IMMUNOMODULATORY AND WOUND-HEALING EFFECTS OF 
THE HOST DEFENCE PEPTIDE LL-37 AND RELATED INNATE 
DEFENCE REGULATORS

by

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Abstract

LL-37, the only known human cathelicidin peptide, possesses a variety of immunomodulatory properties that extend its role in host defence far beyond its original classification as an antimicrobial peptide. Recently, work has been underway to elucidate signalling pathways initiated by LL-37, with the aim of further understanding this peptide’s role in the immune system. The aim of this study was to further uncover the role of transcription factors during the responses of immune cells to LL-37 and related innate defence regulator peptides. Secondary aims were to investigate potential wound-healing properties of these peptides and to compare host defence peptides with chemokines in terms of immunomodulatory function. Here, I demonstrated involvement of AP-1 in LL-37-induced wound healing. I also showed a functional overlap between chemokine CXCL9/MIG and host defence peptide LL-37 and demonstrated similarities between LL-37 and the antibiotic azithromycin.
Preface


Many of the experiments contained within this thesis deal with cells derived from human blood. Blood samples were collected and used according to the University of British Columbia’s Clinical Research Ethics Board certificate # H04-70232, entitled “Exploring Innate Immunity and the Novel Functions of Host Defence Peptides”.
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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Responsive Element Binding Protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DPB</td>
<td>Diffuse Panbronchiolitis</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>FPRL1</td>
<td>Formyl Peptide Receptor-like 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein Coupled Receptor</td>
</tr>
<tr>
<td>HBE</td>
<td>Human Bronchial Epithelial Cells</td>
</tr>
<tr>
<td>HDP</td>
<td>Host Defence Peptide</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>IDR</td>
<td>Innate Defence Regulator</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of Nuclear NF-κB Complex</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 Receptor Associated Kinases</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Protein 88</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated Molecular Pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time Quantitative PCR</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming Growth Factor β Activated Kinase 1</td>
</tr>
<tr>
<td>TBS/T</td>
<td>Tris Buffered Saline + 0.1% Tween 20</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumour Necrosis Factor Receptor Associated Factor 6</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
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CHAPTER 1: Introduction

1.1 Innate Immunity

Humans are under constant threat of infection by potential pathogens, and yet, despite our frequency of contact with such microorganisms, they are rarely successful at causing illness. This accomplishment is due largely to innate immunity, an evolutionarily conserved system that acts as a first line of defence against infections. Microorganisms that are successful in breaching epithelial barriers are engulfed by phagocytes, which release cytokines and chemokines to activate and recruit other cells, thus initiating inflammation. Should this rapid response prove ineffective at controlling the invading pathogen, cells of the innate immune system alert those of the more specific (albeit more delayed) adaptive immune system.

The innate immune system makes use of germline-encoded pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), which recognize specific conserved microbial features known as molecular signatures or pathogen-associated molecular patterns (PAMPs) (1). Such responses are thus fast-acting and relatively non-specific. These characteristics allow for rapid clearance of a large variety of pathogens.

Inflammation must be carefully regulated, since an inappropriate or excessive immune response can have severe deleterious consequences. Septic shock is a life-threatening condition that results from an excessive immune response to a bacterial component, often LPS, which causes blood vessels to become leaky, resulting in tissue damage and multiple organ failure due to lack of perfusion (2). Thus, there is substantial interest in modulating innate immune responses to boost the host’s ability to clear infection while limiting the harmful aspects of inflammation (3).

1.1.1 Chemokines and Innate Immunity

Chemokines are small (8-12 kDa) proteins produced by a range of cell types. Chemokines recruit specific subsets of leukocytes to sites of infection and also activate these cells. Humans have at least 40 chemokines, which have been classified into four subfamilies – CC, CXC, CX2C and CX3C--, according to the arrangement of their N-terminal cysteine residues (4). Chemokines are essential to the regulation of cell trafficking that allows, when appropriate, the triggering of an adaptive immune response. Chemokine receptors belong to the seven-transmembrane-domain, G-protein-coupled receptor (GPCR) family (4). Chemokine-receptor
binding is redundant; that is, several different chemokines may bind one receptor, and one chemokine may bind several different receptors.

While the N-termini of chemokines are responsible for chemoattractant function, other regions may have an altogether different function. At high concentrations, some highly cationic chemokines, including CXCL9/MIG, CXCL10/IP-10, and CCL20/MIP-3\(\alpha\), have been shown to exhibit antimicrobial activity (5), suggesting a potential evolutionary relationship between these molecules and antimicrobial host defence peptides (HDPs, section 1.2), since these peptides also have chemokine activity.

### 1.1.2 Major Signalling Pathways of Innate Immunity

Recognition of microbial signatures by TLRs leads to a cascade of intracellular signalling events that result in expression and release of cytokines and other molecules that are necessary for an effective immune response (6). Specifically, activation of a TLR results in the recruitment of the adaptor molecule myeloid differentiation protein 88 (MyD88) and interleukin-1 receptor associated kinases (IRAKs); this leads to a series of activation steps involving, in sequence, tumour necrosis factor receptor associated factor 6 (TRAF6), transforming growth factor \(\beta\) activated kinase 1 (TAK1) (6), proteolysis of the inhibitor of nuclear NF-\(\kappa\)B complex (I\(\kappa\)B) releasing NF-\(\kappa\)B to migrate to the nucleus, and Activator Protein 1 (AP-1) that is also activated through the mitogen activated protein kinase (MAPK) cascade (6). There are three main branches of MAPK: extracellular signal-regulated kinases (ERK), JUN N-terminal kinases (JNK) and p38. MAPKs can be activated in the cytoplasm or the nucleus; once activated, they activate transcription factors via phosphorylation (7). Transcription factors, including notably NF-\(\kappa\)B and AP-1, regulate the transcription of key genes involved in the innate immune response.

There are five different NF-\(\kappa\)B family members: RelB, c-Rel, p65 (RelA), p100/p52 (NF-\(\kappa\)B2) and p105/p50 (NF-\(\kappa\)B1). These subunits can homodimerize or heterodimerize, with the dimer of p65 and p50 being the most common (8). NF-\(\kappa\)B is held in the cytoplasm by I\(\kappa\)B. Upon activation of the I\(\kappa\)B kinase (IKK), I\(\kappa\)B is phosphorylated and marked for proteosome degradation, allowing NF-\(\kappa\)B to translocate to the nucleus (8). There, it can bind DNA and induce the transcription of a variety of inflammatory genes. Negative regulation of the above-described signalling pathways is necessary for an appropriate balance of pro-inflammatory and
anti-inflammatory responses. The cytoplasmic deubiquitinating enzyme A20 (TNFAIP3) is important for the termination of TLR responses (9). It causes degradation of TRAF6, thus inhibiting IKK activation and subsequent translocation of NF-κB (7).

AP-1 regulates transcription of a variety of genes, including many involved in growth, differentiation and apoptosis. AP-1 can be activated by various pro-inflammatory stimuli, indicative of its role in host defence. It can be a homodimer or heterodimer of members of the \textit{jun} family, \textit{fos} family or activating transcription factor (ATF) family, although it is most commonly a dimer of c-Jun and c-Fos [reviewed in (10, 11)]. AP-1 DNA-binding activity is enhanced by both increased levels of c-Jun and increased phosphorylation of c-Jun. Phosphorylation of c-Jun at Serine-63 and Serine-73 (by c-Jun N-terminal kinase, JNK) allows it to activate transcription either as a homodimer, or as a heterodimer with c-Fos [reviewed in (12)]. The levels and activity of c-Fos can be enhanced by ERK, via phosphorylation and activation of Elk-1 (12). AP-1 can be negatively regulated at several levels. The transcription factor JunD is a negative regulator during cellular proliferation.

