithelium, the production of these chemokines by oral epithelial cells is likely to have a pronounced effect upon inducing inflammatory infiltrate in oral lesions.

MIG, IP-10, I-TAC mRNA expression were upregulated in OLP tissue compared to NOM. These findings suggest that these chemokines are actively involved in the disease

process of OLP. As previously discussed, these chemokines recruit memory T cells (confirmed for IP-10 and CTACK in this study), therefore they probably play a role in the migration of the T cell infiltrate witnessed in OLP. As CXC ELR- chemokines preferentially attract Th1 cells that produce IFN-γ, they may act in a self-recruiting fashion exacerbating OLP inflammation.

The presence of the CXCR3+ cells (the receptor for CXC ELR- chemokines) is often witnessed in association with CCR5+ cells (Qin *et al,* 1998). Therefore it is interesting to note that a recent study found that RANTES (a ligand for CCR5) was produced by epithelial cells in OLP lesions (Little *et al,* 2003). In addition, the production of these chemokines is characteristic of certain types of immune reactions, especially delayed-type hypersensitivity Th1 inflammatory reactions (Qin *et al,* 1998). The particular trigger that causes this initial reaction is unknown, but various sources have been suggested, for example food antigens or local microflora (Thornhill, 2001). In the lichenoid-like reactions there appears to be a number of different sources that can cause such a reaction, for example systemic drugs (Reviewed by Lamey *et al,* 1995) or amalgams.

In addition, CTACK is reported to attract effector T cells which quickly produce pro-inflammatory cytokines after stimulation with antigen (Soler *et al,* 2003). Therefore this chemokine may have a crucial role in initiating pro-inflammatory cell migration in OLP. It is interesting to note that studies have found that the production of this chemokine is limited to skin (Homey *et al,* 2002) and oral epithelial cells (see Chapter 5), thus lichen planus may be initiated in part by the production of CTACK. In oral lichen planus, CLA+

cells are witnessed within the epithelium of lesional tissue (Walton *et al,* 1997), perhaps due to the production of CTACK.

As well as inducing migration of memory T cell populations, MIP3-α is capable of attracting immature Langerhans cells (Dieu-Nosjean *et al,* 2000). Although the exact role of Langerhans cells in OLP is not clear, some studies suggest that there are increased numbers and activation of this cell type (refer to Chapter 1) in OLP, therefore this chemokine may play a crucial role in the initial migration of Langerhans cells into lseional tissue.

The second aim of this study was to investigate the effect and/or presence of possible influences that may affect the production of these chemokines in oral lichen planus. Although the CXC ELR- chemokines were found to be upregulated in this study, IFN-γ mRNA was not detected within OLP lesional tissue. Possible explanations for this finding may be that IFN-γ is a feature of evolving lesions or another cytokine/ factor is involved in the induction and/or synergism of chemokine production in OLP. Other cytokines have been cited as a possible influence in OLP, for example TNF-α (Reviewed by Sugermann *et al,* 1996). This study (and others (Li *et al,* 2000[1]; Li *et al,* 2000[2])) have demonstrated that TNF-α (and Il-1beta) is capable of stimulating chemokine production in oral epithelial cells. Thus this cytokine may also be involved in the disease process by stimulating chemokine production from oral epithelial cells. Furthermore, a recent study has suggested that OLP lesions are dominated by Th1 cytokines (Khan *et al,* 2003), the presence of which is likely to influence chemokine production from oral epithelial cells.

In this study it has been shown that Th1 cytokines are able to stimulate chemokine production from oral epithelial cells and therefore it appears likely that this would also occur in OLP lesions.

Interestingly, it was found that CD40 was expressed in association with epithelial cells in OLP suggesting that ligation of this molecule could also synergise the production of chemokines in this area. In a previous study, CD40 ligation of epithelial cells was found to enhance CXC ELR- chemokine production (Altenberg *et al,* 1999), the chemokines found to be upregulated in OLP. Thus, CD40 expression upon oral epithelial cells in OLP may have relevance in enhancing inflammation in OLP. Expression of CD40 also adds to the evidence that oral epithelial cells are activated during this condtion and are likely to play an active role in the pathogenesis of OLP.

Alternatively, it has been speculated that OLP may be initiated by bacterial flora (Thornhill, 2001), therefore the response to bacterial LPS stimulation was also studied. IP-10 mRNA was induced after LPS stimulation, suggesting that bacteria or bacterial products could have a role in the increase in IP-10 mRNA demonstrated in OLP. In addition, this chemokine can act antimicrobially against an oral bacterium. This suggests that OLP could have a bacterial cause by upregulation of IP-10 chemokine production, and thus attracting T cells into the lesional area. A role of bacteria or specific antigens has also been suggested to occur in OLP due to the presence of an oligoclonal population of T cells in oral lichen planus (Zhou *et al,* 1996).

The last aim of this study was to determine the potential effects of blocking the production of chemokines from oral epithelial cells. The finding that certain chemokines are upregulated provides an appealing opportunity to block the action of these chemokines in a possible option for a therapy for OLP. Opposed to the current treatments for OLP this would act upon the possible cause of T cell infiltration. Interestingly, preliminary results show that blocking IP-10 in arthritis (Saloman *et al,* 2002) or CTACK in experimentally-induced contact hypersensitivity (Homey *et al*, 2002) can produce desirable reductions in inflammation. Furthermore, as CTACK has currently been found to be expressed in only skin (Morales *et al,* 1999) and the oral epithelium this chemokine provides an appealing option for therapeutic blocking in OLP. A CTACK-blocking treatment for OLP would presumably only suppress the response in these tissues whilst other areas of the body would remain unaffected (Reviewed by Mackay *et al,* 2002). This study suggests that such an approach may indeed be effective at blocking inflammatory cell infiltration to epithelial cell-derived supernatants, however, perhaps not to such a large extent as to cutaneous epithelial cells. However, as previously discussed the efficiency of such an approach has been questioned as other chemokines may compensate in the absence of CTACK (Reiss *et al,* 2001). Thus further studies would be required to assess whether such an approach would be fruitful in the treatment of OLP.

Overall this study (and others) suggest that chemokine production is an important feature of oral and cutaneous lichen planus lesions and has a likely role the in pathogenesis of this condition. Chemokine production by oral epithelial cells may recruit cells that intensify inflammatory properties of OLP lesions and /or induce apoptosis of

keratinocytes. Furthermore, determining the involvement of chemokine expression / production in OLP assists in assessing possible factors that may initiate the migration of the T cell infiltrate that is characteristic of OLP lesions and offer a possible avenue of therapy for this condition.

Chapter 8: References

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Appendix 1: Reagents

ELISA blocking buffer: 1% BSA, Fraction V (Sigma, Poole, UK) and 5% sucrose (BDH, Poole, UK) were diluted in PBS (Sigma, Poole, UK) then adjusted to pH7.2-7.4 and 0.2µm filtered. This solution was stored at 4°C until use.

ELISA reagent diluent: 1% BSA was diluted in PBS, then adjusted to pH 7.2-7.4 and 0.2µm filtered. This solution was stored at 4°C until use.

ELISA wash buffer: The following, 2.5mM Na_2HPO_4 (BDH), 0.05mM NaCl and 7.5mM NaH2PO4.2H20 (BDH) were added to distilled water and mixed thoroughly. 0.1% of Tween 20 (BDH) was then added and stored at room temperature.

Phosphate buffered saline (PBS): 1 PBS tablet (Oxoid, Basingstoke, UK) was added to 100ml distilled water and autoclaved at 114°C for 10mins.

3% Trypticase soy broth (TSB): 30g of trypticase soy broth was added to 1 litre of distilled water, autoclaved and stored at room temperature.

Sodium phosphate buffer: 100mM monobasic sodium phosphate (Fisher Scientific UK, Loughborough, UK) and 100mM dibasic sodium phosphate (Fisher Scientific UK, Loughborough, UK) were prepared in deionised water and then mixed together whilst monitoring the pH until pH 7.4 was achieved. This solution was then autoclaved and stored at room temperature.

Antimicrobial assay underlay: To prepare the underlay, 50mls of 100mM dodium phosphate buffer, 5g agarose, low EEO (Sigma, Poole, UK) and 5mls 3% trypticase soy broth were added to 1 litre of distilled water. Then the pH was then adjusted to 7.4, the agarose was dissolved by heating the solution in the microwave. Then the solution was dispensed into 50mls aliquots and autoclaved. The underlay aliquots were then stored at room temperature until use in the radial diffusion assays at which

point they were heated in a microwave until fluid and then stored in a 60°C water bath.

Antimicrobial assay overlay: 10g agarose, low EEO (Sigma, Poole, UK) was added to 6% TSB, aliquoted into 50mls and autoclaved. The overlay aliquots were then stored at room temperature until use in the radial diffusions assays.