### 1.1.3 Wound Healing: an Important Aspect of Innate Immunity

A primary component of intrinsic, non-inducible innate immune defences is the presence of physical barriers. Disruption of such barriers in the form of a wound allows for the introduction of a multitude of potential pathogens. Thus, wound healing is an important aspect of immunity, and the processes of inflammation and wound healing are very much linked. The wound healing process involves four overlapping steps: inflammation, re-epithelialization, tissue granulation and remodelling.

In the very early stages of wound healing, keratinocytes release IL-1, and damage to blood vessels causes clot formation (13). Thus begins the inflammatory phase of wound healing, the goal of which is to remove bacteria and debris, and minimize tissue damage. Growth factors such as transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are released from platelets, resulting in recruitment of fibroblasts, endothelial cells, neutrophils and macrophages to the wound site and the initiation of inflammation (13, 14). Macrophages release cytokines, as well as growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), TGF-β and PDGF (13).
The proliferative phase of the wound healing process begins approximately two to three days post-injury. During this phase, growth factors (FGF, TGF-β and PDGF) recruit fibroblasts to the site of injury (13). Fibroblasts synthesize collagen and release growth factors that aid in angiogenesis. Fibroblasts and endothelial cells then form granulation tissue. Re-epithelialization (which begins within hours of injury) is maximal at this stage and helps restore tissue integrity (15). It is aided by EGF, TGF-α and FGF (13).

Remodelling is the last stage of the wound healing process. It involves the breakdown (mediated by PDGF) and synthesis (mediated by TGF-β) of collagen, which gradually replaces granulation tissue.

AP-1 is known to be important in wound healing. The subunits c-Fos, FosB, c-Jun and JunB are induced in epithelial cells following wounding (16). Mice lacking c-Jun in the epidermis are defective in wound healing (17). c-Jun is phosphorylated, and AP-1:DNA binding is enhanced in keratinocytes in response to wounding (18), while keratinocytes lacking c-Jun are unable to effectively migrate or elongate (17). Conversely however, an inhibitor of c-Jun homodimerization was shown to increase keratinocyte proliferation and reduce cell death, and increase the rate of healing in a murine burn injury model (19).

1.2 Host Defence Peptides

HDPs, also known as antimicrobial peptides, are an important component of the innate immune system in virtually all organisms, ranging from plants to mammals. Generally speaking, they are small (12-50 amino acids), have a net positive charge of +2 to +7 and are amphipathic (20, 21). Based on their three dimensional structure, HDPs can be grouped into four main classes: β-sheet peptides, which are stabilized by disulfide bridges (e.g. defensins), unstructured peptides that become α-helical when in contact with membranes (e.g. human LL-37), extended peptides (e.g. bovine indolicidin) and loop peptides (e.g. bovine bactenecin), which contain a single disulfide bond (21). In humans, there are two main classes of host defence peptides, the defensins and a single cathelicidin (reviewed in (22)).

Defensins are typically 29-35 amino acids in length and are stabilized by three disulfide bridges (23). Based on the structure of these bridges, they can be grouped into α- or β-defensins (23). The α-defensins can be further grouped into those that are found in neutrophil granules (HNP1-4) and those that are produced by Paneth cells (HD5 and 6) (24). Defensins have a
variety of roles in the innate immune system; these have been discussed extensively elsewhere (24).

Cathelicidins, while structurally varied, share, in their precursor form, a common N-terminal “cathelin” domain (of approximately 100 amino acid residues) which is cleaved upon release of the peptide from the cell by proteinase 3, leaving the effector C-terminal portion of the peptide (25, 26). While some authors have tried to ascribe antimicrobial and tissue protective functions to the cathelin domain, it is usually considered to be inactive (27).

Expression of HDPs can be constitutive or inducible (by factors like bacterial products or tissue injury), depending on the cell and tissue type. Most HDPs are stored within granules in an inactive propeptide form and are cleaved upon release from the cell to yield the mature active peptide (20). Some HDPs have been shown to kill bacteria directly via membrane disruption and permeabilization (28). However, this bactericidal capacity is largely dependent on low mono-and di-valent cation concentrations that do not correspond with those found in vivo and peptides can also be inhibited by the presence of human serum and negatively-charged proteins and saccharides such as the glycosaminoglycans (20, 29).

It has become evident over that past several years that many HDPs act, at least in part, by directly affecting innate immune cells and modulating immune processes (30). Such immunomodulatory effects of HDPs include mediating chemotaxis of immune cells, promoting angiogenesis and wound healing, and dampening harmful aspects of inflammation.
Further strengthening the evidence that HDPs exert their antimicrobial effects indirectly by acting on immune cells is the finding by Scott et al. (31) that innate defence regulator (IDR)-1, a peptide modelled on HDPs but without any direct antimicrobial activity, can protect mice against a variety of infections.

1.2.1 The Human Cathelicidin LL-37

LL-37, so named because it contains 37 amino acid residues, beginning with two leucine residues, is the only known human cathelicidin. It has an overall positive change and an α-helical structure that is promoted by interaction with membranes (32). The precursor pro-peptide form of LL-37, hCAP-18, is stored mainly in human neutrophil granules (33, 34), and the mature LL-37 is released upon neutrophil degranulation (35). LL-37 is also produced by a variety of other leukocytes, including B cells, γδ T cells and NK cells (36), as well as epithelial cells and keratinocytes. LL-37 can be found in mucosal secretions, sweat and plasma. Furthermore, LL-37 expression is inducible in response to injury and inflammation/infection (37-39), as well as by 1,25-dihydroxyvitamin D₃ and hypoxia response pathways (40). The physiological concentration of LL-37 has been shown to be >1 µg/ml in human plasma (41), and may reach up to 5 µg/ml (1 µM) at airway and mucosal surfaces, although concentrations may reach much higher levels under pathological conditions (42). Such concentrations correspond with those required for LL-37-induced wound healing, angiogenesis, and anti-endotoxin activity, whereas direct antimicrobial activity requires much higher concentrations in physiological salt conditions (43).

LL-37 appears to play an important role in preventing human diseases and injury. Patients suffering from morbus Kostmann syndrome are deficient in LL-37 and experience severe dental disease (44). Furthermore, mice lacking the gene for CRAMP, the mouse analog of LL-37, have moderately increased susceptibility to bacterial skin infections (45, 46). Conversely, in patients suffering from psoriasis, LL-37 is overexpressed and causes plasmacytoid dendritic cells (DC) to react to self-DNA:LL-37 complexes by producing interferon, which initiates inflammatory reactions (47). Clearly, LL-37 levels must be finely regulated.

LL-37 possesses antimicrobial activity that involves bacterial membrane permeabilization (48). However, this activity is essentially abolished in the presence of physiologically relevant cation concentrations (49). LL-37 can bind and neutralize lipopolysaccharide (LPS) and has been shown to protect against endotoxaemia in vivo in mice.
although these effects also involve the modulation of signal transduction pathways by LL-37 (57). In addition to its limited direct bactericidal activity, LL-37 exhibits other biological activities including modulation of immune cell function and aiding in wound healing; other, potentially pathological, functions include the stimulation of apoptosis in epithelial cells and promotion of histamine release from mast cells (reviewed in (25)). While many functions have been assigned to LL-37, much knowledge remains to be gained regarding how it mediates these functions, particularly in terms of how it initiates signalling within cells of the innate immune system. Such an understanding will be critical to the design of more specific drugs that would cause only a desired subset of LL-37 responses.

1.2.1.1 Immunomodulatory Effects of LL-37

LL-37 appears to play an important role in the innate immune response. It is capable of directly chemoattracting monocytes, neutrophils and CD4+ T cells, and can aid in wound healing and angiogenesis (reviewed in (25)). It can regulate apoptosis, both positively and negatively (52-54), and cause mast cell degranulation (55). LL-37 can increase production of chemokines (e.g. CCL2/MCP-1) and cytokines that are important for an effective immune response (49, 56-58). LL-37 can also influence monocyte-derived DC differentiation by promoting a Th1 phenotype and increasing expression of costimulatory molecules (59), but conversely inhibits DC activation and maturation by TLR ligands (60) and reduces the expression of CD80 on LPS + IFNγ-stimulated monocytes and DC (61).

While some of the above would be considered pro-inflammatory effects, LL-37 also demonstrates selective anti-inflammatory effects and is able to suppress LPS-induced production of the pro-inflammatory cytokines TNF-α and IL-6 by myeloid DC, monocytes, T cells and B cells (50, 58). LL-37 can also suppress IFNγ induction of inflammatory cytokines in peripheral blood mononuclear cells (PBMC) (61) and block macrophage activation by the TLR2 ligand lipoteichoic acid (LTA) (50), but does not have strong effects on PBMC activation by ligands of TLR2/6, TLR5, TLR7 or TLR8 (62). LL-37’s role in limiting the harmful aspects of inflammation is far more sophisticated than simply neutralizing or blocking of TLR ligand responses. Rather, LL-37 selectively modulates TLR signalling to limit certain harmful aspects of inflammation, by down-regulating pro-inflammatory cytokines much more than chemokines (63). In fact, LL-37 itself can transiently activate the IκB-α/NF-κB pathway (64, 65). LL-37 can also cause production of the anti-inflammatory cytokine IL-10 in PBMC (58). Also while LL-37
can block pro-inflammatory cytokine production by M1 macrophages, it does not interfere with other macrophage functions and actually enhances macrophage anti-tumour activity (66). Conversely, LL-37 can promote the uptake of LPS by the basolateral compartment of bronchial epithelial cells (which are normally hypo-responsive to LPS), suggesting that LL-37 can help activate mucosal immune defences (67).

LL-37 is capable of enhancing responses of various immune cells to certain stimuli. Certain molecules, including GM-CSF (29) and IL-1β (64), which would normally be present or induced during an inflammatory response, have been shown to synergize with LL-37 and selectively enhance its immunomodulatory effects. IL-1β and LL-37 synergistically activate AKT, CREB and NF-κB pathways, resulting in increased production of certain cytokines (IL-6, IL-10, but not TNFα) and chemokines (CCL2/MCP-1 and CCL7/MCP-3) in human PBMC (64).

1.2.1.2 LL-37 Receptors

Different receptors on immune cells are responsible for the variety of immunomodulatory activities of LL-37. The receptors identified to date, through which LL-37 functions, are formyl peptide receptor-like 1 (FPRL1), epidermal growth factor receptor (EGFR) and the P2X7 receptor. Furthermore, GAPDH has recently been identified as an intracellular binding partner for LL-37. The receptors for LL-37 are discussed in more detail below.

FPRL1 is a pertussis toxin sensitive, seven transmembrane GPCR with a variety of natural ligands. It is responsible for LL-37-induced chemotaxis of monocytes, neutrophils and T-cells (68), although a recent study suggests that LL-37-induced neutrophil chemotaxis is mediated by CXCR2 (69). LL-37 also mediates angiogenesis through activation of FPRL1 on endothelial cells (70). Conversely, FPRL1 does not appear to be involved in LL-37-induced CCL2/MCP-1 production, as inhibition of FPRL1 does not have a significant effect on CCL2/MCP-1 production by LL-37-treated PBMC (71).

Insertion of LL-37 into the cell membrane of keratinocytes releases metalloproteases, causing cleavage of membrane-bound heparin-binding EGF, thus transactivating EGFR leading to signal transduction and activation of STAT-3, thereby promoting the migration of these cells, a process that contributes to wound healing (72). EGFR is involved in the ability of LL-37 to promote wound healing in epithelial cells (73). EGFR is also involved in metalloproteinase-
dependent LL-37-induced CXCL8/IL-8 release by airway epithelial cells, a process that involves activation of the MAPK ERK1/2 (56).

The P2X\(_7\) receptor belongs to the P2X family of nucleotide-gated channels. The only pore-forming member of this family, the P2X\(_7\) receptor is permeable to large molecules (74). The P2X\(_7\) receptor is expressed on monocytes, macrophages and DC, and its expression is increased in response to inflammatory stimuli (such as LPS and various cytokines) and certain infections (e.g. *Mycobacterium tuberculosis*) (75-77). While the role of this receptor has not been entirely elucidated, it is known to be involved in apoptosis and appears to play an important role in host defences. LL-37 is capable of inducing post-translational processing and release of the pro-inflammatory cytokine IL-1\(\beta\) by LPS-primed monocytes through activation (possibly transactivation) of this receptor (78). LL-37 signals through P2X\(_7\) receptors (as well as FPRL1) to inhibit neutrophil apoptosis (53, 54). Like FPRL1, P2X\(_7\) does not appear to be involved in LL-37-induced CCL2/MCP-1 production (71).

Recently, macrophage GAPDH was shown, using pull-down experiments and mass spectrometry, to be an intracellular receptor for LL-37 (71). Silencing of GAPDH was shown to inhibit LL-37-induced production of chemokines CCL2/MCP-1, CCL4/MIP-1\(\beta\), CCL20/MIP-3\(\alpha\) and CXCL1/Gro-\(\alpha\), and the anti-inflammatory cytokine IL-10 (71).

![Figure 1.2](image)

*Figure 1.2.* Involvement of GAPDH in the responses of macrophages to LL-37. Reproduced from (71) with permission. Copyright 2009. The American Association of Immunologists, Inc.
Other receptors may also mediate some of the effects of LL-37. However, it has been proposed that many of the immunomodulatory effects of LL-37 are not receptor-mediated, due to the observation that the D-form of LL-37 can induce CXCL8/IL-8 production in keratinocytes (57). Nevertheless, it is possible given the great diversity of peptides with activities that overlap those of LL-37, that the activity of the D-form of LL-37 reflects the fact that binding surfaces on receptors for these peptides, rather than binding pockets, mediate downstream signalling, analogous to the situation with the T-cell receptor and that these binding surfaces respond to amphipathic polycations.

1.2.1.3 Signalling Pathways Initiated by LL-37

LL-37 is known to activate a variety of signalling pathways. In its induction of angiogenesis, LL-37 activates the phosphoinositide 3-kinase (PI3K), protein kinase C and NF-κB pathways (70). LL-37 also activates the p38 and ERK-1/2 MAPKs in monocytes, epithelial cells and mast cells, and this signalling is necessary for LL-37-induced expression of the chemokines CXCL8/IL-8, CCL2/MCP-1 and CCL7/MCP-3 (29, 79). LL-37 can also activate the IκB-α/NF-κB pathway in monocytic and epithelial cells by causing transient phosphorylation and degradation of IκB-α, resulting in translocation of p65 and p50 (64, 65).

Recent microarray experiments in the Hancock lab indicated upregulation of genes encoding members of MAPK pathways in monocytes (58). These genes included MAPK3K1 and MAPK2K4, both of which are involved in p38 and JNK signalling (58). MEK 6 was also strongly upregulated (58). Moreover, subsequent studies using pathway inhibitors showed that the PI3K and the MAPK JNK pathways are also essential for LL-37-induced production of some chemokines in PBMC. Furthermore, transcription factor arrays demonstrated that the activities of several transcription factors, including AP-1, AP-2 and NF-κB, were increased in the monocytic cell line THP-1 upon stimulation with LL-37. Microarray data using human CD14+ monocytes also showed an increase in the expression of genes encoding three of the four components of activating signal cointegrator 1 (ASC-1) in signalling responses of monocytes to LL-37. ASC-1, which is composed of ASCC-1, 2 and 3 and TRIP4, binds and co-activates several transcription factors, including AP-1 and NF-κB (80).
1.2.2 Roles of Host Defence Peptides in Wound Healing Processes

Epithelial injury permits invasion of potential pathogens, and so a rapid process of re-epithelialization is crucial to preventing infection. As a complement to their role in immune activation, HDPs appear to also aid in the process of wound healing. Levels of cathelicidins increase in the skin rapidly following injury, produced by keratinocytes and granulocytes (38), and this induction appears to be mediated, at least in part, by growth factors (such as IGF-I and TGF-α) that are expressed at sites of injury (39). Furthermore, LL-37 appears to play an important role in re-epithelialization (81). Conversely, LL-37 is lacking in chronic, non-healing ulcers, and antibody-mediated neutralization of LL-37 results in impaired re-epithelialization (81, 82).