Appendix 2: Data

IP-10, MIG and IL-8 production by primary oral epithelial cells (3.3.1)

Figure 3.4:

a)

Cell treatment	IP-10 produced pg/ml (1)	IP-10 produced pg/ml (2)	IP-10 produced pg/ml (3)	Mean value pg/ml	Standard error	Significance (between ifn and con using Mann-Whitney U test)
ifn	240.167	244.324	221.557	235.3493	1.740977	p=<0.05
con	11.726	9.62	8.793	10.04633	0.614871	
ifn	233.027	222.895	219.764	225.2287	1.316494	p=<0.05
con	11.753	12.063	11.214	11.67667	0.327726	
ifn	206.754	200.946	233.927	213.8757	2.097982	p=<0.05
con	0	0	0	0	0	

b)

Cell treatment	MIG produced pg/ml (1)	MIG produced pg/ml (2)	MIG produced pg/ml (3)	Mean value pg/ml	Standard error	Significance (between ifn and con using Mann-Whitney U test)
ifn	11.8914	10.9069	9.1172	10.6385	0.592967	p=<0.05
con	1.239	1.241	1.206	1.228667	0.070099	
ifn	8.155	8.412	7.623	8.063333	0.317178	p=<0.05
con	1.207	1.221	1.311	1.246333	0.118784	
ifn	9.123	7.344	8.763	8.41	0.484914	p=<0.05
con	1.177	1.188	1.183	1.182667	0.037107	

c)

Cell treatment	IL-8 produced pg/ml (1)	IL-8 produced pg/ml (2)	IL-8 produced pg/ml (3)	Mean value pg/ml	Standard error	Significance (between ifn and con using Mann-Whitney U test)
ifn	305.733	407.692	288.344	333.923	4.245465	p=<0.05
con	184.24	262.885	241.15	229.425	3.186392	
ifn	273.967	324.294	290.9	296.387	2.982724	p=>0.05
con	267.124	332.574	186.681	262.1263	4.27419	
ifn	531.683	702.561	670.233	634.8257	5.496112	p=>0.05
con	535.039	702.337	770.13	669.1687	5.500098	

Temporal effects of IFN-γ stimulation upon MIG, IP-10 and IL-8 production by H357 oral squamous cell carcinoma cell line (3.3.3)

Figure 3.6: Concentration of a) IP-10, b) MIG or c) IL-8 produced by the H357 cell line stimulated with (ifn) or without (con) IFN-γ.

a)

Time post-treatment (hrs)	Cell treatment	IP-10 produced pg/ml (1)	IP-10 produced pg/ml (2)	IP-10 produced pg/ml (3)	Mean value pg/ml	Standard deviation	Significance (between ifn and con using Mann-Whitney U test)
3hrs	ifn	44.668	42.17	38.019	41.619	3.358571	p=>0.05
	con	38.459	31.989	28.51	32.986	5.048877	
6hrs	ifn	47.863	44.157	51.286	47.76867	3.565436	p=<0.05
	con	38.019	38.019	36.728	37.58867	0.745359	
9hrs	ifn	245.47	248.21	213.8	235.8267	19.12479	p=<0.05
	con	47.863	52.481	44.157	48.167	4.170318	
24hrs	ifn	247.99	246.45	237.53	243.99	5.647265	p=<0.05
	con	40.738	39.811	35.892	38.81367	2.57234	
48hrs	ifn	309.03	263.03	319.89	263.03	30.18554	p=<0.05
	con	52.487	40.738	54.325	49.18333	7.371384	
72hrs	ifn	177.83	169.82	169.82	172.49	4.624576	p=<0.05
	con	42.658	40.738	33.384	38.92667	4.895147	

b)

Time post-treatment (hrs)	Cell treatment	MIG produced pg/ml (1)	MIG produced pg/ml (2)	MIG produced pg/ml (3)	Mean value pg/ml	Standard deviation	Significance (between ifn and con using Mann-Whitney U test)
3hrs	ifn	6.22	3.826	3.915	4.653667	1.357214	p=>0.05
	con	4.159	4.013	5.287	4.486333	0.69723	
6hrs	ifn	7.296	6.093	8.087	7.158667	1.004069	p=>0.05
	con	6.472	4.983	6.965	6.14	1.031867	
9hrs	ifn	7.121	8.941	11.164	9.075333	2.024845	p=<0.05
	con	6.216	6.881	6.292	6.463	0.363988	
24hrs	ifn	16.329	13.335	14.605	14.75633	1.502726	p=<0.05
	con	10.132	12.184	12.422	11.57933	1.259064	
48hrs	ifn	25.118	20.502	20.089	21.903	2.791919	p=<0.05
	con	13.43	11.978	14.808	13.40533	1.415161	
72hrs	ifn	10.637	13.16	14.329	12.70867	1.886927	p=>0.05
	con	12.472	9.618	13.516	11.86867	2.017823	

c)