As previously mentioned, LL-37 promotes angiogenesis, an important process in wound healing, by activating endothelial cells through FPRL1 (70). Furthermore, LL-37 has been shown to aid in repair of mechanically induced wounds in airway epithelial cells (83). Low levels of LL-37 have been shown to induce keratinocyte migration via transactivation of EGFR (72), and proliferation and migration of keratinocytes and fibroblasts (84-87).

1.3 Synthetic Immunomodulators

HDPs are clearly attractive candidates for antimicrobial agents. They have an array of beneficial immunomodulatory properties, have weak direct antimicrobial activity and are less likely to elicit antimicrobial resistance than are traditional antibiotics (24). However, the use of HDPs as anti-infective therapeutics is limited by their associated manufacturing costs, susceptibility to proteases and potential cytotoxicity issues (88, 89). Thus, there is substantial interest in creating synthetic immunomodulators that possess some of the immunomodulatory and antimicrobial features of HDPs, while limiting cytotoxicity and production costs. Two synthetic immunomodulators, discussed later in this thesis, are innate defence regulators (IDRs), which were designed and selected for immunomodulatory properties. Other immunomodulators like macrolide antibiotics such as azithromycin, were discovered only after they were in common use clinically as antibiotics.
1.3.1 IDRs, Synthetic Derivatives of HDPs

Efforts have been undertaken to design new peptides (IDRs) with structures based on those of HDPs, but which are smaller (and therefore less costly to produce), are not cytotoxic, and have improved immunomodulatory functions compared to natural HDPs.

A series of peptides were designed in the Hancock laboratory based on the structures of HDPs, with the goal of identifying structural features of HDPs that confer their immunomodulatory functions and making more effective immunomodulators. Bac2A is a linear derivative of the bovine cathelicidin bactenecin (90). It is weakly antimicrobial against Gram-positive and Gram-negative bacteria (90) and has been shown to induce chemotaxis of monocytic cells in vitro as well as CXCL8/IL-8 production by human bronchial epithelial cells (91). The sequence of Bac2A was modified through amino acid substitution and scrambling to yield a variety of peptides, of which peptide HH2 demonstrated a strongly enhanced ability to induce the production of chemokines such as CCL2/MCP-1 by PBMC (i.e. much greater than that of LL-37 or Bac2A) as well as moderate antimicrobial activity against S. aureus and Pseudomonas aeruginosa (92). From HH2, another series of IDRs was designed by a combination of substitutions, scrambling of the peptide sequence and deletion of certain residues. IDR-1018 exhibited the greatest chemokine and cytokine-inducing effects while exerting minimal cytotoxicity toward host cells (93).

To design enhanced directly antimicrobial peptides libraries of 9-amino acid residue peptides were iteratively created and advanced neural network computational methods were used to predict, amongst a virtual library of 100,000 peptides, sequences that would confer optimal activity (94). Of the predicted superior peptides that were synthesized and tested, HHC36 demonstrated particular promise, exhibiting excellent broad spectrum antimicrobial activity against a variety of highly antibiotic resistant pathogens but low levels of hemolysis (94).

The peptide HB-107 was used as a control in some of the later experiments in this thesis involving wound healing functions of IDRs. HB-107 was derived from a fragment of the moth antimicrobial peptide cecropin B (95). HB-107 has no antimicrobial activity but was able to promote cutaneous wound repair by inducing keratinocyte proliferation and CXCL8/IL-8 release by endothelial cells, thereby recruiting leukocytes to the wound site as well (95).
1.3.2 Macrolides as Immunomodulators

Macrolides are a class of antibiotics commonly used to treat airway infections. However, the role of these antibiotics in reducing the pathology of chronic airway infections by *P. aeruginosa* appears to be due largely to their anti-inflammatory properties as opposed to their direct antimicrobial properties, as most macrolides are only weakly active against this bacterium.

It has been known for nearly 20 years that the 15-membered ring macrolide azithromycin possesses immunomodulatory properties (96). Azithromycin is commonly used to treat diffuse panbronchiolitis (DPB), a serious disease of the lower respiratory tract characterized by chronic inflammation (97). Azithromycin treatment of DBP has proven successful even in cases where no bacterial infection was present, suggesting an alternate mechanism of action that extends beyond its traditional role as an antibiotic. Azithromycin has also shown promise in the management of cystic fibrosis (CF), with benefits observed even in patients who were not colonized by *P. aeruginosa* (98, 99). Azithromycin has also shown benefit in other diseases that affect the lungs, such as asthma (98).

It is likely that at least part of the benefit of macrolide treatment of DPB and other lung diseases is due to the ability of these antibiotics to reduce levels of inflammatory cytokines like CXCL8/IL-8 and IL-1β (97) and inhibit IL-12p40 expression by macrophages in response to inflammatory stimuli (100). It has been suggested that such effects are due to suppression of AP-1 and NF-κB (100, 101). Azithromycin reduces TNF-α production by LPS-stimulated THP-1 cells by affecting the p38 MAPK pathway and 70-kDa heat shock protein (HSP-70) (102). Azithromycin has also been shown to modulate DC function by increasing expression of the costimulatory ligand CD80 and the production of IL-10 (103). These immunomodulatory functions clearly parallel those of LL-37, and it is therefore of interest to further compare these two molecules experimentally in terms of their immunomodulatory capabilities.

1.4 Hypothesis and Experimental Goals

The aims of this study were to further expand the understanding of signalling pathways activated by cationic peptides in immune cells and explore the determine whether AP-1 is involved in some of the wound-healing properties of these peptides, and also to investigate similarities between host defence peptides and certain chemokines. Based on microarray and
transcription factor array results, I hypothesized that the transcription factor AP-1 is involved in cellular responses to LL-37, and that ASC-1 may play a role in AP-1 activation during such a response.
CHAPTER 2: Materials and Methods

2.1 Media and Reagents

LL-37, IDR1018, HHC36 and HB107 (sequences of which are listed in table 2.1) were synthesized using N-(9-fluorenyl)methoxy carbonyl (F-moc) chemistry at the Nucleic Acid/Protein Synthesis Unit (University of British Columbia, Canada) or at Genscript (Piscataway, NJ) and dissolved in endotoxin-free water (Sigma-Aldrich). Recombinant human GM-CSF and IL-1β were purchased from Fitzgerald Industries (Acton, MA), and azithromycin was purchased from Sigma-Aldrich; these were also dissolved in endotoxin-free water. The AP-1 inhibitor tanshinone IIA was purchased from Biomol and dissolved in DMSO according to the manufacturer’s guidelines.

Table 2.1 Sequences of peptides used in this study (all peptides are carboxy terminally amidated).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL37</td>
<td>LLGDFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES</td>
</tr>
<tr>
<td>IDR1018</td>
<td>VRLIVAVRIWRR</td>
</tr>
<tr>
<td>HHC36</td>
<td>KRWWKWWRR</td>
</tr>
<tr>
<td>HB107</td>
<td>MPKEKVFLKIEKMGMRNIRN</td>
</tr>
</tbody>
</table>

Rabbit-anti c-Jun, phospho-c-Jun (Ser-63), phospho-c-Jun (Ser-73), c-Fos, phosphor c-Fos (Ser-32) and ASC-1 antibodies, and anti-biotin and anti rabbit antibodies, were purchased from Cell Signalling Technology (Mississauga, Ontario). The anti-phospho-c-Fos (Thr-232) antibody was purchased from Invitrogen.

LPS from *Pseudomonas aeruginosa* H103 was purified using the Hancock-Darveau method (104).

2.2 Analysis of Cytotoxicity

The cytotoxic effects of tanshinone IIA were determined by treating human PBMC with up to 10 µg/ml tanshinone IIA for 24 h. 1% Triton-X-100 was used as a positive control. Lactate dehydrogenase (LDH) levels in the supernatants, indicative of cell permeability, were
determined using a kit from Roche (Mississauga, Ontario, Canada). Tanshinone IIA showed no evidence of cytotoxicity at any of the concentrations tested (data not shown).

2.3 Isolation of Peripheral Blood Mononuclear Cells

Human PBMC were isolated by density centrifugation. Briefly, venous blood was collected from healthy volunteers into sodium heparin-containing Vacutainer® collection tubes (BD Biosciences, Mississauga, Ontario), according to a University of British Columbia Clinical Research Ethics Board approved protocol. Blood was mixed, at a 1:1 ratio, with RPMI 1640 medium (Gibco®, Invitrogen™ Life Technologies, Burlington, ON) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, PAA Laboratories, Etobicoke, ON), 2mM L-glutamine and 1 nM sodium pyruvate (both from Invitrogen™ Life Technologies). PBMC were separated using Ficoll-Paque® Plus (Amersham Pharmacia Biotech, Quebec) and washed with phosphate buffered saline (PBS). PBMC were enumerated using Trypan blue exclusion and resuspended in complete media at a concentration of 1×10^6 cells/ml. Cells were rested for at least one hour at 37°C prior to treatment.

2.4 Selection of CD14+ Monocytes

Monocytes were isolated from PBMC using either positive or negative isolation kits from StemCell Technologies (Vancouver, BC), according to the manufacturer’s instructions. Positive selection was used for the microarray study, and negative selection was used for all other studies involving monocytes. Cells were rested for at least one hour prior to treatment. Flow cytometric analysis of monocytes isolated using these methods revealed monocyte purity (based both on size/granularity and CD14 staining) to be around 80% (data not shown).

2.5 Cell Culture

THP-1 cells are an acute monocytic leukemic cell line that represent a fairly immature stage of monocyte development, but which can be differentiated into cells that mimic monocyte-derived macrophages by treatment with phorbol esters such as PMA (105-107). THP-1 cells (ATCC, Rockville, MD) were cultured in RPMI 1640 (supplemented as described above) and maintained at 10^5-10^6 cells/ml in tissue culture flasks at 37°C and 5% CO₂ in a humidified incubator. Cells were passaged by diluting in fresh medium; this was repeated a maximum of
five times. THP-1 cells were differentiated into macrophage-like cells by treating them with 60 ng/ml PMA for 24 h. Cells were then rested for three days prior to treatment.

The SV40-transformed, immortalized human bronchial epithelial 16HBE4o- (HBE) cell line was a gift from Dr. D. Gruenert (University of California, San Francisco, CA) (108). HBE cells were cultured in minimum essential medium (MEM) with Earle’s salts (Invitrogen), supplemented with 10% v/v heat-inactivated FBS and 2 mM L-glutamine and maintained in tissue culture flasks at 37°C and 5% CO₂ in a humidified incubator and were used between passages 5 and 15.

2.6 Cell Stimulation

For experiments involving inhibitors, cells were pre-treated with inhibitor for 30 minutes prior to treatment with other stimuli.

2.7 Wound Healing Assay

HBE cells were grown to near confluency in 6-well plates. A 6mm strip of cells down the centre of each well was scraped away using a cell scraper and were washed three times with PBS. Treatments were then added to the corresponding wells, and the cells were monitored and photographed daily until the cells grew back to the centre line.

2.8 Western Immunoblotting

THP-1 cells or PBMC were treated with various stimuli for 5 to 60 min. Cells were then washed with ice-cold PBS. For studies using nuclear extracts, these were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kits (Pierce, Rockford, IL) and stored at -80°C. For studies using whole cell lysates, these were prepared by lysing cells in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl pH8, 137 mM NaCl, 10% glycerol and 2mM EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich), and storing lysates at -80°C. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Pierce). Samples were resolved on a 7.5% SDS-polyacrylamide gel, followed by transfer at 90V for 1h to either polyvinylidene (PVDF) or nitrocellulose membranes. PVDF membranes were made hydrophobic by drying with methanol prior to addition of primary antibody, whereas nitrocellulose membranes were blocked in 5% skim milk in TBS/T prior to addition of primary
antibody. Blots were incubated with primary antibody (1:1000 in 5% BSA in TBS/T) overnight at 4°C, washed several times with TBS/T, and then incubated with secondary antibodies (in 5% skim milk in TBS/T) for 1.5h at room temperature. After further washes, the blots were visualized using chemiluminescence peroxidase substrate (Sigma-Aldrich).

2.9 RNA Isolation

RNA was isolated using Qiagen’s RNeasy kit. Following stimulation, cells were washed with cold PBS and lysed with RLT lysis buffer and placed at -80°C. RNA isolation was carried out according to the manufacturer’s instructions, with the addition of an on-column DNase digestion step for approximately 45 min.

2.10 Real-time Quantitative RT-PCR

RNA (0.5 – 1 µg) was reverse-transcribed into cDNA using qScript™ cDNA synthesis kit (Quanta Bioscience, Inc., Gaithersburg, MD). Reverse transcriptase-negative reactions were performed in parallel. 0.25 µL of cDNA (in a total volume of 12.5µL) was amplified using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen). All primers used in this study are presented in Table 2.2. PCR was run on an ABI Prism 7000 or 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 50°C for 20°C minutes, 95°C for 2 min and 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds. GAPDH or β2M was used as an endogenous control.

| Table 2.2 Sequences of primers used in this study. |
|---------------------------------|-----------------|-----------------|
| Gene   | Forward Sequence | Reverse Sequence |
| β2m    | CTCGCCGCTACTCTCTCTCTTCT | TGCTCCACTTTTCAATTCTCT |
| ASCC1  | TGCAGAAAGACACAGCTTTCC | TCTCCAAATATATTCAATTATGC |
| ASCC2  | GTGCACAAGGGCAAGAGC | CGCTTGTGTCAGC AAA |
| ASCC3  | TGCTGTACTTTATATATGATGCTTCTGCT | CCCAGCAGTTCAAATTAGCTCA |
| CCL2   | TCATAGCAGCCACCTTATTCTTCT | TAGCAGCATATTGGTTG |
| EGF    | GTGGGGGTGGTACGATAGAGAC | TCTGCCGCAAATTCCGAGAC |
| FOS    | CCGAGAGTGTAGCAAAACGC | GAGACAGAAGCCGTTG |
| GAPDH  | GGAGGTCAAGGGATTTTGCTGAT | AGGCTCCTCCATGGTGTAAGGC |
| HGF    | GAGGGGAAAAGAAAAGAGAGA | GTGTGGGTATCGGAAGTCCA |
| IGF    | TCAGTCTCTCTTACCTGCGCGTGT | GCAATACATCTCCAGACCTC |
| P2X    | CCCGCCAACAATTCAACCAGAAAC | CCGAAGTGGAGGAGTTGAGAG |
| PDGFRb | AATGTCTCCACACCTTCTG | AGCAGATGGTGTAAGGC |
| TRIP4  | GTGGACCACACAGTTGCAG | GATCCTGGATTCGCAACTTG |
GAPDH or β2M threshold cycle (C_T) values were subtracted from corresponding sample C_T values to obtain ΔC_T. ΔC_T (untreated) was subtracted from ΔC_T (treated) to obtain ΔΔC_T. Fold change (relative to control) was calculated as 2^{-ΔΔCT}.