Time post-treatment (hrs)	Cell treatment	IL-8 produced pg/ml (1)	IL-8 produced pg/ml (2)	IL-8 produced pg/ml (3)	Mean value pg/ml	Standard deviation	Significance (between ifn and con using Mann-Whitney U test)
3hrs	ifn	261.742	247.798	242.299	250.613	10.02251	
	con	286.995	319.973	295.082	300.6833	17.18774	p=<0.05
6hrs	ifn	248.616	264.243	271.807	261.5553	11.8268	
	con	246.058	252.498	245.44	247.9987	3.90877	p=>0.05
9hrs	ifn	295.829	306.176	294.461	298.822	6.405376	
	con	284.393	311.885	307.617	301.2983	14.79516	p=>0.05
24hrs	ifn	509.046	554.806	576.734	546.862	34.53616	
	con	538.286	587.185	579.469	568.3133	26.28906	p=>0.05
48hrs	ifn	867.942	964.175	948.459	926.8587	51.62488	
	con	1034.719	1180.583	1194.369	1136.557	88.46325	p=<0.05
72hrs	ifn	1193.062	1225.825	1222.403	1213.763	18.00934	p=>0.05
	con	1034.719	1180.583	1194.369	1136.557	88.46325	

The profile of T cell chemotactic profiles in response to IP-10 and stromal derived factor-1alpha (SDF-1α) (3.2.5)

Figure 3.8: c) The normalised % migration (to input PBMC) of CD45RAhiCD4+ or CD45RAloCD4+ to IP-10 and SDF1-α. Basal migration is the migration to cell culture medium alone.

Substance	% migration of CD4+CD45RAhi cells			% migration of CD4+CD45RAlo cells			Significance (between CD4+CD45RAhi and CD4+CD45RAlo using Mann-Whitney U test)
basal migration	4.675	4.558	4.627	4.776	4.181	4.912	p>0.05
		Average 4.62			Average 4.623		
IP-10	3.22	7.873	6.549	31.988	38.87	37.976	p<0.05
		Average 5.88			Average 36.28		
SDF	84.734	85.859	87.231	86.622	94.749	89.031	p>0.05
		Average 85.94			Average 90.13		

Levels of IFN-γ, MIG, IP-10 and I-TAC mRNA in normal oral mucosa and oral lichen planus (3.3.6)

Figure 3.11: MIG, IP-10, I-TAC mRNA in oral lichen planus and normal oral mucosa

	Semi-quantatative ratio of of chemokine mRNA: 18S RNA (relative intensity units)		
	MIG	IP-10	I-TAC
olp 1	1	1.05	0.32
olp 2	0.83	0.31	0
olp 3	1.16	0.74	0.35
olp 4	1.09	0.62	0
olp 5	0.45	0.215	0
olp 6	0.88	0.65	0
olp 7	0	0	0
olp 8	0.952	0.67	0
olp 9	0.719	1.4	0
olp 10	1.535	0	0
olp 11	0.978	1.05	0
olp 12	0.386	0.21	0
nom1	0	0.8	0
nom2	0.386	0	0
nom3	0	0	0
nom4	0.619	0	0
nom5	0	0	0
nom6	0	0.36	0
nom7	0.15	0.17	0
nom8	0	0	0
Average ratio of OLP tissue	0.712857	0.493929	0.047857
st dev	0.477989	0.234502	0.121793
Average of NOM tissue	0.144375	0.16625	0
st dev	0.235404	0.269613	0
Significance between OLP and NOM tissue Using Mann-Whitney U test	p<0.05	p=<0.05	p=<0.05

MIG and IP-10 mRNA transcripts in an oral epithelial cell line in response to lipopolysaccharide (LPS) (4.3.1)

Figure 4.3: b) Densitometric analysis of IP-10 mRNA relative to 18S expression with and without LPS treatment

hrs	con			lps			Significance between con and lps Using Mann-Whitney U test
2	0.4918	0.2874	0.3692	0.676	0.7897	1.0869	p<0.05
		Average 0.3828			Average 0.8509		
4	0.9238	0.9059	0.6178	1.6207	1.7795	1.1000	p<0.05
		Average 0.8158			Average 1.5001		
6	1.3651	0.3976	0.6871	1.62397	1.77896	1.98766	p<0.05
			0.816574		Average 1.796852		
8	0.8985483	0.73214725	1.3216	0.5042	0.57234	0.326	p<0.05
		Average 0.984067			Average 0.467451		

Antimicrobial effect of MIG, IP-10, CTACK or MEC upon *S.sanguis* and *E.coli* (4.3.2)

Table 4.1: Antimicrobial activity of 100μg/ml human recombinant MIG, IP-10, MEC and CTACK upon *S.sanguis* and *E.coli* bacterium.

Bacterium tested	Chemokine tested	Antimicrobial units (Number 1)	Antimicrobial units (Number 2)	Antimicrobial units (Number 3)	Mean	Standard deviation
E.coli						
	MIG	32.5	29.1	42.2	34.6	6.797794
	IP-10	13.2	21.2	10.3	14.9	5.645352
	MEC	22.4	12.3	11.6	15.43333	6.043454
	CTACK	20.1	20	21.6	20.56667	0.896289
	Con	0	0	0	0	0
S.sanguis						
	MIG	59.9	40.8	32.5	44.4	14.05027
	IP-10	4.4	10.9	10	8.433333	3.521837
	MEC	15.3	21.1	26.7	21.03333	5.700292
	CTACK	4.6	9.8	15.5	9.966667	5.451911
	Con	0	0	0	0	0

Expression of CTACK mRNA by IFN-γ stimulated and non-stimulated primary oral epithelial cells (5.3.1)

Figure 5.2: RT-PCR of CTACK mRNA in primary epithelial cell lines. ii) Densitometric analysis of the average CTACK mRNA expression (normalised to 18S expression) in the 3 different primary oral keratinocytes treated with IFN-γ (ifn) or non-treated (con).

	Intensity of 18S band (-backround) (idv)	Intensity of CTACK band (-backround) (idv)	Ratio	Average	Standard deviation	Significance (between ifn and con using Mann-Whitney U test)
con 1	36,359.67	4,924.85	0.135448			
con 2	30,352.00	5,396.00	0.177781			
con 3	37,855.59	8,026.75	0.212036	0.175088	0.038365	
ifn 1	13,385.47	2,675.29	0.199865			
ifn 2	34,132.67	8,772.62	0.257015			
ifn 3	40,350.50	13,080.72	0.324177	0.260353	0.062223	>0.05

Production of CTACK by IFN-γ-stimulated and non-stimulated primary oral epithelial cells (5.3.2)

Figure 5.3: The concentration of CTACK produced in 3 different primary oral keratinocyte cell lines stimulated with 1000U/ml interferon-γ or left untreated for 24 hours.

Cell treatment	CTACK produced pg/ml (Experiment 1)	CTACK produced pg/ml (Experiment 2)	CTACK produced pg/ml (Experiment 3)	Mean value pg/ml	Standard deviation	Significance (between ifn and con using Mann-Whitney U test)
Ifn	727.106	471.797	397.556	532.153	172.8668	p<0.05
Con	404.828	367.377	333.514	368.573	35.67204	
Ifn	621.731	645.135	418.649	561.83833	124.5565	p<0.05
Con	138.154	248.506	293.624	226.7613	79.983455	
Ifn	851.165	518.539	367.939	579.21433	247.2609	p>0.05
Con	424.315	292.672	316.725	344.5707	70.09997	

Effects of pro-inflammatory cytokine treatment (IFN-γ or TNF-α with IL-1β) upon the production of CTACK by oral (H357) and cutaneous (UP) keratinocyte cell lines (5.3.3)

Figure 5.4: The concentration of CTACK produced (pg/ml) by i) H357 or ii) UP cells treated either with interferon-γ (IFN) or TNF-α with IL-1β (TNF+IL-1b) for 3, 6 or 24 hours as determined by ELISA.