2.11 Statistical Analysis

Statistical significance was determined using Student’s t-test. A p value ≤ 0.050 was deemed significant.
CHAPTER 3: Initiation of Signalling Pathways in Immune Cells in Response to LL-37

3.1 Alterations in AP-1 Levels and/or Phosphorylation States in Response to LL-37

To determine whether AP-1 levels and phosphorylation states changed in immune cells in response to LL-37, human PBMC or CD14+ monocytes were treated with 20µg/ml LL-37 for up to 1 hour and nuclear extracts or cell lysates were prepared. These samples were used for Western immunoblotting using polyclonal rabbit antibodies against c-Jun, phospho-c-Jun (Ser-63 and Ser-73), c-Fos and phospho-c-Fos (Thr-232 and Ser-32).

Western blots using nuclear extracts indicated that c-Jun levels in the nucleus increased in response to LL-37 in two of three donors (Fig. 3.1). Antibodies against phospho-c-Jun were also used with these samples to determine whether phospho-c-Jun levels increased in response to LL-37, but these antibodies consistently resulted in backgrounds that were too high to determine any differences. Therefore, whole cell lysates were used for further experiments, which yielded much clearer results. It was observed that phospho c-Jun (Ser-73) levels in whole cells lysates increased in response to LL-37, in two of three donors (Fig. 3.2). Phospho-c-Fos levels increased in whole cell lysates in PBMC also in two of three donors (Fig. 3.3).

**Figure 3.1.** Total c-Jun levels in the nucleus increased in the response to LL-37 in 2/3 donors. The lower panel shows the loading control GAPDH. Shown is one representative blot of two. The third donor showed no change in c-Jun levels. PMA (1µg/ml), a known activator of AP-1, was used as a positive control.
3.2 LL-37 Displayed Synergy with Components of the Inflammatory Milieu to Activate AP-1

LL-37 is capable of synergizing with GM-CSF to activate ERK1/2 and p38 (29). More recently, it has also been shown that LL-37 synergizes with IL-1β to activate AKT, CREB and NF-κB pathways, resulting in increased production of certain cytokines (IL-6, IL-10) and chemokines (CCL2/MCP-1, CCL7/MCP-3) in human PBMC (64). Here I demonstrated that LL-37 demonstrated synergy with GM-CSF and IL-1β with respect to c-Jun phosphorylation and total c-Jun production.

**Figure 3.2.** Phospho-c-Jun (Ser 73) levels in PBMC increased in two donors in response to 20 µg/ml LL-37 after 30 min. Shown is one representative blot of two.

**Figure 3.3.** Phospho-c-Fos levels in PBMC increased in response to 20 µg/ml LL-37 at 30 min in two of three donors. Shown is one representative blot of the two positive results.
3.3 AP-1 was Not Essential for LL-37-induced Chemokine Expression by Monocytes

Transcription of IL-6 requires the activity of NF-κB, AP-1, CREB and C/EBP (109), while transcription of CXCL8/IL-8, CCL2/MCP-1, CCL7/MCP-3 and IL-10 requires activity of a complex of transcription factors including NF-κB, CREB and AP-1 (57, 62). Similarly, it has been demonstrated that LL-37 activates groups of transcription factors, and that by altering levels and activation states of various transcription factors, LL-37 can selectively modulate the innate immune response (64).

To determine whether AP-1 activity was required for LL-37-induced chemokine production, inhibitor studies were performed using Tanshinone IIA, which inhibits Fos-Jun-DNA complex formation (110). THP-1 cells were pre-treated with various concentrations of Tanshinone IIA, determined in preliminary studies to demonstrate no cytotoxicity at the doses used. Preliminary experiments showed that pre-treatment of THP-1 cells with TIIA did not blunt LL-37-induced CCL2/MCP-1 or CXCL8/IL-8 production (data not shown). Thus, AP-1 was apparently not essential for CXCL8/IL-8 and CCL2/MCP-1 production in response to LL-37.

Figure 3.4. LL-37 displayed synergy with GM-CSF and IL-1β. PBMC were treated with 20 µg/ml of LL-37, in the presence or absence of either 30 ng/ml GM-CSF, or 10 ng/ml IL-1β, for 30 min. Cell lysates were prepared and used for Western blotting to reveal the presence of the proteins indicated to the right.
3.4 Expression of ASC-1 Components did Not Significantly Increase in Response to LL-37

Recent microarray data from the Hancock lab indicated that three of the four ASC-1 components were upregulated in response to LL-37 in CD14+ monocytes (58). To confirm these findings, primary human CD14+ monocytes from four donors (Fig. 2A) or PBMC (Fig. 2B) from five to seven donors were treated with 20µg/ml LL-37 for various lengths of time as indicated in Fig. 2. Most donors showed modest increases in the levels of these genes, although these levels did not reach statistical significance. Variability between donors is not entirely surprising, particularly in PBMC, which are a mixed population of cells, and may explain why the microarray results are difficult to confirm. Preliminary experiments indicated that ASC-1 did not increase at the protein level (data not shown).
Figure 3.5. Fold increase in gene expression of ASC-1 components in monocytes and PBMC. CD14+ monocytes (A) or PBMC (B) were treated with 20µg/ml LL-37 for 4h (monocytes) or 15min. to 4h. (PBMC). Cells were subsequently lysed for RNA extraction, and qPCR was performed for ASCC1 (Bi), ASCC2 (Bii), ASCC3 (Biii) and TRIP4 (Biv). Fold change is relative to untreated. Shown are averages of 4 donors (A) or 5-7 donors (B) + SEM. Due to donor variability, fold changes did not reach statistical significance.
3.5 Comparison of LL-37 with Azithromycin

The macrolide antibiotic azithromycin shares numerous immunomodulatory properties with LL-37, such as anti-inflammatory and anti-sepsis activity and the ability to modulate DC differentiation. Azithromycin treatment has shown clinical benefit in DBP and CF, even in the absence of bacterial infection. It is likely that at least part of the benefit of macrolide treatment of DPB and other lung diseases is due to the ability of these antibiotics to reduce levels of inflammatory cytokines. It was of interest to further compare LL-37 and azithromycin in terms of immunomodulatory effects, as well as their ability to initiate cell signalling events.

![Fig. 3.6. Azithromycin reduces LPS-induced MCP-1 but not IL-8 release in THP-1 cells.](image)

Intriguingly both LL-37 and azithromycin demonstrated anti-inflammatory activities in suppressing LPS-stimulated CCL2/MCP-1, although LL-37 was clearly more potent. However, unlike LL-37, azithromycin did not reduce LPS-induced CXCL8/IL-8 production by THP-1 cells.
CHAPTER 4: Wound Healing Properties of Host Defence Peptides and Innate Defence Regulators

AP-1 has been demonstrated to have important roles in skin pathology and physiology including in wound healing (111). Therefore in an attempt to discover a possible biological function for AP-1 activation by LL-37, I here investigated one of its alternative functions in wound repair (83), comparing its function to recently described immunomodulatory peptides from our lab.

4.1 Low Concentrations of Cationic Peptides Increased Expression of Genes Involved in Wound Healing

It has been shown previously that LL-37 exerts its wound healing properties at relatively low concentrations (83). To further elucidate the wound healing properties of LL-37 and cationic peptides, changes in the expression levels of genes involved in wound healing processes in response to these peptides were examined in PBMC and HBE. PBMC were treated with low concentrations (0.5 - 1 µg/ml) of LL-37, IDR-1018, HHC36 or HB107 for four hours. The expression of a variety of genes involved in wound healing (EGF, HGF, P2X7 and TGF-β) was quantified using real time PCR.