		CTACK produced by H357 cells (1)	CTACK produced by H357 cells (2)	CTACK produced by H357 cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
3hrs	CON	249.948	198.988	393.258	280.7313	100.7269	
	IFN	452.318	331.559	397.508	393.795	60.46506	p>0.05
	TNF + IL-1b	249.37	265.179	347.358	287.3023	52.60697	p>0.05
6hrs	CON	205.852	267.418	262.603	245.291	34.23992	
	IFN	218.882	238.303	295.003	250.7293	39.55265	p>0.05
	TNF + IL-1b	417.222	563.982	514.475	498.5597	74.66323	p<0.05
24hrs	CON	446.516	412.201	404.463	421.06	22.38247	
	IFN	652.42	522.078	598.421	590.973	65.48942	p<0.05
	TNF + IL-1b	596.858	528.783	596.486	574.0423	39.19617	p<0.05
		CTACK produced by UP cells (1)	**CTACK produced by UP cells (2)**	**CTACK produced by UP cells (3)**	**mean**	**st dev**	**Significance (compared to con using Mann-Whitney U test**
3hrs	CON	380.657	145.673	176.325	234.2183	127.7423	
	IFN	281.904	204.429	331.006	272.4463	63.8163	p>0.05
	TNF + IL-1b	278.012	189.895	169.349	212.4187	57.72693	p>0.05
6hrs	CON	179.527	261.729	318.716	253.324	69.97412	
	IFN	261.44	190.664	235.776	229.2953	35.83217	p<0.05
	TNF + IL-1b	273.696	319.671	233.04	275.469	43.34271	p>0.05
24hrs	CON	536.193	475.682	448.4	486.7583	44.93236	
	IFN	203.404	291.964	192.478	229.282	54.5584	p<0.05
	TNF + IL-1b	581.051	846.082	594.478	673.8703	149.2907	p<0.05

Figure 5.6: The normalised migration of T cell subsets to supernatants of non-treated or TNF-α and IL-1β treated keratinocyte cell lines.
(1) The migration of CD3+CD45ROhi ('memory' T cells) or CD3+CD45ROlo ('naïve' T cells) to oral (H357) cell line supernatants untreated with or without pre-incubation with an anti-CTACK antibody, or with TNF-α and IL-1β incubation with or without pre-incubation with an anti-CTACK antibody.

supernatant	Chemotaxis of CD45RO+ T cells (1)	Chemotaxis of CD45RO+ T cells (2)	Chemotaxis of CD45RO+ T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.404296	0.404296	0.680412	0.496334	0.159416	
H357 cells con	2.046186	1.738693	2.208509	1.997796	0.238616	
H357 cells con Anti-CTACK	2.03416204	1.779409239	2.003041341	1.938870873	0.138971703	p>0.05
H357 cells TNF-α	7.448102698	6.557158035	7.446334477	7.150531737	0.51387746	
H357 cells TNF- α Anti-CTACK	3.725289104	3.265537226	3.579234006	3.523353446	0.234914719	p<0.05

supernatant	Chemotaxis of CD45ROlo T cells (1)	Chemotaxis of CD45ROlo T cells (2)	Chemotaxis of CD45ROlo T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.14196	0.14196	0.079292	0.12107	0.036181	
H357 cells con	0.082878	0.051466	0.081285	0.071876	0.017694	
H357 cells con Anti-CTACK	0.331514	0.370982	0.36857	0.357022	0.022123591	p<0.05
H357 cells TNF-α	0.192851735	0.158685909	0.173327489	0.174955045	0.017140963	
H357 cells TNF- α Anti-CTACK	0.32075547	0.337529218	0.388094195	0.34879296	0.035054	p<0.05

(2) The migration of CD3+CD45ROhi ('memory' T cells) or CD3+CD45ROlo ('naïve' T cells) to cutaneous (UP) cell line supernatants untreated with or without pre-incubation with an anti-CTACK antibody, or with TNF-α and IL-1β incubation with or without pre-incubation with an anti-CTACK antibody.

supernatant	Chemotaxis of CD45RO+ T cells (1)	Chemotaxis of CD45RO+ T cells (2)	Chemotaxis of CD45RO+ T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.404296	0.404296	0.680412	0.496334	0.159416	
UP cells con	0.914878	0.869347	1.088517	0.957581	0.115657	
UP cells con Anti-CTACK	0.82682	0.780917	0.809138	0.805625	0.023152	p<0.05
UP cells TNF-α	1.616154472	1.627042949	1.595289458	1.61282896	0.01613584	
UP cells TNF- α Anti-CTACK	0.781553913	0.839127918	1.071188599	0.897290143	0.153327089	p<0.05

supernatant	Chemotaxis of CD45ROlo T cells (1)	Chemotaxis of CD45ROlo T cells (2)	Chemotaxis of CD45ROlo T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.14196	0.14196	0.079292	0.12107	0.036181	
UP cells con	0.109575	0.128235	0.098418	0.112076	0.015065	
UP cells con Anti-CTACK	0.262183	0.259902	0.362992	0.295025	0.058872	p<0.05
UP cells TNF-α	0.075307806	0.062616602	0.132685181	0.090203196	0.037333711	
UP cells TNF- α Anti-CTACK	0.271745627	0.289494564	0.253416743	0.271552311	0.018039687	p<0.05