IDR-1018 caused significant upregulation of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) expression in human PBMC. EGF is a mediator of wound closure (112). HGF has a variety of roles in wound healing, including inducing angiogenesis, increase keratinocyte migration speed, and regulation of MMPs (113).

HHC36 induced expression of P2X7, which, in addition to serving as a receptor for LL-37, is involved in re-epithelialization (114) and bone repair (115). Neither LL-37 nor HB-107 caused any significant changes in expression of these genes.
Figure 4.1. Low concentrations of 1018 and HHC36 increase the expression, in PBMC, of genes involved in wound-healing. Results shown are averages of results from three donors +SEM. * = p \leq 0.05

A similar series of experiments were performed with HBE. IDR-1018 increased platelet derived growth factor receptor (PDGFR) expression (Fig. 4.2). PDGF is involved in stimulating DNA synthesis and chemotaxis of fibroblasts and smooth muscle cells and stimulated collagen, glycosaminoglycan and collagenase in fibroblasts as well as acting in synergy with other growth factors (116).

Consistent with my original hypothesis, very modest levels of LL-37, IDR-1018 and HHC36 all increased expression of the AP-1 FOS subunit (Fig. 4.2), but HB107 was unable to stimulate FOS production.
4.2 Inhibition of AP-1 Affects Peptide-induced Growth Factor Expression in PBMC

To determine whether AP-1 was involved in growth factor expression increases induced by LL-37, IDR-1018 and HHC36, PBMC were pre-treated, prior to stimulation with these peptides, with Tanshinone IIA (TIIA), which inhibits Fos-Jun-DNA complex formation (110). All three of these peptides induced keratinocyte growth factor (KGF), a member of the FGF family, which is a potent mitogen of epithelial cells (117, 118). In cells that were pre-
treated with TIIA, there was a baseline increase in KGF expression but no significant induction of KGF expression (relative to TIIA-treated cells) by any of the peptides was observed. However, since TIIA itself raised KGF expression, an alternate explanation for these results is that inhibition of AP-1 relieved repression of KGF expression.

4.3 Influence by LL-37 and IDRs on Re-epithelialization in vitro

To determine whether the above-observed changes in gene expression in HBC correlated with enhanced re-epithelialization, an in vitro model of wound healing, the so-called “scratch assay” was employed. Briefly, a portion of adherent HBE were scraped away from the cell culture surface and were allowed to re-grow in the presence or absence of LL-37, IDR-1018, HHC36 or HB107, with or without the AP-1 inhibitor TIIA. Cells were photographed daily until the untreated cells had re-grown over the area that had been scraped away.

Inhibition of AP-1 activity by TIIA completely abrogated re-epithelialization by both peptide-treated and untreated cells, highlighting the importance of this transcription factor in the wound healing process. The results obtained from peptide-treated HBE were more variable. While two biological replicates showed substantial increases in re-epithelialization in IDR-1018-treated HBE, this effect was not observed in two other biological replicates. These differences may be due to the inherent variability in the assay.
CHAPTER 5: Similarities between Host Defence Peptides and Chemokines

Similarities exist between HDPs and chemokines. Certain chemokines possess structural similarity to host defence peptides, including a cationic amphipathic nature, and also possess weak antimicrobial activity in dilute media (5), which is likely to be insignificant in vivo. Nevertheless, since HDPs also have direct chemotactic (chemokine) activity (68, 69), here it was hypothesized that chemokines may also possess some of the immunomodulatory functions of host defence peptides.

5.1 Chemokines CXCL9/MIG and CCL20/MIP-3α Induced CXCL8/IL-8 in PBMC

Peptides like LL-37 are potent stimulators of CXCL8/IL8 production. Here, PBMC were treated with the chemokines CXCL9/MIG, CCL20/MIP-3α, CCL2/MCP-1 or CCL3/MIP-1α for 24 hours. CXCL8/IL-8 release was determined using ELISA. While the non-cationic chemokines CCL2/MCP-1 and CCL3/MIP-1α failed to induce significant levels of CXCL8/IL-8 release, the cationic chemokines CXCL9/MIG and CCL20/MIP-3α did, at concentrations of 1 and 0.3 µM, respectively (Fig. 5.1). These results were similar to those obtained from a similar experiment involving THP-1 cells, in which CXCL9/MIG induced a greater amount of CXCL8/IL-8 release than CCL20/MIP-3α, and CCL2/MCP-1 and CCL3/MIP-1α did not induce any CXCL8/IL-8 release.

To ensure that the observed effects of CXCL9/MIG were not due in part to any cytotoxic effects of this chemokine, LDH levels in supernatants from PBMC from three donors treated with up to 10 µM CXCL9/MIG were quantified. CXCL9/MIG exhibited no cytotoxicity at the concentrations tested (data not shown).
Figure 5.1. CXCL8/IL-8 release by PBMC. PBMC were treated with chemokines for 24 hours and IL-8/CXCL8 release was measured using ELISA. Results shown are averages of results from four donors +SEM. * = p < 0.05

5.2 Chemokine CXCL9/MIG Synergized with GM-CSF in Inducing CXCL8/IL-8 and CCL3/MIP-1α Production

PBMC from four donors were treated with up to 3µM CXCL9/MIG in the presence or absence of 30 ng/ml GM-CSF. CCL3/MIP-1α and CXCL8/IL-8 release were measured by ELISA. In the absence of GM-CSF, 3µM CXCL9/MIG induced CXCL8/IL-8 production at a maximal level of approximately 4 ng/ml and CCL3/MIP-1α production at 12 pg/ml. These levels increased to 27 ng/ml CXCL8/IL-8 and 330 pg/ml CCL3/MIP-1α in the presence of GM-CSF. Synergy between GM-CSF and CXCL9/MIG was statistically significant at p < 0.01 (Fig. 5.2).
Figure 5.2. CXCL9/MIG-induced chemokine production was enhanced in the presence of GM-CSF. Shown are the results from PBMC treated with CXCL9/MIG in the absence or presence of 30 ng/ml GM-CSF for 24h. The presence of GM-CSF enhanced CXCL9/MIG-induced production of CCL3/MIP-1α and CXCL8/IL-8. Data points are an average of results from four donors + SEM. Significance (synergy vs. additive) was determined by 2-way ANOVA followed by Tukey’s post hoc test and is denoted as * p < 0.01.
5.3 Other Data

Our paper (119) provided a broad array of data that was consistent with these observations including microarray data examining the interaction of CXCL9/MIG with human monocytic cells. These gene expression data indicated further similarities with LL-37. Similarly the results indicated that CXCL8/IL-8 production did not occur as a result of chemokine receptor engagement and activation but like LL-37 mediated stimulation of monocytes required the ERK 1/2 and p38 MAPKs.
CHAPTER 6: Discussion and Conclusions

6.1 Introduction

LL-37 is a multi-functional modulator of immune responses. It serves as a chemoattractant for monocytes, neutrophils and CD4+ T cells, through FPRL1 (25). It signals through P2X$_7$ receptors and FPRL1 to inhibit neutrophil apoptosis, but can also promote apoptosis in epithelial cells (52-54, 120). LL-37 can cause mast cell degranulation (55) and chemotaxis (121). It can modulate DC differentiation (59) and inhibits DC activation and maturation by TLR ligands (60). LL-37 can induce IL-1β release by LPS-primed monocytes (78).