(3) The migration of CLA+CD3+ or CLA-CD3+ cells to oral (H357) cell line supernatants incubated as described above.

supernatant	Chemotaxis of CLA+ T cells (1)	Chemotaxis of CLA+ T cells (2)	Chemotaxis of CLA+ T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	1.2223464	1.38056	1.272834	1.291913	0.0808140 17	
H357 cells con	8.92296	12.9863	8.198852	10.035400 13	2.5805270 23	
H357 cells con Anti-CTACK	12.91746	14.94131	12.1112	13.323321 18	1.4580568 5	p>0.05
H357 cells TNF-α	21.9875	22.7652	21.38117	22.04463	0.6937833 84	
H357 cells TNF- α Anti-CTACK	18.537278	16.07644	14.97861	16.530787 4	1.8223183 1	p<0.05

supernatant	Chemotaxis of CLAlo T cells (1)	Chemotaxis of CLAlo T cells (2)	Chemotaxis of CLAlo T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.182009	0.193182	0.18602	0.187070666	0.005660069	
H357 cells con	4.712199	4.7481	4.9453	4.801866255	0.125507502	
H357 cells con Anti-CTACK	3.92948	4.670145	4.54251	4.3807124	0.395954798	p<0.05
H357 cells TNF-α	4.55511	5.15601	4.83909	4.85007	0.300600437	
H357 cells TNF- α Anti-CTACK	6.331455	5.943837	6.504338	6.25987775	0.287024278	p<0.05

(4) The migration of CLA+CD3+ or CLA-CD3+ cells to cutaneous (UP) cell line supernatants incubated as described above.

supernatant	Chemotaxis of CLA+ T cells (1)	Chemotaxis of CLA+ T cells (2)	Chemotaxis of CLA+ T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	1.2223464	1.38056	1.272834	1.291913	0.080814017	
UP cells con	9.002943	9.212939	9.36475	9.193544	0.181681578	
UP cells con Anti-CTACK	5.348939	6.34011	5.093822	5.594293	0.658379157	p<0.05
UP cells TNF-α	15.354398	14.19498	13.99278	14.51405	0.734751075	
UP cells TNF- α Anti-CTACK	10.232559	9.768143	9.461858	9.820853	0.388045018	p<0.05

supernatant	Chemotaxis of CLA lo cells (1)	Chemotaxis of CLA lo cells (2)	Chemotaxis of CLAlo T cells (3)	mean	st dev	Significance (compared to con using Mann-Whitney U test)
med only	0.182009	0.193182	0.18602	0.187070666	0.005660069	
UP cells con	1.98826	1.694137	1.694658	1.792352	0.169661678	
UP cells con Anti-CTACK	2.12083762	2.494613	1.892846	2.169432	0.303812423	p>0.05
UP cells TNF-α	2.029848	1.938457	1.804081	1.924129979	0.113563411	
UP cells TNF- α Anti-CTACK	2.18384	2.221454	2.273955	2.226416	2.226416942	p<0.05

Production of MIP-3α by oral (H357) and cutaneous (UP) keratinocyte cell lines (6.3.1)

Figure 6.2: The concentration of MIP-3α produced in a) H357 cells or b) UP cells treated with IFN-γ (IFN), TNF-α and IL-1β (TNF + IL-1b) or untreated in culture (CON) for 3, 6 or 24 hours.

a)

	Cell treatment	Production of MIP-3a by H357 cells (1)	Production of MIP-3a by H357 cells (2)	Production of MIP-3a by H357 cells (3)	mean	st dev	Significance Between treated and control cells at same time-point
3hrs	CON	774.154	684.199	648.068	702.1403	64.92949	
	IFN	1108.976	1235.955	1064.75	1136.56	88.87328	p<0.05
	TNF + IL-1b	953.884	871.961	989.775	938.54	60.3872	p<0.05
6hrs	CON	1175.011	1072.665	1072.665	1106.78	59.08949	
	IFN	859.306	880.837	788.106	842.7497	48.53188	p<0.05
	TNF + IL-1b	1825.014	1983.445	2059.208	1955.889	119.504	p<0.05
24hrs	CON	450.443	650.89	649.02	583.451	115.1921	
	IFN	1700.687	1510.91	1770.707	1660.768	134.4201	p<0.05
	TNF + IL-1b	2341.76	2307.51	2339.66	2329.643	19.19677	p<0.05

b)