LL-37 is able to suppress immune cell responses to ligands of TLR2 and TLR4 (50, 58). LL-37 selectively modulates TLR signalling to limit certain harmful aspects of inflammation (63). In fact, LL-37 itself can activate the IκB-α/NF-κB pathway (64, 65). LL-37 can increase production of chemokines (e.g. CCL2/MCP-1) and cytokines that are important for an effective immune response (49, 56-58). GAPDH was shown to be an intracellular receptor for LL-37, and appears to be required for LL-37-induced production of chemokines CCL2/MCP-1, CCL-4/MIP-1β, CCL-20/MIP-3α and CXCL-1/Gro-α, and the anti-inflammatory cytokine IL-10 (71). FPRL1 and P2X$_7$ do not appear to be involved in LL-37-induced CCL2/MCP-1 production (71).

GM-CSF and IL-1β have been shown to synergize with LL-37 and selectively enhance its immunomodulatory effects. IL-1β and LL-37 synergistically activate AKT, CREB and NF-κB pathways, resulting in increased production of certain cytokines (IL-6, IL-10, but not TNFα) and chemokines (CCL2/MCP-1, CCL7/MCP-3) in human PBMC (64).

Here, the role of transcription factors in immune cells responses to LL-37 was further elucidated, with particular attention to the wound healing properties of LL-37 and indicated that AP-1 was activated particularly by combinations of LL-37 and GM-CSF or IL-1β and that it likely has a role in wound healing. Furthermore I was able to elucidate a more profound functional overlap between LL-37 and certain cationic chemokines.
6.2 Signalling Pathways and Transcription Factors Activated by LL-37

LL-37 activates various signalling pathways in immune cells. In its induction of angiogenesis, LL-37 activates PI3K, protein kinase C and NF-κB pathways (70). LL-37 activates p38 and ERK1/2 in monocytes, epithelial cells and mast cells, and this signalling is necessary for LL-37-induced expression of the chemokines CXCL8/IL-8, CCL2/MCP-1 and CCL7/MCP-3 (29, 79). LL-37 can also activate the IκB-α/NF-κB pathway in monocytic and epithelial cells and cause translocation of p65 and p50 (64, 65).

LL-37 causes upregulation of genes encoding members of p38 and JNK MAPK pathways in monocytes (58). These genes included MAPK3K1 and MAPK2K4 and MEK 6 (58). PI3K and the MAPK JNK pathways are also essential for LL-37-induced production of some chemokines in PBMC. Furthermore, transcription factor arrays demonstrated that the activities of several transcription factors, including AP-1, AP-2 and NF-κB, were increased in the monocytic cell line THP-1 upon stimulation with LL-37. The data presented here reinforce the induction of AP-1 but imply that it is not per se involved in chemokine induction by LL-37 but rather has a role in growth factor production. Although previous data implicated at least 11 different transcription factors downstream of LL-37 stimulation (58), this is the first observation that implies that an individual transcription factor may be involved in some but not all functions of this multifunctional peptide.

6.3 LL-37 as a Mediator of Wound Healing

In addition to its more direct immunomodulatory roles, LL-37 appears to play an important part in various wound healing processes, thus providing further strength to its anti-infective role. LL-37 exerts many of its wound-healing functions by trans-activation EGFR. This activation results in the migration of keratinocytes through STAT-3 (72), epithelial wound healing (73) and MAPK ERK1/2-dependent CXCL8/IL-8 release by airway epithelial cells (56). My data indicate that LL-37 demonstrated an ability to induce growth factors in epithelial cells and PBMC, and appeared to induce KGF in PBMC in an AP-1-dependent manner. This is consistent with the published effects of LL-37 on wound healing.
6.4 The Potential of IDR as Therapeutics in Wound Healing

LL-37 (as well as many other HDPs) clearly has important roles in various aspects of innate immunity and wound healing, although its activities tend to be quite modest. Moreover, issues of production costs and potential cytotoxicity are limiting factors in developing HDPs like LL-37 into therapeutics. IDRs were developed to counter these issues. Their structures are loosely based upon those of HDPs and the optimal peptides were selected by screening for immunomodulatory activity. While the primary focus of this thesis was on the naturally occurring LL-37, IDRs were included in wound healing studies as data from a collaborator Lars Steinstraesser has shown, for example, that IDR-1018 is far superior to LL-37 in terms of wound healing activity in a mouse model (Figure 6.1).

![Figure 6.1: Quantification of re-epithelialization in wounds treated with IDR-1018. Re-epithelialization was assessed with either IDR-1018 (1.7 and 17 μg doses), LL-37 (17 μg dose) or PBS. The bars represent standard error of the mean wound closure (*,+ p<0.05; **,++ p<0.01 (*IDR-1018 17 μg doses; +IDR-1018 1.7 μg doses) compared to vehicle control). Data from collaborator Lars Steinstraesser, Ruhr University Bochum.](image)

6.5 The Role of Chemokines in Host Defence, beyond Chemotaxis

The N-termini of chemokines contain the chemokine receptor binding sites; this region is associated with chemotactic activity of chemokines (122). The structure of CXCL9/MIG differs somewhat from that of most chemokines in that it is slightly larger than most (103 amino acids as opposed to 70 amino acids) and has a more extended C-terminal tail (123, 124). Further distinguishing itself from typical chemokines, CXCL9/MIG has a strong overall positive charge and possesses antimicrobial activity (125). Here I investigated if it shared other properties with cationic HDPs, notably immunomodulatory properties.

I demonstrated that CXCL9/MIG induced the expression, by a monocytic cell line and PBMC, of CXCL8/IL-8, while other studies in our manuscript (119) performed by others...
showed induction of a variety of other chemokines by a monocytic cell line including CCL3/MIP-1α, CCL4/MIP-1β, CCL2/MCP-1 in a pertussis toxin-insensitive manner. Similarly, another cationic chemokine CCL20/MIP3α, but not the non-cationic chemokines CCL2/MCP-1 or CCL3/MIP-1α, stimulated monocytic cells to produce substantial amounts of CXCL8/IL-8 and CCL3/MIP-1α. I was also able to show that GM-CSF enhanced CXCL9/MIG’s immunomodulatory properties, as indeed these components have been demonstrated to enhance chemokine induction by LL-37 (29). These results collectively point to a strong overlap in function between the cationic chemokine and HDPs and indicate that CXCL9/MIG may serve a more central role in immunity than previously thought, by synergizing with other inflammatory components to induce the production of a broad array of other chemokines.

6.6 Azithromycin

Azithromycin is often of clinical benefit in the absence of bacterial infection, owing to its anti-inflammatory/immunomodulatory properties. Many of azithromycin’s immunomodulatory properties parallel those of LL-37, and a comparison between the immune responses to the two molecules was undertaken here. It was clearly demonstrated that, while azithromycin was capable of reducing LPS-induced CCL2/MCP-1 production, this occurred to a lesser extent than with LL-37. Furthermore, azithromycin, unlike LL-37, did not reduce LPS-induced CXCL8/IL-8 production. Thus, this series of experiments not only further elucidated the immunomodulatory actions of azithromycin, but also provided a valuable comparison to LL-37.

6.7 Conclusions and Future Directions

I consider these studies to be a launching point rather than completely finalized. One serious focus of future studies will be to establish more reliable in vitro systems to enable the investigation of wound healing in vitro. If this proves impossible and I was unable to obtain reliable results in the scratch model, despite many trials, it may be necessary to use in vivo transgenic models, employing conditional knockouts in AP-1 subunits (since each is a essential protein) to manipulate this transcription factors in specific cell types. One exciting new area that is opened up by this thesis is to understand mechanistically the relationship between various immunomodulators. Here I show a functional overlap between a chemokine and a HDP while the antibiotic azithromycin and LL-37 shared anti-inflammatory properties. It will be very
interesting to understand whether these functional overlaps stem from common or overlapping mechanisms and the accumulating mechanistic data on the HDPs, particularly LL-37 will provide an excellent basis for comparison.
References


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