	Cell treatment	Production of MIP-3a by UP cells (1)	Production of MIP-3a by UP cells (2)	Production of MIP-3a by UP cells (3)	mean	st dev	Significance Between treated and control cells at same time-point
3hrs	CON	0	0	0	0	0	
	IFN	0	0	0	0	0	
	TNF + IL-1b	0	0	0	0	0	
6hrs	CON	0	0	0	0	0	
	IFN	0	0	0	0	0	
	TNF + IL-1b	31.888	0	163.249	65.04567	86.52822	p<0.05
24hrs	CON	168.316	169.837	278.861	205.6713	63.38867	
	IFN	598.049	122.095	146.865	289.003	267.9281	
	TNF + IL-1b	306.771	403.244	1137.459	615.8247	454.3166	p<0.05

MIP-3α mRNA expression in oral and cutaneous keratinocyte cell lines with stimulation by TNF-α with IL-β or IFN-γ (6.3.2)

Figure 6.3: a) The expression of 18S and MIP3-α in H357 or UP cells 3, 6, 24 hours after no treatment (1-3 respectively), after treatment with 1000U/ml IFN-γ (4-6) or with TNF-α and IL-1β (7-9).

	con	ifn	tnf
H357 3hrs	1.4939 1.4463 1.0297 Average 1.3233	1.1342 1.5543 1.2380 Average 1.3088 p>0.05	0.9855 1.4710 1.6958 Average1.3841 p>0.05
H357 6hrs	0.7047 0.9817 0.6949 Average 0.7937	0.4667 0.9656 0.7236 Average 0.7186 p>0.05	1.6123 1.3248 1.6166 Average 1.5174 p<0.05
H357 24hrs	1.008 1.3811 0.5983 Average 0.9958	0.9087 0.9119 1.8298 Average 1.2168 p>0.05	1.3891 1.4235 1.50763 Average 1.44 p<0.05
	con	ifn	tnf
UP 3hrs	0	0	0.8726 0.6245 0.6857 Average 0.727631 p<0.05
UP 6hrs	0.2123 0.7141 0.4997 Average 0.4753	0.8791 0.7682 0.6705 Average 0.7726 p>0.05	0.7114 0.8034 1.1884 Average 0.90128 p>0.05
UP 24hrs	0.1928 0.3342 0.4593 Average 0.3287	0.2497 0.5103 0.5092 Average 0.42301681 p>0.05	0.9897 1.2111 1.075 1.091946 p<0.05

Determine MIP-3α and CCR6 mRNA expression levels in normal oral mucosa and oral lichen planus (6.3.3)

Table 6.1: Densitometric analysis of the MIP-3α and CCR6 mRNA expression in oral lichen planus (OLP) and normal oral mucosal (NOM) tissue.

	Intensity of 18S band (-backround)(idv)	**Intensity of MIP-3a band (-backround)(idv)**	**Ratio**	**Average**	**Standard deviation**	**Significance** (between olp and nom using Mann-Whitney U test)
olp1	49,839.33	15,438.50	0.3097654			
olp 2	63,228.93	15,635.35	0.247281585			
olp 3	67,416.46	3,249.00	0.048192978			
olp 4	70,686.21	6,997.95	0.099000215			
olp 5	63,118.70	2,986.00	0.047307692			
olp 6	68,953.77	2,968.50	0.043050583	0.132433075	0.116693	
nom 1	34,697.68	6,908.00	0.199091121			
nom 2	40,959.66	10,839.69	0.264643066			
nom 3	61,358.78	6,619.22	0.107877308			
nom 4	68,238.72	7,480.00	0.109615186			
nom 5	69,544.88	2,851.11	0.040996692			
nom 6	59,262.47	3,147.50	0.053111185	0.129222426	0.086761	p>0.05
	Intensity of 18S band (-backround)(idv)	**Intensity of CCR6 band (-backround)(idv)**	**Ratio**	**Average**	**Standard deviation**	**Significance** (between olp and nom using Mann-Whitney U test)
olp1	32,906.87	3,786.99	0.115082048			
olp2	37,964.50	5,721.75	0.150713166			
olp3	21,980.41	6,605.14	0.300501219			
olp4	30,729.37	0	0			
olp5	29,156.44	7,959.92	0.273007267			
olp6	31,123.30	7,083.33	0.227589298	0.177815	0.112142	
nom1	20,404.24	6,484.52	0.317802574			
nom2	29,527.34	0	0			
nom3	33,028.75	6,221.81	0.188375582			
nom4	26,718.80	6,830.83	0.255656317			
nom5	20,262.18	0	0			
nom6	18,522.53	3,580.69	0.193315384	0.159192	0.132033	p<0.05